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Biochimica et Biophysica Acta 1195 (1994) 267–275

BBA Biochimica et Biophysica Acta

Assembly and molecular organization of self-assembled lipid bilayers on solid substrates monitored by surface plasmon resonance spectroscopy

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Received 28 January 1994

Abstract

The structural properties of lipid films, made from a squalene/butanol solution containing varying amounts (0–15 mg/ml) of egg phosphatidylcholine and deposited on a thin metallic silver layer, were investigated using surface plasmon resonance (SPR) spectroscopy. Optical parameters (thickness, refractive index and extinction coefficient) of such supported self-assembled lipid membranes were obtained from a theoretical analysis of the experimental SPR curves. The mass of the lipid membrane and the area and volume occupied by one lipid molecule were also calculated. The results were consistent with the formation of durable and homogeneous lipid bilayers on the solid substrate, and indicated similarities in structural properties between the present lipid bilayers and freely suspended and Langmuir-Blodgett bilayer membranes. Such bilayers represent a simple model for biological membranes, as well as providing a means of immobilizing proteins for various practical applications, including receptor-based sensors and molecular devices. The results confirm the value of the SPR technique for investigating the properties of thin biomolecular dielectric films deposited on a metal surface.

Keywords: Lipid membrane; Self-assembled lipid bilayer; Langmuir-Blodgett film; Vesicle; Surface plasmon resonance

1. Introduction

The present consensus view of biomembrane structure includes the concept of a lipid matrix (usually in the form of a bilayer) into which membrane proteins are embedded via hydrophobic and electrostatic interactions [1,2]. Although the detailed roles of the lipids in modulating the functional properties of these protein components is still largely unknown, the earlier static view of biomembranes based upon electron microscopy has now been replaced by a more dynamic picture [1,2]. In many cases the lipids and proteins are able to undergo rotational and lateral diffusion within the plane of the lipid matrix, and signaling via transduction processes takes place across the lipid bilayer. At present, there is a great deal of emphasis on obtaining a detailed understanding of biomembrane-protein structure/function relationships, especially with regard to integral membrane proteins [2].

A reconstituted freely suspended planar lipid bilayer membrane (BLM) separating two aqueous phases has proven to be an excellent model for biomembranes [3]. Although these model membranes are well suited for some types of experimental characterization (particularly electrical measurements), because of the smallness of the planar bilayer (typically less than 1 mm^2) and the generally low protein densities which can be reconstituted into them, they have the drawback that corroborative spectroscopic assays of biochemical activities and determination of membrane structural features cannot be carried out. However, several techniques have been developed to incorporate membrane proteins into lipid films at the interface between water and a non-aqueous phase [3–9]. Such systems offer the advantage of a larger geometry, which can be manipulated to allow a range of electrical, chemical, and structural measurements to be made on the same film. This extension of available experimental techniques makes these interfacial films well suited for investigating the coupling of

Abbreviations: SPR, surface plasmon resonance; BLM, bilayer lipid membrane; PC, phosphatidylcholine; L-B, Langmuir-Blodgett.

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SSDI 0005-2736(94)00197-9

structural and biochemical reactions to electrogenic events [3,9–14].

In addition to the biological sciences, many technological fields have recently become more concerned with organic interfaces. The characterization and engineering of assemblies of partially disordered and highly anisotropic supramolecular organic structures has considerable potential in optical and molecular electronics. This is especially true for the development of novel biosensors based on the incorporation of functional membrane-associated enzymes and receptors into devices which can use changes in film electrical (or photoelectrical) and optical properties to detect charge-transfer reactions or substrate binding, as well as for the development of useful interfacial properties (such as wettability, adhesion, tribology, and corrosion, to mention only a few). The techniques presently available for the construction of such systems include Langmuir-Blodgett (L-B) [15] and self-assembly [16] methods, by which ordered, monomolecular layers can be formed on solid surfaces. By incorporating specific functional groups into such systems, it is possible to design constructs with increasing complexity and multiple selectivities [3].

In previous work from this laboratory [17–21] involving electrochemical measurements with a series of redox proteins, we have demonstrated that a novel approach based on the formation of self-assembled BLMs deposited on solid substrates can effectively provide two important characteristics: (i) a biocompatible means of binding and immobilizing both peripheral and integral membrane proteins in such a way as to retain their functional properties; and (ii) generation of a thin (probably bimolecular in thickness, i.e., approx. 10 nm, with an arbitrarily large surface area), dynamic, and appropriately oriented system whose composition and properties can be systematically varied. We believe that understanding the complexity of the structural and dynamic interactions between lipid molecules, and between lipid and protein molecules, in such a model system is an important step towards understanding the molecular basis for the highly organized and complex functions of biomembranes.

In the present communication, we report the use of surface plasmon resonance (SPR) spectroscopy to investigate the process of lipid bilayer formation on a metallic solid surface, and to characterize such self-assembled structures, which in the present case are composed of egg phosphatidylcholine (PC) deposited on a thin silver film. Following the pioneering work of Abeles et al. [22,23], SPR has been used to study the optical properties of thin film coatings [24,25], metal-electrolyte properties [23,24], specific recognition reactions at self-assembled monolayers on gold [9,26–31] and very recently on optical immunosensors [32–34]. Our ultimate goal in this work is to develop the techniques for depositing and characterizing functional lipid bilayer-immobilized receptors and other integral membrane proteins on solid substrates, which will permit opto-electrochemical studies of the structure and dynamics of these systems as they relate to functional properties such as ligand–receptor interactions or energy transduction.

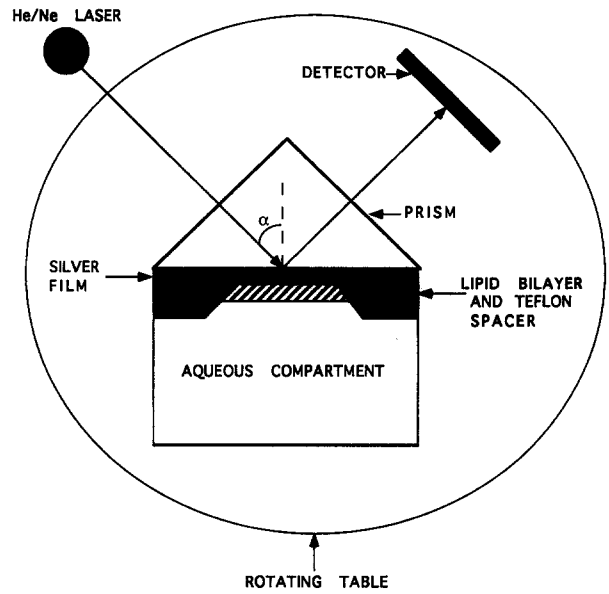

Fig. 1. Cross-section view from top of experimental geometry employed for surface plasmon resonance studies of self-assembled lipid bilayer membranes deposited on a metal surface.

2. Materials and methods

2.1. Lipid film formation and cell design

Lipid films were formed from a solution containing varying amounts of egg PC in squalene (Fluka)/butanol (0.5:10, v/v). SPR measurements were performed using a previously described cell [35], which also permits electrical and spectroelectrochemical measurements, and which can thus be used to observe both the formation of molecular assemblies at solid surfaces, and to investigate redox reactions and ligand–receptor interactions taking place at such interfaces. In the experiments reported below, the optically transparent indium oxide electrode used in our previous photoelectrochemical measurements [35] has been replaced with a right angle glass prism coated with a thin metallic silver film. The silver was vacuum-deposited onto the prism (BK-7 glass) with a deposition rate of 1 nm per second. Before deposition, the prisms were cleaned with reagent grade solvents and blown dry with nitrogen gas. Fig. 1 shows a schematic diagram of the cell, together with the experimental geometry employed for SPR studies of self-assembled lipid bilayer membranes.

A lipid membrane was formed on the metal surface, using a procedure described previously [35]. This method of membrane formation is in principle quite similar to that used to form freely suspended (conventional) lipid bilayer membranes separating two aqueous solutions [36–38]. It

involves spreading a small amount of lipid solution (≈ 4 μl) via a Hamilton microsyringe across an orifice in a Teflon sheet (4 mm in diameter) which separates a metal support (i.e., a silver film) from the aqueous phase (Fig. 1). Conceptually, the technique is based on interactions between a nascent metallic surface and amphipathic lipid molecules; the interaction forces can be described in terms of the metal/polymer interface theory (see Ref. [39] for a review). The surface of the metal, being highly hydrophilic, attracts the polar groups of the lipid molecules, thus forming an adsorbed lipid monolayer with the hydrocarbon chains oriented toward the bulk lipid solution phase. As our previous electrochemical results [17–21] demonstrated, the surface packing density of the ordered lipid molecules in this first lipid monolayer depends mainly on two factors, i.e., the lipid concentration in the membrane-forming solution and the nature of the lipid solvent. Subsequent to this first step of lipid membrane formation, the main body of the sample cell is filled with the appropriate aqueous solution. This initiates the second step, which involves a thinning process which probably is the same as in the formation of conventional freely suspended lipid bilayers (see Refs. [36–38] for reviews), i.e., gravitational forces in association with capillary action at the Teflon border causes excess lipid and solvent to move out of the aqueous phase. A Plateau-Gibbs border is provided by the lipid solution between the Teflon spacer and the metal surface. When the film approaches molecular dimensions in thickness, London-van der Waals forces accelerate the thinning process until these forces are opposed at equilibrium by the steric repulsion of the hydrocarbon chains. Also involved is the hydrophobic component of the lipid solvent, which fills both the space between the hydrocarbon chains, resulting in dilution of the final concentration of the lipid molecules in the monolayer, as well as the space between the two monolayers thereby increasing the thickness of the lipid film; see below for further discussion). It is important to emphasize that if the border conditions determined by a smooth-edged hole in the Teflon sheet are fulfilled, and a proper choice of lipids and solvent is made (see Ref. [37] for a review), lipid bilayer membrane formation will be a self-assembling process, as it is in the case of freely suspended bilayers.

2.2. Surface plasmon resonance measurements

A surface plasmon, described as a fluctuation of electron density propagating along a metal surface, can be generated either by electrons or by photons. When light passes from an incident medium under total reflection conditions, an evanescent electromagnetic field (usually on the order of 100 nm in depth) is created in the latter material. When a thin metallic film (thickness ≈ 50 nm) is imposed between the media, the electromagnetic field polarized parallel to the incident plane excites collective oscillations of the free electrons within the metal (i.e., surface plasmons). This generates the necessary electromagnetic field, which propagates along the interface between the metal and the emergent medium, with an amplitude that decays exponentially on both sides of that interface, a situation which can be described theoretically by Maxwell's equations [40,41]. A resonance condition exists for the excitation of surface plasmons which is fulfilled by varying the incidence angle (α; see Fig. 1) at a fixed excitation light wavelength. Resonance causes the energy of the incident light to be absorbed by the surface plasmon wave, and less light is thereby reflected by the interface between the higher incident medium and the metal film. At resonance the reflected energy reaches its minimum. Thus, the measurement of reflectance of the beam as a function of the incident angle produces the SPR curve (see Appendix and Refs. [40,41] for details). Electromagnetic theory (see Appendix and Refs. [40,41]) shows that the properties of the SPR curve depend on three optical parameters: the refractive index (n), the extinction coefficient (k), and the thickness (t) of the metal film and the dielectric medium in close contact with it. Therefore, the resonance effect, together with the fact that the electromagnetic field which excites surface plasmons penetrates the interface to the depth of only a fraction of the exciting light wavelength, makes this phenomenon exceedingly sensitive to the optical conditions at the metal surface. Any surface modification in the immediate vicinity of the metal, such as depositing a lipid bilayer will change the resonance condition by shifting the position of the minimum and altering the shape of the resonance curve.

In our experiment, the incident medium is a glass prism (with an index of refraction $n = 1.515$) upon which the layer of metallic silver is deposited. The emergent medium is water which is in contact with the outer metal surface. Deposition of a lipid bilayer membrane on the silver surface introduces a new interface, which will influence the resonance properties of the plasmon wave.

In order to evaluate the n, k and t parameters of an adsorbed lipid membrane using the SPR method one can either: (i) obtain SPR spectra excited at different wavelengths and angles of incidence and analyze them by solving Maxwell's equations [40–42]; or (ii) obtain an SPR spectrum excited at one wavelength and analyze it by fitting a theoretical resonance curve (Eq. (A-2) in the Appendix) to the experimental one, using non-linear least squares procedures which define a global minimum for the fitting error. In the present study, the second procedure has been followed, using optimization methods developed for SPR measurements on thin-solid film optics [43–45]. The layers were assumed to be isotropic and homogeneous compared to the wavelength of the exciting laser light (see below for discussion). The fitting procedure was followed for the bare silver film in contact with an aqueous medium (i.e., number of layers deposited on the glass prism, $r = 1$; see Eq. (A-2) in the Appendix), and the optical parameters thereby calculated were used to obtain the best fit to the SPR curves of the lipid bilayer-coated silver film (assum-

ing a one-dielectric layer system deposited on the silver film, i.e., total number of layers in the system, $r = 2$; see Eq. (A-2) in the Appendix).

In the work reported here, steady-state SPR measurements were performed using the attenuated total reflection technique, which involves rotation of a prism using a rotating table with a programmable controller (Model 855C, Newport), and a light detector (silicon solar cell) and a laser light source (helium-neon cw laser; wavelength 6328 Å) mounted on the fixed arm of a goniometer [45] (Fig. 1). This arrangement allows us to measure the incidence angle with an accuracy of 10^{-2} degrees. The photocurrent output from the light detector was digitized by a Heath-Zenith SD 4850 digital oscilloscope and the data were transferred to the hard disk of a computer. The optical arrangement described above allows the SPR spectrum to be measured over a 20 degree rotation with an acquisition time of 10 s. All measurements have been done at room temperature (23 ± 0.5°C).

There are three sources of experimental error which need to be considered in these experiments. First there is an error in the measurement of the incident angle ($\Delta\alpha$). As indicated above, $\Delta\alpha \approx 0.01$ degrees, which translates into thickness and refractive index errors of 0.5 Å and 0.005, respectively. Second, there is the error which is related to the precision of the theoretical fitting (standard deviation is between 0.010 and 0.035), which is relatively small. Probably the largest error in the absolute values of the parameters derives from variations in the lipid bilayer structure. This is reflected in the scattering of the experimental shifts and shape changes of the SPR spectra between different samples. Such scattering produced errors in thickness of $\Delta t = \pm 1$ Å, in refractive index of $\Delta n = \pm 0.02$, and in extinction coefficient of $\Delta k = \pm 0.02$.

3. Results and discussion

3.1. SPR spectra for egg PC deposited on silver films

Typical experimental and theoretical curves for a bare silver film in contact with an aqueous solution, as well as for silver-supported lipid layers generated with different PC concentrations in the lipid layer-forming solution, are shown in Fig. 2. As can clearly be seen in panels A–C, a lipid layer deposited on a silver film changes the SPR spectrum as follows: (i) the position of the resonance minimum is shifted towards larger incident angles; (ii) the

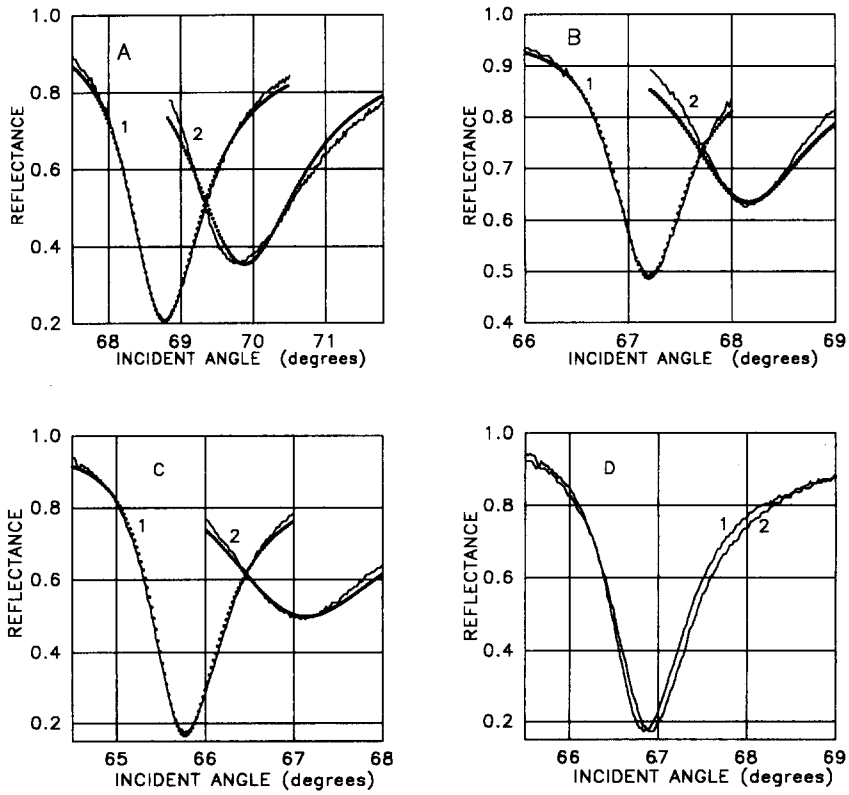

Fig. 2. Typical experimental (line) and theoretical (dots) SPR spectra of: (A) a 64 nm thick silver film, bare in curve 1, and in curve 2 coated with a lipid bilayer made from a solution containing 0.5 mg/ml egg PC in squalene/butanol (0.5:10, v/v). The fitting procedure yielded the following parameters for the lipid bilayer: $n = 1.446$, $k = 0.027$ and $t = 8.65$ nm; (B) a 67 nm thick silver film, bare in curve 1, and in curve 2 coated with a lipid bilayer made from a solution containing 2.5 mg/ml egg PC (with the fitting parameters: $n = 1.472$, $k = 0.043$ and $t = 7.59$ nm); (C) a 48 nm thick silver film, bare in curve 1, and in curve 2 coated with a lipid bilayer made from a solution containing 10 mg/ml egg PC (with the fitting parameters: $n = 1.637$, $k = 0.134$ and $t = 7.17$ nm); (D) a 53 nm thick silver film, bare in curve 1, and in curve 2 coated with the lipid solvent (squalene/butanol).

reflected light intensity at the resonance minimum is increased; and (iii) the SPR curve is broadened. The amplitudes of these changes are related to the PC concentration; this will be discussed further below. Fig. 2 also indicates the changes which occur in the bare silver film SPR curves as the thickness of the metal film varies. For comparison, Fig. 2 (panel D) shows the change in the silver film SPR spectrum after addition of the solvent (squalene/butanol, 0.5:10, v/v) used to dilute PC in the lipid layer-forming solution. Only very small changes are observed (primarily a small shift of the SPR spectrum without an alteration of shape) when lipid is absent, in comparison to the changes caused by the presence of PC (compare D with panels A–C). These observations clearly demonstrate that PC adsorbs on a silver film, and that this adsorption is readily detected by SPR spectroscopy.

There is another very important qualitative observation based on the results presented in Fig. 2 (panels A–C); i.e., for all measured PC concentrations only one resonance minimum is observed. This indicates that the coverage of the silver surface with PC molecules is homogeneous in comparison to the wavelength of the laser beam. In other words, even at the lowest PC concentration (Fig. 2, panel A), one does *not* have a situation in which patches of lipid are arranged in different types of structures, with the large 'empty' holes between them filled up with lipid solvent or water. On the contrary, the whole illuminated surface area of the silver film (which in our case is ≈ 4 mm in diameter) seems to function like a uniform layer without any measurable signs of discontinuity in surface coverage. Furthermore, if multiple layers were deposited on the silver film at high PC concentrations, one would also expect to observe a complex SPR spectrum (i.e., with more than one minimum shifted considerably from one another). Such spectra have not been obtained. It is also important to note that the changes in the resonance curve described above saturate at high PC concentrations; i.e., further increases in the lipid content of the solvent do not alter the SPR spectrum at all. Such results strongly indicate that there is only one type of lipid structure deposited on the metal film.

The optical parameters obtained as a result of the theoretical fits to the experimental curves for different PC concentrations, using the above-noted condition that the lipid membrane structure is homogeneous, are shown in Fig. 3 (panels A–C). The standard deviations in the fitting were very small in all cases (between 0.01 and 0.035), indicating, as discussed above (see Materials and methods), rather good accuracy in the optical parameter estimations. There are several interesting quantitative conclusions which can be drawn from these results:

(i) As noted above, the values of all three parameters saturate at a PC concentration in the lipid layer-forming solution of approximately 7.5 mg/ml.

(ii) The thickness of the lipid film reached at saturating PC (Fig. 3, panel A) is approximately 7.2 nm.

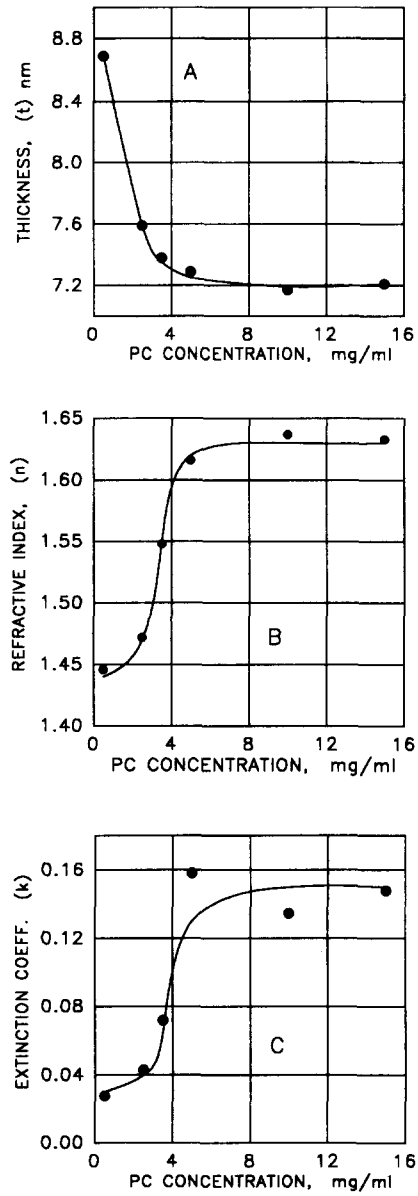

Fig. 3. Dependence on lipid concentration of the optical parameters obtained by fitting SPR spectra of egg PC bilayers deposited on a silver film. (A) Thickness (t); (B) refractive index (n); (C) extinction coefficient (k).

(iii) The refractive index parameter (see Fig. 3, panel B) reaches the value of 1.63 at PC saturation.

(iv) The quite high value of k obtained at saturation (Fig. 3, panel C) indicates some significant losses of light energy due to processes other than SPR itself. Such losses cannot be the result of absorption of light by the lipid membrane, because the long wavelength helium-neon laser (632.8 nm) is far away from the absorption bands of PC (which lie below 450 nm). The most likely possibility is scattering of light by the lipid structure, probably as a consequence of imperfections in the alignment of both the internal fatty acid chains and the lipid membrane surface. A similar effect has been observed in SPR studies of

Langmuir-Blodgett films of long chain fatty acid derivatives [46].

(v) The lipid membrane structure apparently begins to be formed at very low concentrations of PC in the lipid solutions, with values of n closer to that of the solvent itself ($n = 1.405$), relative to the n value obtained from a fully formed membrane at higher PC concentrations (see Fig. 3, panel A). Further, the value of t which is obtained at these low PC concentrations is larger than that for the fully formed lipid bilayer structure (see Fig. 3, panel B).

3.2. Structural characteristics of the lipid films

In order to characterize the structure of the deposited lipid membrane, it is helpful to calculate the mass of the lipid material forming the membrane, and the surface area and volume occupied by a lipid molecule, using the optical constants obtained from the SPR measurements. As described in the Appendix, in order to calculate the adsorbed lipid mass (m_l) when the membrane is formed from pure lipid material (i.e., without solvent), one can use a Lorentz-Lorenz relationship (Eq. (A-5)), in which the mass is expressed in terms of the molar mass (M_l), the molar refractivity (D_l), the thickness of the adsorbed layer (t), and the refractive index of the pure material (n). For a mixture of materials (in our case lipid plus the squalene/butanol solvent), one may use Eq. (A-8) from the Appendix, where the mass is related to the partial specific volume of the lipid molecules (v_l), and the refractive indices of the lipid and the solvent. Based on n values obtained for PC in freely suspended lipid bilayer membranes (between 1.56 and 1.66 [36,47]), we assume that the value for n obtained in the present experiments at saturating PC concentrations ($n = 1.63$ from Fig. 2, panel A) represents a pure PC membrane (i.e., with no appreciable solvent incorporated; note that for the solvent, $n = 1.405$). For all experimental situations in which n is smaller than the saturation value, we assume that the membrane contains both lipid and solvent. The calculations were performed assuming $M_l/D_l = 3.6$ [48] and $v_l = 0.9$ ml/g [48,49], and using the values of t and n obtained from the SPR experiment (see Fig. 3, panel A and B). From the calculated mass values and *assuming a bilayer structure* for the membrane, we can estimate average values for both the surface area per lipid molecule (A_l), and the volume of a single lipid molecule plus its associated water and excess solvent molecules (V_l). The results of such calculations are presented in Table 1.

In order to define a structural model for the solid-supported lipid membranes, the results in Table 1 have to be compared to the comparable parameters obtained for other known lipid membrane systems. There are two such membrane structures which need to be considered (see Ref. [50] for a review). The first of these, the planar bilayer lipid membrane, is formed either across a small pinhole orifice as a freely suspended film [36–38,50], or on solid supports

Table 1
The optical parameters (t and n) and the area (A_l) and volume (V_l) occupied by one lipid molecule calculated for different concentrations of PC in the lipid bilayer-forming solution

	[PC] in mg/ml					
	0.5	2.5	3.5	5.0	10.0	15.0
t (Å)	87	76	73	73	72	72
n	1.44	1.47	1.54	1.62	1.63	1.63
A_l (Å^2)	110	60	35	30	30	30
V_l (Å^3)	4500	2400	1300	1200	1200	1200

from Langmuir-Blodgett monolayers formed at the water/air interface [2,3,15,50–52]. The second type, the closed bilayer vesicle (liposome), is formed by swelling of a lipid in aqueous dispersions [49,50,53]. Both the L-B films and the aqueous dispersions have been widely studied using different physico-chemical methods, including neutron and X-ray diffraction, and NMR techniques [2,3,15,49–53]. On the other hand, the freely suspended bilayer membranes, being much more fragile, are less well characterized structurally [37].

One of the most important physical characteristics of lipid bilayer dispersions is the thermotropic phase transitions which occur between three different phases: (i) crystal, called L_C (or C) phase; (ii) gel, L_β (or G) phase; and (iii) fluid, L_α (or F) phase. The most direct physical characterization of a L-B monolayer film at the water/air interface is the surface pressure (defined as the film-induced change in surface tension) vs. surface area curve. The shapes of these curves are a function of several factors, including molecular structure, effective molecular diameter, and the packing of molecules in the film [2,3,51,52]; such curves provide an experimental definition of the physical state of the film. Thus, at a constant temperature in the range of the critical or melting temperature, by applying external pressure the phase of a lipid monolayer film can be changed from solid-condensed (SC) through liquid-condensed (LC), to liquid-expanded (LE) through liquid-condensed (LC) [2,3,51,52].

As Phillips and Chapman [54] have pointed out, a correlation exists between the lipid monolayer properties at the water/air interface and the properties of the multilamellar lipid bilayers formed in aqueous dispersions. Thus, the 'condensed monolayer' correlates with the 'crystalline' or 'gel' phase, while the 'expanded' state of the monolayer correlates with the 'fluid' or 'melted' state which occurs above the lipid transition temperature. It is important to note that similar thermotropic phase changes occur with both monolayers and lipid bilayers [2,3,51,52].

Although, as noted above, freely suspended lipid membranes are not as easily accessible to the variety of experimental techniques used to characterize aqueous dispersions and L-B films, there is still a large body of experimental data available concerning parameters such as thickness (measured by electrical capacitance), or packing of lipid

Table 2
Summary of literature parameters obtained with various phospholipid bilayer structures

	Bilayer dispersions [a]			L-B films [b]			Freely suspended BLM [c]
	L_C	L_β	L_α	SC	LC	LE	
t (Å)	59.5	64.0	67.0	–	–	–	60.0–90.0
n	–	–	–	–	–	–	1.56–1.66
A_1 (Å^2)	45.8	48.5	70.9	45.0	60.0	90.0	–
V_1 (Å^3)	1363	1552	2374	–	–	–	–

[a] Data from [49]: L_C, crystal phase; L_β, gel phase; L_α, fluid phase.
[b] Data from [3]: SC, solid-condensed phase; LC, liquid-condensed phase; LE, liquid-expanded phase.
[c] Data from [47].

molecules in the bilayer membrane (measured by electrical resistance). These two parameters describe the structural organization of such lipid membranes, and demonstrate that the presence of solvent within the membrane is the fundamental distinction between this and the other two types of membrane [36–38,55–57]. The trapped solvent increases the average thickness of the membrane (see Tables 1 and 2, and also Refs. [36–38,55–57]), and seems to play a role similar to compression and expansion by external pressure in the case of the L-B films, i.e., the solvent dilutes the lipid molecules, decreasing their packing within the membrane. This depends only on the type of solvent and lipid and the temperature, and is independent of the technique of bilayer formation [36,37,55–57].

Table 2 presents some of the literature parameters which describe the physical state of the abovementioned types of lipid membrane. In order to compare these with the present results, it is important to emphasize that the SPR technique 'sees' the deposited lipid membrane as a single material (which includes lipid hydrocarbon chains and polar groups, as well as molecules of associated water and excess solvent), and averages the values of the optical parameters. As can be seen from Tables 1 and 2, the values of both thickness and refractive index obtained with a self-assembled lipid membrane deposited on a silver film agree very well with those of freely suspended lipid bilayers. Although there are no reliable results in the literature with freely suspended membranes to compare with the present values of A_1 and V_1, there is reasonable agreement with A_1 values obtained for L-B films. We conclude from this that the dilution of the lipid membrane by the solvent produces a similar effect as does the expansion of a L-B film, changing the self-assembled lipid bilayer phase from SC at the highest lipid concentration to the LE phase at the lowest lipid concentration.

Although the fully formed silver-supported lipid bilayers show clear similarities with freely suspended lipid bilayers (i.e., similar values of n and t, as well as a comparable range of PC concentrations in the lipid membrane-forming solution from which fully developed bilayers can be formed), there are two very important differences between these two types of membranes:

(i) The supported lipid bilayers show an exceptional long-term stability. Once such a membrane is formed, no change in the optical properties could be detected over a period of 12 h.

(ii) A supported lipid bilayer membrane can be formed even from a solution containing a concentration of PC below the concentration required to generate a fully developed membrane. This cannot be accomplished with freely suspended bilayers, because at very low PC concentrations the lipid membrane becomes so fragile that is not stable for long periods. These differences clearly indicate a role of the solid support in controlling both the process of membrane formation and the mechanical properties of the lipid film, and demonstrate some additional similarities with the L-B films.

4. Conclusion

The above results definitively demonstrate that a simple method for forming self-assembled lipid membranes on large solid surfaces, which has been developed in this laboratory [17–21,35], generates durable and homogeneous lipid *bilayers*, with parameters similar to both freely suspended and L-B films which indicate the formation of different physical phases of the membrane produced from different membrane-forming solutions. These parameters can easily be varied by changing the lipid concentration, and probably the lipid composition as well, although this has not been studied in the present series of experiments. The composition and structural features of the membrane, as indicated by the optical parameters (thickness and refractive index) and the surface area and volume occupied by one lipid molecule, confirm the view of these self-assembled lipid membranes which has been developed as a consequence of our electrochemical studies with redox proteins and lipid-coated electrodes [17–21,35].

The lipid bilayer membrane is formed as a consequence of various driving forces. Creation of a highly hydrophilic solid surface which attracts the polar groups of the lipid molecules seems to be the first and critical requirement for successful formation of a bilayer membrane. As has been shown with hydrocarbon polymers, evaporated metal surfaces provide exceptionally good conditions for such hy-

drophilic interactions [39]. This first lipid monolayer adsorbed on the metal surface, together with an aqueous solution on the other side of the lipid layer and a Plateau-Gibbs border provided by the lipid solution between the Teflon spacer and the metal film, creates conditions similar to that observed with a freely suspended lipid bilayers leading eventually to a spontaneous phase transition to the lowest stable bilayer state [37].

The results presented in this report also confirm the value of the SPR technique in investigating the properties of supported lipid films. The measurements allow the determination of optical parameters (n and t), based on which one can calculate the mass of the lipid material involved in membrane formation, and then estimate other physical quantities (the area and volume per lipid molecule) which characterize the average structure of the lipid layer. Although only egg PC has been used in the experiments reported here, it is evident that the method can be used for any lipid structure and/or composition, as long as stable layers are formed on the metal surface. We are presently extending these measurements to include other lipids, as well as proteins which can be either bound to the bilayer surface or incorporated into the membrane interior.

Acknowledgements

This work was supported by a grant from the Vice-President for Research at the University of Arizona. The authors express their gratitude to Dr. T. Zhao for providing the silver coatings. Technical assistance of R. Schmidt in constructing the SPR apparatus is also appreciated.

Appendix

Lipid membrane structure determination by SPR spectroscopy

Surface plasmon resonance excited by totally reflected light (see Materials and methods) can be described in terms of the optical admittance, Y, which is defined as the ratio of the magnitudes of the magnetic and electric fields of the electromagnetic wave [40,41]. In general, the optical admittance of a surface containing a deposited multilayer is the ratio of the total fields for the surface and the multilayer:

$$Y = C/B \tag{A-1}$$

where

$$\begin{bmatrix} B \\ C \end{bmatrix} = \prod_{r=1}^{p} \begin{bmatrix} \cos\delta_r & \mathrm{i}(\sin\delta_r)/y_r \\ \mathrm{i}\, y_r \sin\delta_r & \cos\delta_r \end{bmatrix} \begin{bmatrix} 1 \\ y_{r+1} \end{bmatrix}$$

In this matrix equation, $\delta_r = 2(n - \mathrm{i}k)_r\, t_r(\cos\alpha_r)/\lambda$, $y_r = (n - \mathrm{i}k)_{\mathrm{ir}}/\cos\alpha_r$, n is the refractive index, t is the thickness, k is the extinction coefficient, α is the incident angle, and p is the number of thin films deposited on the incident medium (in our case, the medium for which $r = 0$ is a glass prism, whereas $(r + 1)$ is an aqueous solution; see Fig. 1). If y_0 is the admittance of the incident medium (i.e., the glass prism), then the reflectance, R, is given by:

$$R = (y_0 - Y)^2/(y_0 + Y)^2 \tag{A-2}$$

This equation, which describes the surface plasmon resonance phenomenon, has been used in this work to evaluate the optical parameters of the silver and lipid layers (see Materials and methods).

Calculation of lipid mass from the refractive index and thickness

The Lorentz-Lorenz relation for the refractive index n of a mixture of substances can be written as follows [58]:

$$(n^2 - 1)/(n^2 + 2) = D_1N_1 + D_2N_2 + D_3N_3 + \ldots\ldots \tag{A-3}$$

where D_j and N_j are the molar refractivity of substance j and the number of moles of substance j per unit volume, respectively. For a pure substance (in our case lipid), one may write:

$$\sigma_1^\circ = M_1N_1 = M_1/D_1(n^2 - 1)/(n^2 + 2) \tag{A-4}$$

where σ is the mass density per unit volume and M_1 is the molar mass. If one considers a solid-supported membrane of refractive index n and thickness t, then the mass of a pure substance (lipid) which forms the membrane can be calculated from the following equation:

$$m_1 = \sigma_1^\circ t = 0.1(M_1/D_1)t(n^2 - 1)/(n^2 + 2) \tag{A-5}$$

where t is expressed in nm and m_1 in $\mu g/cm^2$. For a mixture of lipid (l) and lipid solvent (s), Eq. (A-3) can be rewritten as follows:

$$\begin{aligned}(n^2 - 1)/(n^2 + 2) &= D_1N_1 + D_sN_s \\ &= D_1/M_1(\sigma_1) + D_s/M_s(\sigma_s)\end{aligned} \tag{A-6}$$

Assuming that we have an ideal mixture, the volume fraction of lipid is $(v_1)(\sigma_1)$, where v_1 is the partial specific volume of lipid at the temperature of the experiment, and $(v_1)(\sigma_1^\circ) = 1$, when σ_1° is the mass density of the pure lipid. The remaining volume fraction $(1 - v_1\sigma_1)$ has the mass density of pure solvent, i.e., σ_s°. Thus, the density of solvent in the mixture is $\sigma_s = \sigma_s^\circ(1 - v_1\sigma_1)$ and Eq. (A-6) can be rewritten as follows:

$$\begin{aligned}&(n^2 - 1)/(n^2 + 2) \\ &\quad = D_1/M_1(\sigma_1) + D_s/M_s(\sigma_s^\circ)(1 - v_1\sigma_1) \\ &\quad = D_1/M_1(\sigma_1)(n_s^2 - 1)/(n_s^2 + 2)(1 - v_1\sigma_1)\end{aligned} \tag{A-7}$$

where n_s is the refractive index of the pure solvent. Using Eq. (A-7), one can calculate the mass of lipid in $\mu g/cm^2$

in a self-assembled solid-supported lipid membrane formed from a mixture of lipid and solvent, as follows [48]:

$$m_l = \sigma_l t = 0.3(f(n))(n - n_s)/(D_l/M_l) - v_l(n^2 - 1)/(n^2 + 2) \quad \text{(A-8)}$$

where $f(n) = (n + n_s)/(n^2 + 2)(n_s^2 + 2)$. Thus, Eqs. (A-5) or (A-8) allow the estimation of the amount of lipid in a membrane formed either from pure lipid or from a lipid/solvent mixture, respectively, knowing the molar mass, the molar refractivity and the partial specific volume of the lipid material.

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