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THE THICKNESS OF MONOOLEIN LIPID BILAYERS AS DETERMINED FROM REFLECTANCE MEASUREMENTS

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Measurements of the reflectance of monoolein/*n*-alkane and monoolein/squalene lipid bilayers have been made. The total thickness of the bilayer was calculated from the dependence of reflectance on the refractive index of the aqueous salt or sucrose solution surrounding the bilayer. The total thickness was then compared to the thickness of the hydrocarbon chain region as determined from capacitance measurements. From this comparison, we found that the thickness of each polar region of the bilayers in salt solutions was 0.5 ± 0.1 nm, independent of the hydrocarbon solvent used. When the aqueous solutions contained sucrose, each polar region was approx. 0.9 nm thick. When *n*-tetradecane and *n*-hexadecane were used as solvents, microlenses of solvent trapped in the monoolein bilayer increased the reflectance. After about one hour, the coalescence of microlenses into larger lenses allowed the reflectance of the bilayer alone to be measured. The use of reflectance to measure the thickness of monoolein bilayers appears to be consistent with other methods and to give useful information about the structure of lipid bilayers.

Introduction

A planar lipid bilayer (black film) is an important model system for the study of the properties of membranes. Measurements of the capacitance per unit area are used to determine the thickness of these black films [1]. The popularity of this technique for calculating thickness is due to the simplicity of the measurement and the clear interpretation of the results. Most, if not all, of the electrical capacitance is due to the nonpolar region of the film [2,3]. This region can be treated as being a homogeneous, isotropic layer with a dielectric constant between 2.1 and 2.2. The calculation of thickness from capacitance per unit area is not very sensitive to the assumptions made, and it is considered to be accurate to within a few percent [4].

The thickness of a lipid bilayer can also be calculated from its optical reflectance. To achieve comparable accuracy, however, the refractive index of the bilayer must be known to a fairly high precision, about 0.15%. Early attempts to measure the refractive index were not successful [5]. Reliable determinations of refractive indices and thicknesses were first obtained by Cherry and Chapman [6]. They produced accurate measurements of reflectance and Brewster angles of phosphatidylcholine/*n*-decane bilayers in aqueous solutions having various refractive indices. It was not possible to interpret this data in terms of a single-layer or triple-layer model. As an alternative, they proposed that the bilayer had the optical properties of a birefringent uniaxial crystal. In this model, the bilayer is characterised by two refractive indices (parallel and perpendicular to the optic axis) and one thickness, the total thickness of the film. The uniaxial crystal model did provide a good description of the experimental data [6]. By making both optical and electrical measurements, Cherry and Chapman calculated the thickness of both the polar and hydrocarbon regions of the film. One assumption made in their work, that the properties of the lipid bilayer were independent of the composition of the surrounding aqueous solutions, remained untested.

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There has been only one systematic comparison between the two methods of measuring the thickness of lipid bilayers. Pagano et al. [7] measured the reflectance of films formed from glyceryl monooleate (monoolein) dispersed in three different *n*-alkane solvents. The variation of thickness with alkane solvent did not parallel the variation observed with capacitance measurements. Pagano et al. suggested that the presence of microlenses of alkane solvent in the bilayer might affect the reflectance measurements and account for this discrepancy, but there has been no subsequent work to test this possibility. Recently, a further discrepancy between the thicknesses determined by the reflectance and capacitance techniques was noted for films made from monoolein dispersed in squalene [8]. Capacitance measurements indicate that these bilayers are very thin (2.5 nm), due to the almost total exclusion of squalene from the film [9]. From their optical measurements, Bach and Miller [8] calculated a thickness of 5.7 nm which, even after allowing generously for the polar group dimensions (say 1–2 nm) seems inconsistent with the capacitance. They proposed that these black films may not be absolutely planar, but may exhibit fluctuations in thickness. If that were the case, capacitance and reflectance measurements would detect differently weighted average thicknesses of the bilayer. This, they believed, could account for some of the discrepancy between the two methods of measuring thickness.

In an attempt to resolve these problems, we have made reflectance and capacitance measurements on monoolein lipid bilayers. We examine the assumptions needed to interpret the reflectance data and try to determine if reflectance measurements can be used to derive additional information about the structure of lipid bilayers.

Materials and Methods

NaCl, $CaCl_2$ and sucrose were of AnalaR grade (BDH, Poole, U.K.). The salts were roasted at 500°C to remove organic impurities. Water used for making solutions was distilled twice. Solutions of $CaCl_2$ and sucrose greater than 1 M were cloudy; they were treated by adding charcoal (Nuchar-C-190-N, Kodak Ltd., London, U.K.), shaking the solution and removing the charcoal by filtration. Monoolein (Nu-Chek Prep, Elysian, MN, U.S.A.) was dissolved in *n*-alkanes or squalene at 3 mg/ml concentration. The alkanes (puriss grade, Koch-Light Ltd., U.K.) and squalene (Sigma, St. Louis, MO, U.S.A.) were passed through columns containing aluminium oxide (BHD). Experiments were performed at room temperature, 21–23°C, unless otherwise noted.

The apparatus used to measure reflectance was similar to that described by Cherry and Chapman [6]. Light from a 1 mW He-Ne laser (Coherent, Palo Alto, CA, U.S.A.) was polarized perpendicular to the plane of incidence and focussed to a spot 60–100 μm in diameter on the black film. The angle of incidence was 7.5°. The reflected light was detected by a photomultiplier tube (955B, EMI, Ruislip, U.K.). The photocurrent was measured by a Vibron electrometer (Electronic Instruments Ltd., Richmond, U.K.) as the voltage drop across a 10^6 or 10^8 ohm resistor and was recorded on a Servoscribe pen recorder (Smith's Industries, London, U.K.). The apparatus was calibrated by measuring the intensity of light reflected from a piece of fused quartz. The black film cell was made of Teflon impregnated with black glass to reduce the intensity of scattered light. The cell windows were made of fused quartz. Vertical films were formed in a 1 mm diameter hole by the feeder tube technique as described by Fettiplace et al. [10]. Capacitance was measured at 500 Hz and 40 mV (peak-to-peak) [1]. The area of the film was measured with a calibrated graticule. Refractive indices at 632.8 nm were measured with an Abbe refractometer (60/ED, Bellingham and Stanley, Ltd., Tunbridge Wells, U.K.) and ranged from 1.332 (0.1 M NaCl) to 1.419 (4.0 M $CaCl_2$) or 1.407 (1.5 M sucrose).

Measurements were made of the film reflectance, $R_\perp$, in aqueous solutions of various refractive index, n_0. For small angles of incidence, the relationship between reflectance and the thickness, d, and refractive index, $n_\perp$, of the film can be written [6] as *:

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[*] Eqn. 1 is valid for both the single-layer and uniaxial crystal models of the film; measurements of the reflectance of perpendicularly polarized light do not give any information about film anisotropy. Unless Brewster angle measurements are also made, $n_\perp$ can be interpreted as either the average refractive index of an isotropic film or the refractive index perpendicular to the optic axis of a birefringent film.

$$R_\perp^{1/2} = \frac{\sin\beta}{n_\perp}\,(n_\perp - n_0 + \Delta) \tag{1}$$

where

$$\beta = \frac{2\pi n_\perp d \cos\phi}{\lambda} \tag{2}$$

and

$$\Delta = \frac{(\mathrm{n}_\perp - \mathrm{n}_0)^2}{(\mathrm{n}_\perp + \mathrm{n}_0)} \tag{3}$$

λ is the wavelength of incident light (in air) 632.8 nm, and ϕ is the angle of refraction in the film. Since $\Delta \lesssim 0.006$, an initial estimate for $n_\perp$ can be obtained by setting $\Delta = 0$ in Eqn. 1 and making a linear extrapolation of a graph of $R_\perp^{1/2}$ vs. n_0 to zero reflectance (Fig. 1a). This value of $n_\perp$ is then used to graph $R_\perp^{1/2}$ vs. $n_0 - \Delta$ (Fig. 1b); a refined value for $n_\perp$ is obtained by a similar extrapolation, and d is then calculated from the slope of the line. If this method of calculating $n_\perp$ and d is to be valid, the properties of the film must be independent of the composition of the aqueous phase. This will be discussed further in the next section.

Results

Fig. 1 illustrates the linear relationship predicted by Eqn. 1, between the square root of the reflectance of the film, $R_\perp^{1/2}$, and the refractive index of the aqueous phase, n_0 (Fig. 1a), and between $R_\perp^{1/2}$ and $n_0 - \Delta$ (Fig. 1b) for monoolein/*n*-decane black films. Each point represents the average reflectance from at least three films; the reflectance was monitored continuously for more than 20 min. The reflectance did not vary with time. In both graphs there are two lines: the upper one was obtained when the aqueous solution bathing the film contained chloride salts (NaCl or $\mathrm{CaCl_2}$) and the lower one when the aqueous solutions contained sucrose. A linear regression analysis of the data in Fig. 1b produced values of $n_\perp = 1.442$, $d = 5.77$ nm for films in aqueous salt solutions, and $n_\perp = 1.430$, $d = 6.66$ nm for films in aqueous sucrose solutions. The correlation coefficient of the linear least squares fit, r^2, was greater than 0.999 in both cases. The uncertainty in the calculated value of $n_\perp$ is 0.003 and that of d is

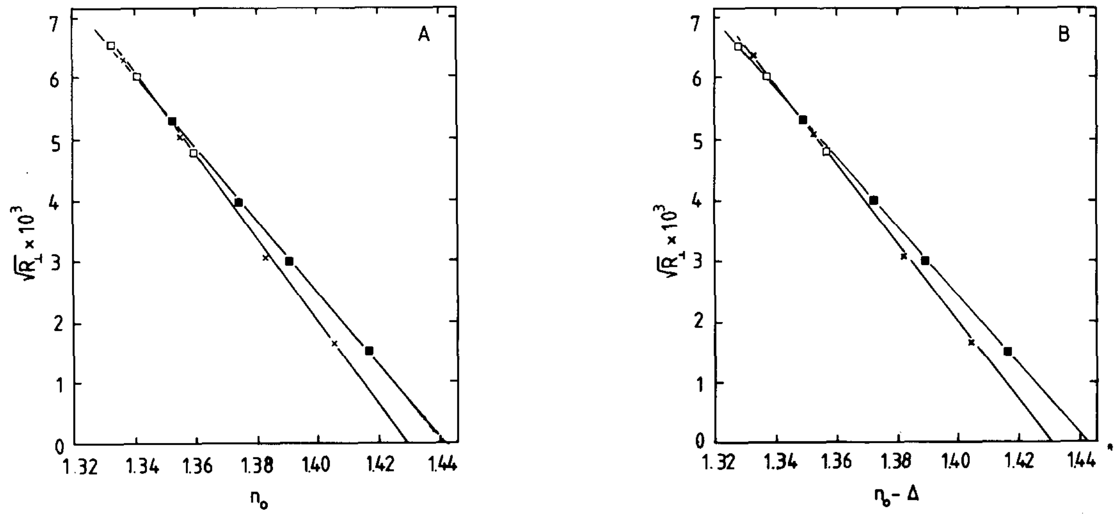

Fig. 1. A. The square root of the reflectance, $R_\perp$, of monoolein/*n*-decane black films vs. the refractive index of the aqueous solution, n_0. This graph is used to obtain an estimate of the refractive index of the black film, $n_\perp$, by making a linear extrapolation to $R_\perp^{1/2} = 0$. The different symbols represent experiments done with different solutes in the aqueous phase: NaCl (□), $\mathrm{CaCl_2}$ (■) and sucrose (X). B. A graph of the same data as in (A), expressed as $R_\perp^{1/2}$ vs. $n_0 - \Delta$ (Δ is defined by Eqn 3). A linear relationship is expected if the thickness, d, and refractive index, $n_\perp$, of the film are independent of the aqueous solute concentration. $n_\perp$ is obtained by linear extrapolation to $R_\perp^{1/2} = 0$ and d is proportional to the slope of the line.

TABLE I

A. The thickness, d, and refractive index, $n_\perp$, of monoolein black films as derived from reflectance in chloride salt solutions. The thickness of the hydrocarbon region, d_h, is from capacitance measurements [4,9]. The thickness of the polar regions, $d_p = \frac{1}{2}(d - d_h)$, and $n_\perp{}^c$ is calculated from the composition of the film [4] and the refractive index of the components.

Solvent	d (nm) (±3%)	d_h (nm) (±2%)	d_p (nm) (±20%)	$n_\perp$ (±0.003)	$n_\perp{}^c$
n-Decane	5.77	4.81	0.48	1.442	1.439
n-Tetradecane	5.18	4.07	0.56	1.448	1.451
n-Hexadecane	4.03	3.27	0.38	1.461	1.458
Squalene	3.66	2.50	0.58	1.470	1.467

B. The thickness and refractive index of monoolein black films as derived from reflectance in sucrose solutions. The symbols have the same meaning as in A.

Solvent	d (nm) (±3%)	d_h (nm) (±2%)	d_p (nm) (20%)	$n_\perp$ (±0.003)	$n_\perp{}^c$
n-Decane	6.66	4.81	0.92	1.430	1.439
Squalene	4.23	2.50	0.87	1.451	1.467

about 3% (0.18 nm) **. Similarly, reflectance of black films formed from monoolein dispersed in squalene was measured (Fig. 2). Again, the values of $n_\perp$ and d calculated from the data depended on whether salt or sucrose was used to change the refractive index of the aqueous phase (Table I). As in the case of monoolein/*n*-decane films, the smaller value of d, 3.66 nm, was obtained when the aqueous solutions contained chloride salts.

Finally the reflectance of monoolein/*n*-tetradecane and monoolein/*n*-hexadecane black films was measured. Unlike the cases where *n*-decane or squalene were used as solvents, the reflectance of these films was time dependent. The reflectance decreased by as much as 25% before reaching a steady state value one hour after formation of the film (Fig. 3). The values of thickness and refractive index listed in Table Ia for these films were calculated from the steady state values of reflectance.

To test the assumption that the properties of the black films are independent of the composition of the aqueous phase, measurements of the film capacitance per unit area were made. It was found that accurate measurements of film area could not be made when $R_\perp$ was less than 10^{-5} due to the low reflectance of the Gibbs-Plateau border in these

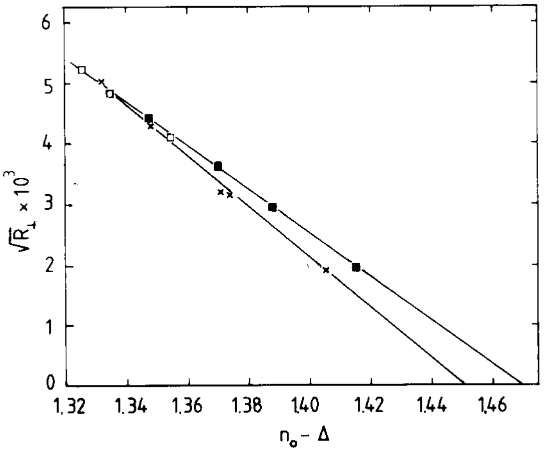

Fig. 2. $R_\perp^{1/2}$ vs. $n_0 - \Delta$ for monoolein/squalene black films. The symbols have the same meaning as in Fig. 1.

** The uncertainties in $n_\perp$ and d were estimated by reanalysing the reflectance data including only those points for which capacitance measurements could also be made ($R_\perp > 10^{-5}$). In estimating the error in d, we also considered the errors arising from the calculation of $R_\perp$ from the current in the photomultiplier tube (about 1%).

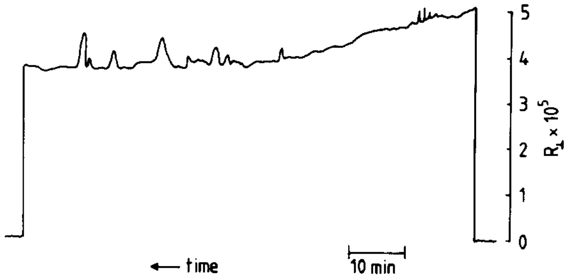

Fig. 3. A tracing of the signal recorded from the photomultiplier (which is proportional to $R_\perp$) as a function of time for monoolein/*n*-tetradecane black films in 0.1 M NaCl. Time progresses from right to left. At $t = 0$, the film is totally black and the signal corresponds to a reflectance of $5.0 \cdot 10^{-5}$. After about 10 min, the signal begins to decrease and one observes the appearance of small lenses in the film. Reflected light from the lenses is detected by the photomultiplier as they pass through the section of the film illuminated by the laser. Near the end of the trace, the reflectance of the film has decreased to a steady-state value of $3.7 \cdot 10^{-5}$. Finally, the film was broken.

TABLE II

The capacitance per unit area, *C/A*, of monoolein/*n*-decane and monoolein/squalene black films in various aqueous solutions. Sucrose solutions also contained 0.1 M $CaCl_2$ as the electrolyte. T = 25°C. The errors represent the standard deviation of at least 20 measurements on three films.

Aqueous solution	n_0	*C/A* (nF/mm²) monoolein/ *n*-decane	*C/A* (nF/mm²) monoolein/ squalene
0.1 M NaCl	1.332	3.71 ± 0.09	7.44 ± 0.09
3.0 M NaCl	1.359	3.81 ± 0.11	
4.0 M NaCl	1.367		7.51 ± 0.08
0.1 M $CaCl_2$	1.335	3.70 ± 0.06	7.46 ± 0.07
2.0 M $CaCl_2$	1.378	3.79 ± 0.09	
3.0 M $CaCl_2$	1.402		7.47 ± 0.15
0.1 M sucrose	1.339	3.60 ± 0.09	7.42 ± 0.09
0.8 M sucrose	1.373	3.56 ± 0.16	7.53 ± 0.06
1.2 M sucrose	1.385		7.45 ± 0.13

solutions *. As shown in Table II, the capacitance per unit area for monoolein/squalene films was independent of the composition of the aqueous phase. In the case of monoolein/*n*-decane films, the capacitance per unit area depended on whether chloride salts or sucrose was present in the aqueous phase, but was nearly independent of the concentration of the solutes.

Discussion

The capacitance data in Table II provides evidence that the composition of monoolein black films is nearly independent of the concentration of solute in the aqueous phase. We note several points here. Firstly, monoolein/*n*-decane films may be slightly thinner (0.1 nm) in solutions of high salt concentrations. Andrews et al. [11] observed that the thickness of these films decreases by 0.4 nm in going from 0.1 M NaCl to 5.92 M NaCl, but we did not use NaCl concentrations greater than 3 M for our reflectance measurements (Fig. 1). Secondly, the greater thickness of monoolein/*n*-decane films in sucrose solutions is similar in magnitude to the effect observed by Requena and Haydon [12] in the thickness of these films in glycerol solutions. Finally, it is unlikely that the difference in capacitance per unit area for monoolein/*n*-decane in salt solutions and in sucrose solutions is due to any contribution to the capacitance from the polar regions, since this effect would be more pronounced in the case of the thinner monnolein/squalene films, and that is not observed. Thus, we think that the use of Eqn. 1 to calculate thicknesses and refractive indices of monoolein films from reflectance data is justified.

The results of the reflectance experiments performed with monoolein black films in aqueous salt solutions are summarised in Table Ia. There is a monotonic decrease in thickness, d, as the molecular weight of the solvent increases; this parallels the decrease in d_h, the thickness of the hydrocarbon region as determined from the capacitance per unit area. Values of the polar region thickness, d_p, included in the table were calculated from $1/2(d - d_h)$. It can be seen that the thickness of the polar region is a constant (0.5 ± 0.1 nm) independent of the hydrocarbon solvent. This thickness corresponds

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* The area of monoolein/squalene films was determined in transmitted light, but when $R_\perp < 10^{-5}$, the transmittance of light was too high to get enough contrast between the film and the border.

to the thickness of the polar end of a monoolein molecule lying approximately in the plane of the bilayer.

Table Ia also contains the values of the refractive index of the film, $n_\perp$, calculated from the reflectance experiments. There is a monotonic increase in the refractive index of the films as the molecular weight of the solvent increases. The refractive index of monoolein/squalene films (1.470) is nearly the same as that of pure monoolein bulk liquid (1.467 at 24°C), as would be expected for a nearly solventless film. For the monoolein/*n*-alkane films, the values of $n_\perp$ can be compared to the refractive index, $n_\perp^c$, which would be expected assuming an homogeneous film and using the volume fraction of monoolein in the film derived from its capacitance per unit area [4] as well as the known refractive indices of the film components. The measured refractive indices of the films agree with $n_\perp^c$ to within 0.003. This agreement is remarkable in view of the obviously non-isotropic structure of the thin film.

The properties of monoolein films in sucrose solutions (Table Ib) are different from their properties in salt solutions. The thickness of the polar region is greater, about 0.9 nm (as compared with 0.5 nm), and the refractive indices are considerably less than would be expected for the known composition of the film. It is possible that the polar end of the monoolein molecule assumes a different orientation when there is sucrose in the aqueous solution such that it extends further into the aqueous phase. If this occurred, it might act to decrease the area per molecule of monoolein in the film and thus tend to thicken the hydrocarbon chain region. Consistent with this, capacitance measurements (Table II) do indicate that monoolein/*n*-decane films are perhaps 0.2 nm thicker in sucrose solutions than in salt solutions, although this is not so for monoolein/squalene films. The latter films also differed in that their stability in sucrose solutions was quite poor.

Studies of the reflectance of egg phosphatidylcholine/*n*-decane films also reveal a difference in thickness depending on the aqueous solute; $d = 6.2$ nm when salts are used [6] and $d = 6.6$ nm when sucrose is used [13], but there is no difference in refractive index. No measurements of capacitance per unit area were made for these films in sucrose solutions.

The initially high reflectance of monoolein films containing either *n*-tetradecane or *n*-hexadecane (Fig. 3) may be due to reflection of light by microlenses of trapped solvent in these films. As the film ages, the reflectance decreases and visible lenses (>2 μm) are detected as they pass through the illuminated area of the film. It has been long suspected [10,14] that visible lenses may arise when microlenses coalesce with each other, and these experiments appear to support that interpretation. Since some microlenses probably coalesce with the Gibbs-Plateau border, it is not possible to determine the initial fraction of the film area covered by microlenses, but it is probably small since the capacitance per unit area shows no time dependence [10]. We have found no evidence for the presence of microlenses in monoolein films when either *n*-decane or squalene is used as the solvent.

The results presented in this paper differ somewhat from the published data on reflectance experiments on monoolein films. Pagano et al. [7] measured thicknesses of 6.2, 6.2 and 4.9 nm for films formed with *n*-decane, *n*-tetradecane and *n*-hexadecane, respectively. They noted that these values were significantly higher than the thicknesses expected on the basis of capacitance measurements and the assumption of 0.9 nm for the polar regions. They suggested that the discrepancy was due to the presence of microlenses in the films. If their measurements of reflectance were made on films which had not aged, then their interpretation is in agreement with our observations on monoolein/*n*-tetradecane and monoolein/*n*-hexadecane films.

The reflectance experiments of Bach and Miller [8] indicated that monoolein/squalene films in sucrose solutions had a thickness of 5.7 nm and a refractive index of 1.431, which is significantly different from our results ($d = 4.23$ nm, $n_\perp = 1.451$). We note, however. that there is considerable scatter in their data and that $d = 4.5$ nm and $n_\perp = 1.45$ appear to give an equally reasonable fit to their data. This is comparable to our results. If our measurements are correct, there is no need to postulate that monoolein/squalene bilayers have a structure which is not planar.

Reflectance measurements on lipid bilayers appear to be a viable technique for the calculation of bilayer thickness. When used in conjunction with capacitance measurements, they can be used to obtain further information about the structure of the film, in particular the thickness of the polar regions, the presence of microlenses and the planarity of the film. It is hoped that reflectance measurements may also provide information about the structure of lipid bilayers containing small polar or amphipathic molecules.

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