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Optical Anisotropy in Lipid Bilayer Membranes: Coupled Plasmon-Waveguide Resonance Measurements of Molecular Orientation, Polarizability, and Shape

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ABSTRACT The birefringence and linear dichroism of anisotropic thin films such as proteolipid membranes are related to molecular properties such as polarizability, shape, and orientation. Coupled plasmon-waveguide resonance (CPWR) spectroscopy is shown in the present work to provide a convenient means of evaluating these parameters in a single lipid bilayer. This is illustrated by using 1–10 mol % of an acyl chain chromophore-labeled phosphatidylcholine (PC) incorporated into a solid-supported PC bilayer deposited onto a hydrated silica surface. CPWR measurements were made of refractive index and extinction coefficient anisotropies with two exciting light wavelengths, one of which is absorbed by the chromophore and one of which is not. These results were used to calculate longitudinal and transverse molecular polarizabilities, the orientational order parameter and average angle between the longitudinal axis of the lipid molecule and the membrane normal, and the molecular shape factors of the lipid molecules. The values thereby obtained are in excellent agreement with parameters determined by other techniques, and provide a powerful tool for analyzing lipid-protein, protein-protein, and protein-ligand interactions in proteolipid films.

INTRODUCTION

The complexity of biological membranes derives in large part from their heterogeneity in structure and composition, which places mixtures of various lipids and integral membrane proteins in irregularly shaped microscopic regions with differing dielectric properties in close proximity to each other, in a mosaic of fluid and solid domains. Additional complications are introduced by the fact that, during the functional activity of membrane-associated proteins, the structural organization of the proteolipid membrane undergoes significant changes (Brown, 1993; Dowhan, 1997). As a consequence of this, biochemical and biophysical studies have mainly focused on characterizing proteins and lipids as separate entities, with less emphasis being placed upon structural and functional investigations of proteolipid assemblies. Although this has led to impressive progress in high-resolution x-ray crystallography of integral membrane proteins and in medium-resolution structural descriptions of both the gel and the liquid-crystal phases of phospholipid bilayers (Shipley, 2000; Nagle and Tristram-Nagle, 2000), we still have only a superficial understanding of the interplay between lipid structure and dynamics and protein function in biological membranes (Bloom et al., 1991; Mouritsen and Kinnunen, 1996; Dowhan, 1997). Consequently, there is a need for both new theoretical models and novel experimental tools that will allow the characterization of such complex anisotropic structures.

One such recently developed experimental methodology is coupled plasmon-waveguide resonance (CPWR) spectroscopy, which, combined with techniques for forming solid-supported proteolipid membranes, allows kinetic, thermodynamic, and structural characterization of anisotropic thin films (Salamon et al., 1997a, 1999; Salamon and Tollin, 1999a). This technology possesses several important advantages that make it especially well suited for studies of proteolipid membranes. First, it is based on the measurement of reflected light intensity, using a simple optical detection system whose geometrical arrangement permits a complete isolation of the optical probe from the system under investigation. Second, spectral analysis yields a full set of thin film optical parameters, i.e., refractive index n and extinction coefficient k as well as the film thickness t. Furthermore, measurements can be performed with exciting light polarized either parallel or perpendicular to the plane of the membrane, thereby allowing the characterization of systems that are both birefringent and dichroic. Third, the methodology involves the use of evanescent waves (surface-bound electromagnetic waves) that are generated under resonance conditions, which provides an exceptionally high sensitivity.

As a consequence of these properties, CPWR spectroscopy is able to characterize lipid-protein and protein-protein interactions occurring either within or at the surface of single anisotropic proteolipid membranes, as well as structural changes resulting from such interactions (Salamon et al., 1999). Recent examples of the application of this technique include the incorporation into lipid bilayers of proteins such as cytochrome b_6f (Salamon et al., 1998), phosphatidylserine synthase (Salamon et al., 2000b), and the human δ-opioid receptor (Salamon et al., 2000a) and the characterization of subsequent protein-protein and receptor-ligand interactions.

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All of the previous CPWR studies have been performed using plasmon excitation wavelengths outside of the absorption bands of the membrane components, which results in the loss of information about the extinction coefficient parameter. Thus, the analysis of such spectra can yield values only for t and the refractive indices n_p and n_s. Although this allows monitoring of changes in packing density of the proteolipid membrane and of structural features of the membrane such as surface area per molecule and overall molecular orientation (described by the anisotropy of n), such experiments can be even more informative when they are performed within absorption bands of individual membrane components. As will be shown below, the sensitivity of the CPWR method is sufficient to measure the optical absorption anisotropy of a single bilayer membrane containing only a few mole percent of chromophore-labeled molecules (lipid in the present case). Thus, by using the appropriate labels and exciting wavelengths, this capability can be used to follow structural changes, monitored by optical dichroism, occurring in either lipid or protein molecules during lipid-protein or protein-ligand interactions, and thus to distinguish between changes occurring in these two phases. Our results further show that CPWR spectroscopy can yield additional structural information, namely, molecular polarizability and molecular shape within ordered thin films, which are otherwise difficult to determine. This provides another tool for analyzing lipid-protein, protein-protein, and protein-ligand interactions, in which these two molecular quantities are often altered.

MATERIALS AND METHODS

Principles of optical anisotropy as applied to proteolipid membranes

Nature of optical anisotropy

The refractive index is one of the optical parameters describing the response of matter to an electromagnetic field. This is a macroscopic quantity and is related to the properties of individual molecules through the molecular polarizability tensor as well as to the environment in which these molecules are located (e.g., packing density and organization; for details see below) (De Jeu, 1978). Environmental properties are especially important when molecules are located in a matrix that has a nonrandom organization and thus induces long-range spatial molecular order. In the case of biological membranes, such molecular ordering creates an optically anisotropic system with a uniaxial optical axis having two (different) principal refractive indices, n_e (or $n_\parallel$) and n_o (or $n_\perp$) (De Jeu, 1978). The first of these indices is observed for an extraordinary ray, associated with a light wave in which the electric vector is polarized parallel to the optical axis. The ordinary index (n_o or $n_\perp$) is observed for linearly polarized light in which the electric vector is perpendicular to the optical axis. The difference between the values of these two refractive indices results in so-called form birefringence:

$$\Delta n_f = n_\parallel - n_\perp \tag{1a}$$

In general, the observed birefringence is considered to be the sum of an intrinsic birefringence (Δn_i) of the molecule itself (resulting from the asymmetry in the molecular polarizability tensor) and the form birefringence:

$$\Delta n = \Delta n_i + \Delta n_f \tag{1b}$$

It is important in this context to emphasize that the form birefringence also includes a contribution from molecular shape, either due to individual molecules or as modified by the formation of molecular aggregates, and is described by the ratio of the main axes of the molecule or aggregate. In principal, these two types of birefringence can be experimentally separated, and therefore molecular and environmental factors can be sorted out (Bragg and Pippard, 1953). This is the fundamental basis upon which measurements of refractive indices with polarized light can lead to the evaluation of the structural parameters of anisotropic systems. This can be an especially fruitful approach in studies of lipid membranes interacting with integral membrane protein molecules. As will be discussed more fully below, the ability of CPWR spectroscopy to evaluate both the n and k parameters provides a further advantage in elucidating structural parameters for systems that are both birefringent and dichroic.

Relationship of the anisotropic refractive indices to the molecular polarizability tensor and the molecular shape factor

To correlate this macroscopic parameter to molecular properties, including the influence of the environment in which the molecule resides, one has to evaluate the electric field that is induced at the molecule of interest (i.e., the local field, E^{local}) by an averaged macroscopic electric field (E), which accompanies light propagation in a dielectric environment. Such a local field is, in general, a sum of the averaged macroscopic field and an additional component that is produced by the molecule itself. The relationship between the macroscopic and local fields (E_i and E_i^{local}) in a dielectric environment can be written as follows (Böttcher, 1952; den Engelsen, 1976):

$$E_i^{\mathrm{local}} = E_i + L_i P_i, \tag{2a}$$

where $i = x, y$, or z and P_i is the polarization per unit volume. L_i is a form factor (molecular shape in this specific example) that describes the influence of asymmetry in the shape of the molecule on the creation of the local electric field. In the case of isotropic media, $L_x = L_y = L_z = 4\pi/3$, and Eq. 2a becomes the well-known Lorentz formula for the local field. The polarization is related to the dielectric properties of the environment in which the molecule is placed and the averaged electric field E_i by

$$P_i = (\epsilon_i - 1)E_i/4\pi, \tag{2b}$$

where ϵ_i is the dielectric constant along the i axis and is related to the molecular properties by

$$P_i = \alpha_i E_i^{\mathrm{local}}/V, \tag{2c}$$

where α_i is the ii-component of the molecular polarizability tensor whose principal axes are assumed to coincide with x, y, and z, and V is the molecular volume. For a dielectric environment that does not absorb light energy, i.e., for which the extinction coefficient $k = 0$, $\epsilon_i = n_i^2$ where n_i is the refractive index.

Combining Eqs. 2a, 2b, and 2c results in

$$n_i^2 - 1 = 4\pi\alpha_i/(V - \alpha_i L_i). \tag{3}$$

Eq. 3 links the anisotropic refractive indices to the components of the molecular polarizability tensor and the shape factors. From this equation it is clear that optical anisotropy can be due either to the anisotropy of the molecular polarizability tensor or to the asymmetry of the shape factors or to both. Eq. 3 therefore allows one either to evaluate the effect of changing environmental conditions and state of aggregation on the anisotropy of the refractive index if the molecular polarizability tensor is known or to calculate the molecular polarizability from the anisotropy of the refractive index and the shape factors. The latter calculation will be done in the present work.

There are two fundamentally different methods of calculating the shape factors L_i, which as indicated by Eq. 2a affect the local electric field (den Engelsen, 1976). The first approach is based on the assumption that the field at the reference molecule results from both the applied field and the induced-dipole field of the other molecules in the sample. The second approach is essentially based on a calculation of the cavity field in a continuous dielectric (Onsager, 1936). As has been demonstrated by den Engelsen (1976), good agreement between experimental and theoretical values of refractive indices can be obtained using a modification (by Lorentz) of Onsager's theory, assuming that the hydrophobic lipid membrane interior constitutes a continuous, nonpolar medium. We will employ the same approach in this work.

The Lorentz calculation (Böttcher, 1952) gives an expression for the cavity field caused by the apparent surface charges on the wall of the cavity. In the present context, the long acyl chains of lipid molecules can be represented as cylindrical cavities (Fig. 1). If the optical axis of the uniaxially anisotropic medium coincides with the cylinder axis and the electric field E is applied in the i-direction, either parallel to the cylinder axis or normal to it, the shape factors L_i (Eq. 3) can be written as

$$L_z = 4\pi[1 - l/(d^2 + l^2)^{1/2}] \tag{4}$$

and

$$L_x = L_y = 2\pi l/(d^2 + l^2)^{1/2}, \tag{5}$$

where l represents the average length of the acyl chains and d is the diameter of the cylinder (see Fig. 1; den Engelsen, 1976).

Eq. 3 describes an ideal case when there is no spatial distribution of molecular orientation and the principal axes of the molecular polarizability tensor coincide with the x, y, and z axes. In the more practical case when there is a spatial distribution of molecular orientation, one should replace α_i in Eq. 3 with an average value of the polarizability, α_i^{av}, and L_i with an average value of the shape factor, L_i^{av}. Furthermore, if $\alpha_x = \alpha_y = \alpha_t$, and $\alpha_z = \alpha_l$ (which is the case for rod-like molecules such as lipids; see Fig. 1), then

$$\alpha_z^{av} = \alpha^{av} + 2/3(\alpha_l - \alpha_t)S \tag{6}$$

$$\alpha_x^{av} = \alpha^{av} - 1/3(\alpha_l - \alpha_t)S, \tag{7}$$

where

$$\alpha^{av} = 1/3(\alpha_l + 2\alpha_t) \tag{8}$$

and α_l and α_t are the longitudinal and transverse polarizabilities of the rod-like molecule. The average value of the shape factor presented in Eqs. 4 and 5 can be obtained by projecting the long axis of the molecule onto the z axis (i.e., the optical axis; see Fig. 1), and by projecting the cross section of the cylinder onto the membrane plane, i.e., the x-y plane in Fig. 1. This will result in the replacement of the l and d parameters in Eqs. 4 and 5 with l' and d', which have the following form:

$$l' = l\cos\theta \tag{9}$$

$$d'^2 = d^2/\cos\theta, \tag{10}$$

where θ is the angle that the long axis of the molecule makes with the optical axis of an anisotropic system, i.e., the z axis in Fig. 1. In principle, this angle represents an average value that can be obtained from the angular distribution of the long axes of the molecules. In practice, this average value can be very well approximated by the degree of orientational order (S), which is defined as follows (Saupe, 1964):

$$S = 1/2(3\langle\cos^2\theta\rangle - 1), \tag{11}$$

where the brackets ($\langle$ and $\rangle$) denote an average over all abundant orientations. As will be discussed below, the experimental conditions employed in this work allow us to safely use such an approximation. Replacing l and d in Eqs. 4 and 5 by Eqs. 9 and 10, and knowing the value of the S parameter, one can evaluate the average values of the shape factors. Furthermore, these values can then be used in Eq. 3, which also has to be modified by substituting individual polarizabilities (e.g., α_i) with their averaged values (i.e., α_i^{av}), using Eqs. 6 and 7. This will result in the following modified equations:

$$(n_z^2 - 1)/4\pi = \alpha_z^{av}/(V - \alpha_z^{av}L_z^{av}) \tag{12}$$

and

$$(n_x^2 - 1)/4\pi = \alpha_x^{av}/(V - \alpha_x^{av}L_x^{av}), \tag{13}$$

from which one can evaluate the molecular polarizabilities along the principal axes of the molecule (α_l and α_t), knowing the value of the degree of orientational order (S) and the experimental values of the refractive index obtained with exciting light both parallel (s-polarized component; yielding n_x, or n_s) and perpendicular (p-polarized component; yielding n_z, or n_p) to the membrane plane. In the present work, the value of d' has been obtained from the surface area occupied by one molecule, which has been calculated using the experimentally determined average value of the refractive index, and l' has been calculated assuming the average length of the acyl chains to be equal to 19 Å.

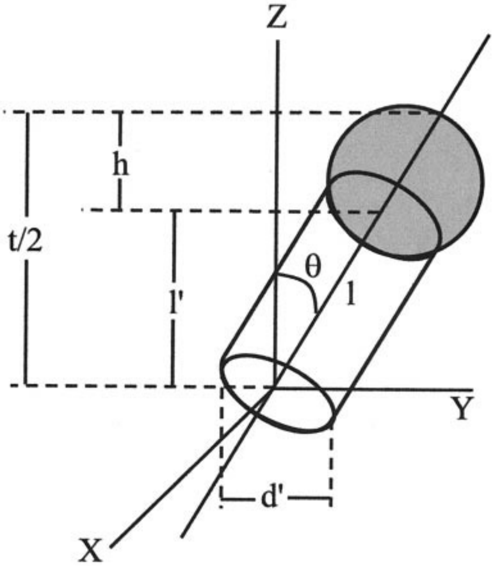

FIGURE 1 Geometry used in the calculation of the orientational angle (θ) for an idealized cylindrical shape of the two hydrophobic chains of a lipid molecule of length l and a cross-section diameter d placed within a planar lipid membrane (parallel to the x-y plane). The value of $t/2$ is equal to ($l' + h$), where h and l' represent the average dimensions of the headgroup and the length of the acyl chains, respectively, projected onto the z axis (see Fig. 2).

Correlation between the degree of orientational order and linear dichroism

Let us consider a uniaxially anisotropic medium with its optical axis along the z axis (Fig. 1), which contains guest chromophore groups having a well-defined orientation angle of the absorbing dipoles (transition moments). The anisotropy in the spatial distribution of the absorption transition moments is defined either by the linear dichroism (Norden et al., 1977):

$$D = k_{\|} - k_{\perp} \tag{14a}$$

or by the anisotropy of the absorption (extinction) coefficient:

$$A_{\mathrm{k}} = (k_{\|} - k_{\perp})/k^{\mathrm{av}}, \tag{14b}$$

where $k_{\|} = k_z$ represents the absorption of light polarized parallel to the optical axis, $k_{\perp} = k_x = k_y$ denotes the absorption obtained with light polarized perpendicular to the optical axis, and $k^{\mathrm{av}} = 1/3(k_{\|} + 2k_{\perp})$. If, in addition, the absorption transition moments are parallel to the long axis of molecule (see Fig. 1), then Eqs. 14a and 14b can be rewritten in the following form (Norden et al., 1977):

$$D = 3k^{\mathrm{av}}[1/2(3\langle\cos^2\theta\rangle - 1)] \tag{15a}$$

$$A_{\mathrm{k}} = 3/2(3\langle\cos^2\theta\rangle - 1) \tag{15b}$$

Taking into account the definition of the degree of orientational order (Eq. 11), one can obtain from Eqs. 14a and 14b the following relationships:

$$S = D/3k^{\mathrm{av}} \tag{16a}$$

$$S = A_{\mathrm{k}}/3 \tag{16b}$$

The above relationships between either linear dichroism or absorption anisotropy and the degree of orientational order will be used in these experiments as a measure of the order of the whole ensemble of molecules. Such an approach, i.e., using chromophore-labeled molecules as guests to derive the orientation of the host molecules, has been used in numerous experimental designs. In particular, this procedure applies well in this work where the chromophore group has been covalently attached to one of the two fatty acid chains of the lipid molecule and therefore represents the average fatty acid chain orientation.

CPWR spectroscopy

The concept of surface plasmons originates from the plasma formulation of Maxwell's theory, where the free electrons of a metal are treated as a high-density liquid. Plasma oscillations in metals are collective longitudinal excitations of the conductive electron gas, and plasmons are the quanta corresponding to these charge-density oscillations. Such oscillations can be localized on an interface between metallic and dielectric surfaces, along which they propagate as electromagnetic waves with the electric field normal to this interface and decay exponentially with penetration distance from the interface. These properties are the same as those describing the guided surface waves (also known as evanescent waves) generated optically under total internal reflection conditions.

From Maxwell's equations it can be shown that surface plasmon electromagnetic waves occur only when the following conditions are fulfilled. First, one of the adjacent media (i.e., the surface-active medium that generates the plasmon waves) has a negative value for the real part of its complex dielectric constant. Second, a component of the wave vector along the interface between these two media satisfies an equation that involves the dielectric constants of both media. These constraints require that surface plasmons can be excited only with certain materials (gold and silver are the best examples) and only by evanescent waves (i.e., they cannot be excited directly by incident light) when the wave vector of the evanescent wave is in resonance with that of the surface plasmon wave. Furthermore, plasmon excitation can be achieved only by an evanescent wave generated by transverse magnetic (TM-) or *p*-polarized incident light (for details see Salamon and Tollin, 1999a,b; Salamon et al., 1999). However, when the metal film is covered with a thin layer of dielectric that supports waveguide modes, there is the possibility of coupling the incident light into both the plasmon mode of the metal film and the guided mode of the dielectric overlayer. This allows resonance excitation using transverse electric (TE-) or *s*-polarized light, because exciting the guided mode reverses the phase shift of the light and the metal sees it as if it were a *p*-polarized excitation. By precisely adjusting the thickness and refractive index of the dielectric film, the guided mode conditions and surface plasmon conditions can be made coincident for both polarizations at two different incident angles, resulting in two very sharp resonances. The idea of coupling the *s*-polarized incident light to plasmons has been previously described by Macleod (1986, 1992) and was later developed into the present version of CPWR spectroscopy (Salamon et al., 1997a).

The experimental arrangement for CPWR measurements is shown in Fig. 2. The details of the procedures and methods of data analysis have been described elsewhere (Salamon et al., 1997a,b, 1999; Salamon and Tollin, 1999a,b). Here we will provide a brief summary. The CPWR technique combines advantages of both plasmon resonance and waveguide spectroscopies. Such a combination uses a simple light detection system that involves the measurement of the intensity of totally reflected *p*- and *s*-polarized light and that is characterized by a high sensitivity due to the resonance phenomenon upon which the measurement is based. These properties, taken together with a proper analysis of resonance spectra, allow the evaluation of the *n*, *k*, and *t* parameters of anisotropic proteolipid membranes (Salamon et al., 1999, 2000a,b; Salamon and Tollin, 1999a,b).

CPWR spectra can be described by three parameters: the light incident angle α (or light wavelength λ), the spectral width, and the resonance depth, which, in turn, depend on the *n*, *k*, and *t* values of the sample. Thin-film electromagnetic theory based on Maxwell's equations provides an analytical relationship between the spectral parameters and the sample

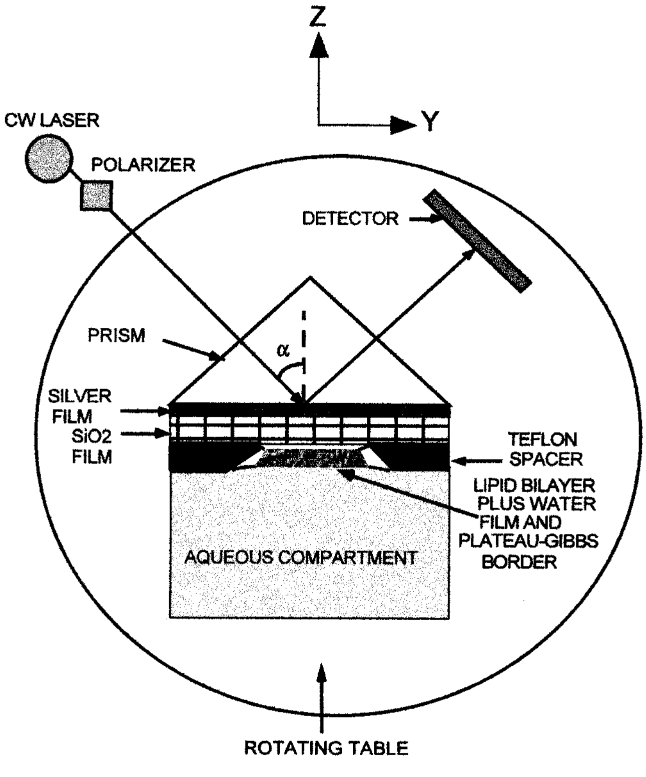

FIGURE 2 Side view of a CPWR experimental device containing a glass prism coated with a silver layer overcoated with a SiO_2 film. Also shown are a deposited lipid bilayer held in place by a Teflon spacer and the excitation and measuring systems.

properties (Salamon et al., 1997b), where the sample is modeled as a uniform layer (or layers) of anisotropic dielectric material. This allows a unique evaluation of these parameters by fitting theoretical curves to the experimental ones.

Preparation of lipid membranes

In the present study, self-assembled solid-supported lipid membranes have been used (Salamon and Tollin, 1991, 1996). The method of preparation uses the principles that govern the spontaneous formation of a freely suspended lipid bilayer membrane (a so-called black lipid membrane) (Mueller et al., 1962). This involves spreading of a small amount of lipid solution across an orifice in a Teflon sheet that separates the thin dielectric film (SiO_2) on the surface of the plasmon generator from the aqueous phase. The hydrophilic surface of hydrated SiO_2 (Gee et al., 1990; Silberzan et al., 1991) attracts the polar groups of the lipid molecules, thus forming a lipid monolayer deposited on a layer of adsorbed water, with the hydrocarbon chains oriented toward the droplet of excess lipid. Filling the main body of the CPWR cell with the appropriate aqueous solution initiates the second step, which involves a thinning process, i.e., formation of both the second monolayer and plateau-Gibbs border that anchors the bilayer film to the Teflon spacer, allowing the excess of lipid and solvent to move out of the orifice (Salamon et al., 1999). In the present experiments, the lipid films were formed on the SiO_2 surface from the following membrane-forming solution: 8 mg/ml 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in squalene/butanol (0.1:10, v/v). Varying amounts of POPC (1–10 mol %) with a chromophore group attached to one of its hydrocarbon chains were incorporated into this solution.

Materials

The chromophore used in these experiments is a fatty acid analog in which a dipyrrometheneboron difluoride (BODIPY) chromophore forms a segment of the fatty acyl methylene chain. This nonpolar substituent has a strong first absorption band in the green region of the visible spectrum (535 nm). The absorption is due to a single electronic transition lying within the plane of the chromophoric group and is used in this work as a probe of acyl chain orientation within a planar lipid membrane. BODIPY-labeled phosphatidylcholine was purchased from Molecular Probes (Eugene, OR). POPC was purchased from Avanti Polar Lipids (Birmingham, AL).

RESULTS AND DISCUSSION

Evaluation of spectral parameters

Fig. 3 shows a representative example of CPWR spectra obtained with a lipid membrane containing 10 mol % of acyl chain BODIPY- labeled lipid, using either red (λ_r = 632.8 nm; *A* and *B*) or green (λ_g = 543.5 nm; *C* and *D*) exciting light and employing both *p*- (*A* and *C*) and *s*-

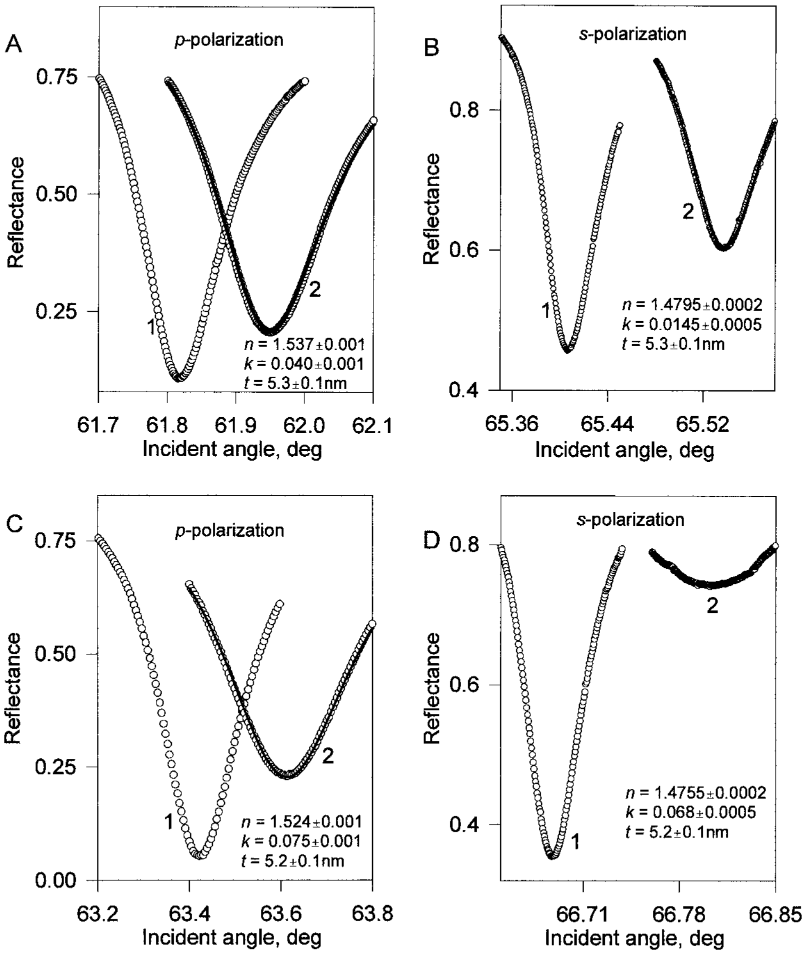

FIGURE 3 CPWR spectra obtained with bare SiO_2 (*curve 1*) and with a self-assembled solid-supported lipid membrane (*curve 2*), formed on the SiO_2 surface from a solution of 8 mg/ml POPC containing 10 mol % BODIPY-PC in squalene/butanol (0.1:10, v/v). The spectra were measured with either 632.8 nm (red; *A* and *B*) or 543.5 nm (green; *C* and *D*) laser exciting light, using both *p*-polarization (*A* and *C*) and *s*-polarization (*B* and *D*). Solid lines represent theoretical fits to the experimental data (○). The buffer was 10 mM Tris-HCl, pH 7.3.

polarized excitation (*B* and *D*). As indicated in the preceding section, the chromophore absorbs light in the green spectral range (with maximum absorption around 535 nm), which coincides with λ_g. On the other hand, the red excitation light (λ_r) is not absorbed by the chromophore and therefore can be used as a baseline to calculate the correct extinction coefficient for the green light. This use of multiwavelength excitation of CPWR spectra provides another advantage by allowing a global fitting of the theoretical to experimental resonance curves, which is employed in CPWR spectroscopy (Salamon et al., 1997b, 1999; Salamon and Tollin, 1999a,b) to obtain the optical parameters, to be performed in a much more efficient and precise way. As a result, the fits are very good, as demonstrated by the examples shown in Fig. 3. The *n* and *t* values generated with such fits (see Fig. 3 and Table 1) are very similar for both red and green excitations, whereas the extinction coefficient *k* is much higher for green than for red light. This is as expected for an absorption process that occurs only with green light excitation.

The *k* values obtained from the fitting procedure with *p*-polarized ($\Delta k_{p(g)} = [k_{p(g)} - k_{p(r)}] = 0.035 \pm 0.001$) and *s*-polarized light ($\Delta k_{s(g)} = [k_{s(g)} - k_{s(r)}] = 0.0535 \pm 0.0005$) have to be corrected for the differences in signal amplitude for a given *k* value of CPWR measurements with *p*- and *s*-polarized components (Salamon et al., 1999; Salamon and Tollin, 1999a,b). These result from the different coupling of *p*- and *s*-polarized plasmon resonances to the waveguide modes within the SiO_2 layer, which create a different pattern of optical propagation of the two evanescent waves within the SiO_2 waveguide system. As a consequence, the two polarizations produce much different electromagnetic field amplifications, resulting in resonances occurring at different angular positions and characterized by different optical properties (see Fig. 3). In the context of absorption (extinction) measurements it is important to note that the different patterns of electromagnetic wave propagation for each polarization within the dielectric layer results in different values for the numbers of internal reflections per unit surface area of the waveguide. Although this property of the CPWR device does not influence the calculation of the refractive index value (because this parameter does not depend on the optical pathway), it is important in evaluation of the absorption coefficient (because this parameter is directly proportional to the length of the optical pathway). Therefore, a calibration of the device is necessary to correct for this effect. In this work, we have used calculations of the amplitudes of the electric field within the SiO_2 waveguide system as a measure of such a calibration. Comparison of the electric field intensity produced by either *p*- or *s*-polarized light for the CPWR device used in this work indicates that the latter polarization produces an electric field that is 5.0 ± 0.1 times larger than the *p*-component (Salamon and Tollin, 1999a,b). Therefore, to normalize the extinction values, we have divided the *s*-component value by a factor of 5, which yields $\Delta k_{s(g,N)} = 0.0107 \pm 0.0001$. Note also that the values of n_p obtained either with red or green light are very similar and significantly larger than those of n_s (Table 1). Inasmuch as the optical parameters obtained with *p*-polarization and *s*-polarization refer to the perpendicular and parallel directions with respect to the bilayer membrane surface, respectively (Salamon et al., 1997a), the *k* parameters measure the dichroism of the lipid membrane and the *n* parameters the birefringence of the membrane. These properties are a result of the ordering of the long axes of the lipid molecules along the normal to the membrane plane.

Lipid membrane structure

The most important structural parameters of a lipid membrane are the thickness, the average surface area per lipid molecule (or molecular cross-sectional area), and the degree of long-range molecular order. In previous work, these parameters have been evaluated by conventional structural techniques such as x-ray diffraction and NMR (for review see Nagle and Tristram-Nagle, 2000) and have been used to compare bilayers and to validate quantitative theory and membrane structural simulations. As described above, CPWR spectroscopy allows all three of these parameters to be characterized. The film thickness *t* can be obtained directly from the fitting of the CPWR resonance spectrum. Inasmuch as this quantity represents an average value for an interfacial region characterized by a refractive index different from that of the bulk solution, for a deposited lipid membrane this will include both hydrocarbon and headgroup regions, together with water molecules located at both the silica surface and bound to the lipid membrane. A value for the surface area per molecule can be obtained from the

TABLE 1 Average values of the refractive index (*n*) and thickness of a self-assembled solid-supported POPC bilayer containing small amounts of a BODIPY-labeled PC, obtained from CPWR spectra excited either by *p*- or *s*-polarized light

Labeled lipid (mol %)	n_p		n_s		t (nm)	
	$\lambda_r \pm 0.001$	$\lambda_g \pm 0.001$	$\lambda_r \pm 0.0005$	$\lambda_g \pm 0.0005$	$\lambda_r \pm 0.1$	$\lambda_g \pm 0.1$
0	1.526	1.522	1.4704	1.4652	5.2	5.2
1.5	1.526	1.521	1.4700	1.4650	5.2	5.2
4.0	1.525	1.523	1.4702	1.4662	5.2	5.2

mass m deposited per unit surface area, calculated using the average value of the refractive index n_{av} (Cuypers et al., 1983; Salamon et al., 1999):

$$m = 0.1(M/R)t(n_{av}^2 - 1)/(n_{av}^2 + 2), \tag{17}$$

where $n_{av}^2 = 1/3(n_p^2 + 2n_s^2)$, M is the molecular weight, R is the molecular refractivity of the material, and mass is expressed in μg/cm^2. The refractive indices n_p and n_s are obtained directly from the fitting of the resonance spectra as described above (see Table 1). The average values of these two structural parameters (i.e., hydrophobic chain length plus the length of the hydrated polar groups, and surface area per lipid molecule) obtained with the bilayers used in this work are 26 Å per lipid monolayer (i.e., 52/2; see Fig. 1 and Table 1) and 50 Å^2, respectively.

To compare these results with those obtained using conventional methodologies, it is important to emphasize that there is still a large uncertainty in structural characterizations of lipid membranes. Although recent applications of new theoretical approaches (see Shipley, 2000) have allowed medium-resolution structural descriptions of both the gel and the liquid-crystal phases of membrane phospholipid bilayers, the uncertainty in these parameters for the benchmark lipid dipalmitoylphosphatidylcholine (DPPC) is still at the 100% level (Nagle and Tristram-Nagle, 2000), as it was ~30 years ago (Chapman et al., 1967; Engelman, 1971; Buldt et al., 1978). This degree of uncertainty not only makes it difficult to compare bilayers of different lipids but also presents even more problems in comparing different kind of bilayers. Whereas x-ray and NMR structural measurements have been made with lipid suspensions in water, the results presented in this paper have been obtained with a single solid-supported lipid bilayer, created in the same manner as so-called black (or freely suspended) bilayers. The major difference between these two types of membranes results from two factors: 1) the black lipid membranes contain long-chain aliphatic solvent molecules (in our case squalene) and 2) the deposition of the lipid membrane is on a solid surface rather than as a dispersion in water. Inasmuch as lipids are known to behave as both thermotropic and lyotropic liquid crystals, one might expect that incorporation of hydrocarbon molecules would result in alteration of the liquid crystalline state of the membrane, in a similar manner to a temperature change, i.e., affecting long-range order. Our previous results with solid-supported lipid membranes indicate (Salamon et al., 1994, 2000b) that the presence of hydrocarbon indeed induces both a larger ordering of hydrocarbon chains, producing a higher packing density (i.e., a decrease in surface area per lipid molecule) and a larger thickness. These observations have been confirmed by results from other laboratories using both ellipsometry and x-ray methods (Plant, 1993; Thoma et al., 1996). Furthermore, such studies have also demonstrated positive birefringence of lipid membranes, clearly indicating that the lipid molecules are ordered with their long axis perpendicular to the surface of the membrane. The high birefringence values obtained show that the long-range order in such membranes is rather high, which agrees with previous results obtained in our laboratory as well as with the results presented below.

As noted above, the membrane thickness obtained from the CPWR spectrum (52 Å) includes the headgroup region and bound water molecules. This will be comparable to the so-called steric thickness of the membrane, as defined in the x-ray diffraction measurements, which is approximately 47 Å for the fully hydrated DPPC liquid phase (see Nagle and Tristram-Nagle, 2000). The low value obtained here for the area per lipid molecule, 50 Å^2 (the range of values obtained with conventional methodologies extends from ~48–72 Å^2 for DPPC; Nagle and Tristram-Nagle, 2000) and the somewhat higher value of the thickness are consistent with the idea that the present type of lipid membrane has a higher packing density and a larger overall thickness, indicating a higher degree of molecular order as compared with lipid lamellar suspensions in water. The suggestion of increased molecular order is strongly substantiated by the high value of the positive birefringence obtained here, which is on average equal to 0.053, as can be calculated (Eq. 1a) from the refractive indices given in Table 2 obtained with light polarized normal to the membrane surface ($n_p = n_\parallel$) and parallel to the membrane ($n_s = n_\perp$). This value is larger than those of either freely suspended black lipid membranes (den Engelsen, 1976) or Langmuir-Blodgett bilayers (Ramsden, 1999) and agrees very well with other measurements of solid-supported lipid membranes containing hydrocarbon solvent molecules (Thoma et al., 1996).

To calculate the average degree of orientation (S), one has either to know the distribution function that characterizes

TABLE 2 Values of the individual (n_p and n_s) and average (n_{av}) refractive indices, obtained with a lipid membrane containing 10 mol % BODIPY-PC, using two exciting light wavelengths (red and green) and two polarizations (*p* and *s*)

Average value from red and green light				L^{av}		α^{av} (Å^3)		α (Å^3)	
n_p	n_s	$(n_{av})^2$	θ	L_z^{av}	L_x^{av}	α_z^{av}	α_x^{av}	α_l	α_t
1.530	1.4775	2.236	38°	1.38	5.57	68	46	85	37

The average angle of orientation of the longitudinal lipid molecule axis with respect to the normal to the membrane plane (θ) obtained from the degree of orientational order (S; see Eqs. 11, 16a, and 16b) is used in the calculation of molecular polarizabilities (α) and shape factors (L).

the spatial distribution of the long molecular axes around the normal to the membrane surface or to experimentally measure this parameter using a guest chromophore group (or molecule) that orients itself in a similar way as does the host molecule. The latter can be accomplished by determining the linear dichroism, from which one can obtain the degree of orientation (see Eqs. 16a and 16b). Although the former is theoretically a more precise technique to obtain S, the latter is more frequently used in practical applications because it is a simple and effective experimental methodology proven in various liquid crystal materials (Johansson et al., 1978; Johansson and Lindblöm, 1980; Kozielski et al., 1987; Salamon and Skibinski, 1983). In this work, we have used the latter technique, using a chromophore label attached to the acyl chain of the lipid molecule.

Fig. 4 shows the calculated values of the extinction coefficient of the labeled membranes as a function of the amount of chromophore incorporated. The average value of the extinction k^{av} has been calculated from the two components obtained from the resonance measured with excitation light polarized either normal ($\Delta k_{p(g)}$) or parallel ($\Delta k_{s(g,N)}$) to the surface plane of the membrane (see above). These results demonstrate the following. First, the linear relationship between k^{av} and the amount of chromophore label present in the lipid membrane is a good experimental confirmation that the extinction values obtained from the CPWR resonance spectra reflect the quantity of chromophore. This is despite the fact that the measurement is made with a single lipid bilayer membrane containing labeled molecules present to only a small fraction of the total lipid mass (1–10 mol %). Second, the consistently higher values of $\Delta k_{p(g)}$ as compared with $\Delta k_{s(g,N)}$ strongly indicate that the chromophore in the membrane has its average transition moment oriented along the normal to the surface plane of the membrane, which in the planar lipid bilayer constitutes the optical axis. If in addition one assumes that this transition moment has a similar angular distribution around the normal to the surface plane of the membrane as does the long molecular axis of the lipid molecule (which is expected for a rigid planar aromatic chromophore such as BODIPY covalently attached to the closely packed acyl chains), then the linear dichroism D (or anisotropy of extinction A_k) calculated from the experimental results yields the degree of orientational order S (see Table 1 and Eqs. 14a–16b). As noted above, this describes the order of the bulk lipid molecules as well and therefore can be used to determine molecular parameters of the lipid membrane such as molecular polarizability and/or molecular shape (see Eqs. 12 and 13).

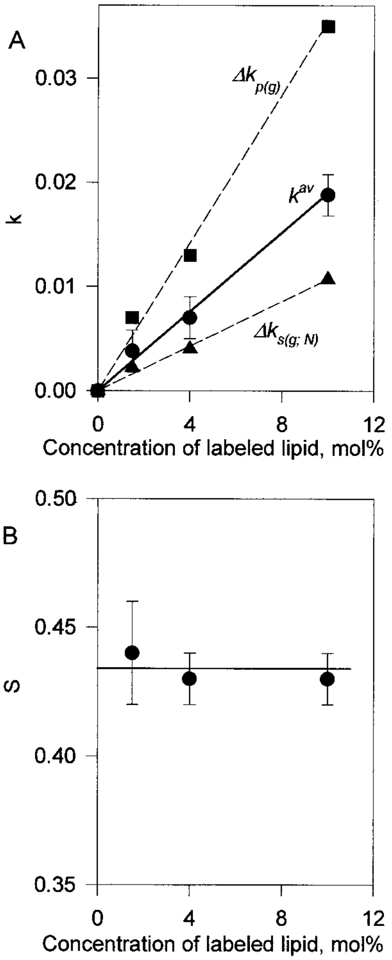

FIGURE 4 (*A*) Plots of the measured values of the extinction coefficient (k) for both p- and s-polarized components of exciting green light and of the calculated average extinction coefficient value (k^{av}) versus the chromophore-labeled lipid concentration; (*B*) Plot of the calculated degree of orientational order (S) of the lipid molecules within the membrane versus the chromophore-labeled lipid concentration.

Molecular properties of the membrane

A unique advantage of the CPWR methodology lies in the fact that it yields both birefringence, i.e., anisotropy of refractive index (Fig. 3 and Table 1), and dichroism (Fig. 4) in a single measurement using a single lipid bilayer membrane. As noted above, birefringence involves contributions from both the shape of the molecule and its molecular polarizability tensor. In the case of lipids, the shape can be approximated by a cylinder whose length is that of an extended hydrocarbon chain (as indicated by l in Fig. 1), with a cross-sectional area of two such chains. It seems reasonable to exclude the polar headgroups from these calculations, because the polar groups are commonly assumed to have a much more isotropic polarizability tensor than the nonpolar chains (den Engelsen, 1976), and therefore they have a much smaller effect on the refractive index anisotropy. Furthermore, the shape of a polar headgroup is much more symmetrical than that of the elongated acyl chains. As has been pointed out above, the cross-sectional area of a lipid molecule in the present system (representing the cross section of two hydrocarbon chains) is $\sim$50 Å^2, which indicates a rather high molecular packing density. Such a well-packed lipid membrane structure implies that the acyl chains have a rather small motional freedom, which will result in a high value of the degree of orientational order and a narrow angular distribution of the hydrocarbon chains. Thus, the majority of lipid chains are distributed narrowly around an angle θ (see Fig. 1 and Table 2), which can be evaluated from the order parameter S obtained from the dichroism measurement (see Table 1, Fig. 4, and Eq. 6). To verify this value one can calculate the hydrocarbon thickness of a monolayer (l' in Fig. 1) using $l = 19$ Å. This yields $l' = 15$ Å and a headgroup dimension $h = 11$ Å (see Fig. 1). These two parameters (l' and h) are in the range of values obtained with x-ray and NMR techniques (see, e.g., Nagle and Tristram-Nagle, 2000).

The value of the average angle allows us to calculate the average values of the shape factors ($\mathrm{L}_{\mathrm{l}}^{\mathrm{av}}$ and $\mathrm{L}_{\mathrm{t}}^{\mathrm{av}}$; see Eqs. 4, 5, 10, and 11). Furthermore, the results of these calculations, together with the refractive indices, allow us to obtain from Eqs. 12 and 13 the averaged values of the molecular polarizabilities (α_z^{av} and α_x^{av}), which finally can be used to determine the longitudinal, α_{l}, and transverse, α_{t}, molecular polarizabilities. The results of such calculations, together with the experimental values of the refractive indices and the order parameter, are shown in Table 2.

The lack of experimental and theoretical values for the molecular polarizabilities of molecules such as lipids makes it difficult to verify the results presented in Table 2. As a consequence, we have used the following three procedures to establish the validity of these values. First, we have calculated theoretical values of refractive indices from Eq. 3, taking the α_{l} and α_{t} values from Table 2 and assuming an ideal ordering of lipid molecules; i.e., $S = 1$. This allows us to determine whether such theoretical values are reasonable by direct comparison with other experimental results. The assumption of an ideal molecular order will result in the following changes of the shape factors: $\mathrm{L}_{\mathrm{l}}^{\mathrm{av}} = L_{\mathrm{l}}$ and $\mathrm{L}_{\mathrm{t}}^{\mathrm{av}} = L_{\mathrm{t}},$ and such changes will be reflected in the macroscopic optical parameters of the lipid membrane, i.e., refractive indices and thickness. The results of such calculations are shown in Table 3. The values presented in Table 3 fall within the wide range of results obtained previously for a variety of different lipids (den Engelsen, 1976; Thoma et al., 1996; Ducharme et al., 1990; Salamon et al., 2000a,b), which vary from 1.44 (for the lowest measured value of n_x) to ~1.67 (for the highest value of n_z). Furthermore, as can be seen from Tables 2 and 3, the increase in the value of the refractive index anisotropy coincides with an appropriate expansion of the membrane thickness. Such a relationship between thickness and molecular order has been observed many times when a solid lipid bilayer is melted to a liquid bilayer by raising the temperature. Typically, thickness decreases of 0.5–1.0 nm are observed (Engelman, 1971; Chapman et al., 1967; Buldt et al., 1978), which is in good agreement with the Δt value shown in Table 3. Variations in membrane thickness of this magnitude correlated with refractive index anisotropies have also been recently observed in measurements with different lipid bilayer membranes (Thoma et al., 1996; Plant, 1993; Salamon et al., 2000b). Second, we have calculated an average value of the molecular polarizability α^{av} (see Table 3), i.e., the value that one would obtain with molecules characterized by the presently calculated α_{l} and α_{t} values under completely isotropic conditions. Such an average polarizability value for a lipid molecule compares very well with those obtained with small hydrocarbon molecules (Miller, 2000). Third, the values of α_{l} and α_{t} obtained here agree rather well with those calculated by den Engelsen (1976) for five different fatty acids: from myristic (C_{12}), which has $\alpha_{\mathrm{l}} = 40.1$ and $\alpha_{\mathrm{t}} = 22.1$, through palmitic, stearic, arachidic, and behenic (C_{22}), the latter characterized by $\alpha_{\mathrm{l}} = 62.9$ and $\alpha_{\mathrm{t}} = 33.9$. It should be noted that the values given in Table 3 correspond to two fatty acid chains.

TABLE 3

α (Å^3)		n		Δt
α_{l}	α_{t}	n_{p}	n_{s}	(nm)
85	37	1.69	1.40	0.9

Values of refractive index (n) were calculated using the values of molecular polarizability (α; see Table 2) and assuming an ideal ordering of the lipid molecules; i.e., $S = 1$ and $\theta = 0$. The change in lipid bilayer membrane thickness (Δt) from that obtained with a real bilayer membrane having a measured thickness of 5.2 nm and a degree of orientational order equal to 0.43 (see Table 1 and Fig. 3 *B*) is also given.

CONCLUSIONS

The major objective of this work has been to demonstrate the ability of CPWR spectroscopy to simultaneously measure the optical dichroism and refractive index anisotropy in the same anisotropic thin-film sample, thereby permitting evaluation of structural and molecular parameters of the film. Such systems are usually difficult to study by conventional structural techniques, as clearly illustrated by the rather wide range of experimental values in the literature for the average area per lipid molecule and bilayer thickness (Shipley, 2000; Nagle and Tristram-Nagle, 2000). As we have shown, CPWR spectroscopy is able to provide a convenient means of evaluating structural parameters, including surface area per lipid molecule, thickness, and molecular orientation of a single lipid bilayer, as well as additional properties of lipid molecules such as molecular polarizability and shape. These capabilities provide an important tool for analyzing lipid and proteolipid membranes and for investigating structural changes due to lipid-protein, protein-protein, and protein-ligand interactions.

The experimental results presented here demonstrate that CPWR spectroscopy is able to measure the optical dichroism of small amounts of light-absorbing material contained within a single anisotropic lipid bilayer membrane. It is important to point out that such chromophores can be either covalently attached to membrane components (either lipid or protein) or noncovalently incorporated into the bilayer. This allows one to evaluate both the quantity of the light-absorbing material as well as the degree of its molecular ordering, as calculated from the absorption anisotropy. As indicated above, the measured value of the refractive index of an anisotropic film depends on the molecular polarizability tensor α, the shape factors L, and the degree of orientational order S (see Eqs. 12, 13, and 7–9). These are three quite independent parameters, with α being a molecular quantity, independent of aggregation and environment, and L and S both strongly affected by aggregation and molecular environment. The effect of the environment on these latter two parameters is well illustrated by many experimental examples of phosphatidylcholine bilayers in which small quantities of either hydrocarbons or cholesterol have been incorporated, thereby causing large alterations in their optical anisotropies (Thoma et al., 1996; Tardieu et al., 1973; McIntosh, 1980). Such results clearly indicate that the proper characterization of anisotropic films requires evaluation of a full set of optical parameters. Any attempt to understand such systems based on changes in only one parameter is inadequate and can lead to completely incorrect conclusions. This is an important limitation of conventional surface plasmon resonance methods, which can measure only refractive index in the perpendicular direction relative to the film plane. The simultaneous measurement of both birefringence and dichroism of a single lipid bilayer membrane allows one to independently evaluate S and therefore to separate L from S. It is also important to emphasize that CPWR dichroism measurements can be extended to other spectral ranges, including the ultraviolet and infrared regions. This permits the independent monitoring of the amounts of material and the degree of order of different constituents of a thin film, thereby separating structural alterations that may occur within the various components of the membrane. Such measurements can be especially beneficial in understanding lipid-protein, protein-protein, and receptor-ligand interactions, which usually result in binding accompanied by changes in molecular structure of various membrane components (Salamon et al., 1994, 1996, 1998, 1999, 2000a; Zakharov et al., 1998).

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REFERENCES

Bloom, M., E. Evans, and O. G. Mouritsen. 1991. Physical properties of the fluid-bilayer component of cell membranes: a perspective. *Q. Rev. Biophys.* 24:293–397.

Böttcher, C. J. F. 1952. Theory of Electric Polarisation, Elsevier, Amsterdam.

Bragg, W. L., and A. B. Pippard. 1953. The form birefringence of macromolecules. *Acta Cryst.* 6:865–867.

Brown, M. F. 1993. Modulation of protein function by properties of the membrane bilayer. *Chem. Phys. Lipids.* 73:159–180.

Buldt, G., H. U. Gally, A. Seelig, J. Seelig, and G. Zaccai. 1978. Neutron diffraction studies on selectively deuterated phospholipid bilayers. *Nature.* 271:182–184.

Chapman, D., R. M. Williams, and D. B. Ladbrooke. 1967. Physical studies of phospholipids. Thermotropic and lyotropic mesomorphism of some 1,2- diacyl-phosphatidylcholines (lecithins). *Chem. Phys. Lipids.* 1:445–475.

Cuypers, P. A., J. W. Corsel, M. P. Janssen, J. M. M. Kop, W. T. Hermens, and H. C. Hemker. 1983. The adsorption of prothrombin to phosphatidylserine multilayers quantitated by ellipsometry. *J. Biol. Chem.* 258: 2426–2431.

De Jeu, W. H.1978. Physical properties of liquid crystalline materials. *In* Liquid Crystal Monographs, Vol. 1. G. Gray, editor. Gordon and Breach, Amsterdam. 34–48.

den Engelsen, D. 1976. Optical anisotropy in ordered systems of lipids. *Surf. Sci.* 56:272–280.

Dowhan, W. 1997. Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu. Rev. Biochem.* 66:199–232.

Ducharme, D., J. J. Max, C. Salesse, and R. M. Leblanc. 1990. Ellipsometric study of the physical states of phosphatidylcholines at the air-water interface. *J. Phys. Chem.* 94:1925–1932.

Engelman, D. M. 1971. Lipid bilayer structure in the membrane of *Mycoplasma laidlawii. J. Mol. Biol.* 58:153–165.

Gee, M. L., T. W. Healy, and L. R. White. 1990. Hydrophobicity effects in the condensation of water films on quartz. *J. Colloid. Interface Sci. U.S.A.* 83:6258–6262.

Johansson, L. B. A., A. Davidsson, G. Lindblöm, and B. Norden. 1978. Linear dichroism as a tool for studying molecular orientation in membrane systems. II. Order parameters of guest molecules from linear dichroism and nuclear magnetic resonance. *J. Phys. Chem.* 82: 2604–2609.

Johansson, L. B. A., and G. Lindblöm. 1980. Orientation and mobility of molecules in membranes studied by polarized light spectroscopy. *Q. Rev. Biophys.* 13:63–118.

Kozielski, M., D. Bauman, M. Drozdowski, and Z. Salamon. 1987. Study of the guest-host effect in thin aligned liquid crystal films by Raman spectroscopy. *Mol. Cryst. Liquid Cryst.* 142:1–13.

Macleod, H. A. 1986. Thin Film Optical Filters. Hilger, Bristol, and Macmillan, New York.

Macleod, H. A. 1992. Unconventional coatings. *In* Tutorials in Optics. D. T. Moore, editor. Optical Society, Washington, DC. 121–135.

McIntosh, T. J. 1980. Differences in hydrocarbon chain tilt between hydrated phosphatidylethanolamine and phosphatidylcholine bilayers: a molecular packing model. *Biophys. J.* 29:237–246

Miller, T. M. 2000. Atomic and molecular polarizabilities. *In* Handbook of Chemistry and Physics. D. R. Lide, editor. CRC Press LLC, New York. 10160–10174.

Mouritsen, O. G., and P. K. J. Kinnunen. 1996. Role of lipid organization and dynamics for membrane functionality. *In* Biological Membranes. K. Merz, Jr., and B. Roux, editors. Birkhäuser, Boston. 463–502.

Mueller, P., D. O. Rudin, H. T. Tien, and W. C. Wescott. 1962. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature.* 194:979–980.

Nagle, J. F., and S. Tristram-Nagle. 2000. Lipid bilayer structure. *Curr. Opin. Struct. Biol.* 10:474–480.

Norden, B., G. Lindblöm, and I. Jonas. 1977. Linear dichroism spectroscopy as a tool for studying molecular orientation in model membrane systems. *J. Phys. Chem.* 81:2086–2093.

Onsager, L. 1936. Electric moments of molecules in liquids. *J. Am. Chem. Soc.* 58:1486–1493.

Plant, A. L. 1993. Self-assembled phospholipid/alkanethiol biomimetic bilayers on gold. *Langmuir.* 9:2764–2767.

Ramsden, J. J. 1999. Molecular orientation in lipid bilayers. *Phil. Mag. B.* 79:381–386.

Salamon, Z., M. F. Brown, and G. Tollin. 1999. Plasmon resonance spectroscopy: probing interactions within membranes. *Trends Biochem. Sci.* 24:213–219.

Salamon, Z., S. Cowell, E. Varga, H. I. Yamamura, V. J. Hruby, and G. Tollin. 2000a. Plasmon resonance studies of agonist/antagonist binding to the human δ-opioid receptor: new structural insights into receptor-ligand interactions. *Biophys. J.* 79:2463–2474.

Salamon, Z., D. Huang, W. A. Cramer, and G. Tollin. 1998. Coupled plasmon- waveguide resonance spectroscopy studies of the cytochrome b_6f/plastocyanin system in supported lipid bilayer membranes. *Biophys. J.* 75:1874–1885.

Salamon, Z., G. Lindblöm, L. Rilfors, K. Linde, and G. Tollin. 2000b. Interaction of phosphatidylserine synthase from *E. coli* with lipid bilayers: coupled plasmon-waveguide resonance spectroscopy studies. *Biophys. J.* 78:1400–1412.

Salamon, Z., H. A. Macleod, and G. Tollin. 1997a. Coupled plasmon-waveguide resonators: a new spectroscopic tool for probing proteolipid film structure and properties. *Biophys. J.* 73:2791–2797.

Salamon, Z., H. A. Macleod, and G. Tollin. 1997b. Surface plasmon resonance spectroscopy as a tool for investigating the biochemical and biophysical properties of membrane protein systems. I. Theoretical principles. *Biochim. Biophys. Acta.* 1331:117–129.

Salamon, Z., and A. Skibinski. 1983. Fluorescence emission of guest molecules as a tool of surface alignment study in nematic liquid crystals. *Mol. Cryst. Liquid Cryst.* 90:205–215.

Salamon, Z., and G. Tollin. 1991. Interfacial electrochemistry of cytochrome c at a lipid bilayer modified electrode: effect of incorporation of negative charges into the bilayer on cyclic voltammetric parameters. *Bioelectrochem. Bioenerg.* 26:321–334.

Salamon, Z., and G. Tollin. 1999a. Surface plasmon resonance: theoretical principles. *In* Encyclopedia of Spectroscopy and Spectrometry, Vol. 3. J. C. Lindon, G. E. Tranter, and J. L. Holmes, editors. Academic Press, New York. 2311–2319.

Salamon, Z., and G. Tollin. 1999b. Surface plasmon resonance: applications. *In* Encyclopedia of Spectroscopy and Spectrometry, Vol. 3. J. C. Lindon, G. E. Tranter, and J. L. Holmes, editors. Academic Press, New York. 2294–2302.

Salamon, Z., Y. Wang, M. F. Brown, H. A. Macleod, and G. Tollin. 1994. Conformational changes in rhodopsin probed by surface plasmon resonance spectroscopy. *Biochemistry.* 33:13706–13711.

Salamon, Z., Y. Wang, J. L. Soulages, M. F. Brown, and G. Tollin. 1996. Surface plasmon resonance spectroscopy studies of membrane proteins: transducin binding and activation by rhodopsin monitored in thin membrane films. *Biophys. J.* 71:283–294.

Saupe, A. 1964. Kernresonanzen in kristallinen Flüssigkeiten und kristallinflüssigen Lösungen. *Teil I. Z. Naturfoschung.* 19A:161–171.

Silberzan, P., L. Leger, D. Ausserre, and J. J. Benattar. 1991. Silanation of silica surfaces. A new method of constructing pure or mixed monolayers. *Langmuir.* 7:1647–1651.

Shipley, G. G. 2000. Bilayers and nonbilayers: structure, forces and protein crystallization. *Curr. Opin. Struct. Biol.* 10:471–473.

Tardieu, A., V. Luzzati, and F. C. Reman. 1973. Structure and polymorphism of the hydrocarbon chains of lipids: a study of lecithin-water phases. *J. Mol. Biol.* 75:711–733.

Thoma, M., M. Schwendler, H. Baltes, C. A. Helm, T. Pfohl, H. Riegler, and H. Möhwald. 1996. Ellipsometry and x-ray reflectivity studies on monolayers of phosphatidylethanolamine and phosphatidylcholine in contact with *n*-dodecane. *n*-hexadecane, and bicyclohexyl. *Langmuir.* 12:1722–1728.

Zakharov, S. D., M. Lindeberg, Y. Griko, Z. Salamon, G. Tollin, F. G. Prendergast, and W. A. Cramer. 1998. Membrane-bound state of the colicin E1 channel domain as an extended two-dimensional helical array. *Proc. Natl. Acad. Sci. U.S.A.* 95:4282–4287.