Fluorescence Spectroscopy of an Oriented Model Membrane

(probes/polarization/oxidized cholesterol bilayer/spherical membranes)

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ABSTRACT We have devised a simple method that makes it feasible to apply fluorescence techniques to lipid bilayer membranes to elucidate aspects of their structure and dynamics. Fluorescence excitation, emission, and polarization spectra were obtained from a single spherical bilayer membrane consisting of oxidized cholesterol and a fluorescent probe. The emission transition moments of *N,N'*-di(octadecyl)oxacarbocyanine and 12-(9-anthroyl)-stearic acid were found to be aligned parallel to the plane of the bilayer, whereas that of *p*-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene was aligned in a perpendicular direction. All three probes exhibited appreciable rotational mobility, parallel to the plane of the bilayer, in durations of nanoseconds. An attractive feature of this model membrane is that fluorescence measurements can be made at the same time as electrical measurements and perturbations. Also, it may be possible to incorporate functional protein assemblies into this model and to use fluorescence spectroscopy to delineate some aspects of their assembly and function.

The structure and dynamics of biological membranes are challenging areas of inquiry: replication, energy transduction, nerve impulse propagation, sensory reception, and hormonal integration exemplify the diversity and richness of function mediated by organized molecular assemblies in biological membranes. The unraveling of these complex processes may be facilitated by the use of well-defined model systems and by the application of techniques that provide insight into the conformational correlates of membrane function. The synthetic lipid membrane, introduced by Mueller and Rudin, is a pertinent model because its conformation is similar to that of lipid bilayer regions of biological membranes (1). Furthermore, it acquires distinctive functional properties, such as selective ionic permeability, excitability, and active transport, on the addition of specific small molecules or proteins. The study of conformational aspects of this model membrane and of biological membranes by fluorescence spectroscopy appears promising in view of the sensitivity of fluorescence techniques and their utility in elucidating facets of the structure and dynamics of biological macromolecules in solution (2, 3). In particular, the emission characteristics of responsive fluorescent chromophores provide information concerning proximity, polarity, and rotational mobility (4-6).

We have devised a simple method (7) that makes it feasible to apply fluorescence techniques to the Mueller-Rudin model membrane. In this paper, we show that (a) the fluorescence emission from a single lipid-bilayer membrane containing a fluorescent probe can readily be observed if a favorable geometrical arrangement is used, (b) that the orientation of a fluorescent chromophore located in the bilayer membrane can be determined from fluorescence polarization measurements,

and (c) that rotational motions parallel and perpendicular to the plane of the bilayer that occur in nanoseconds can be resolved.

MATERIALS AND METHODS

Lipid bilayer membranes can have a planar or spherical configuration. In the planar arrangement, the bilayer membrane is stretched across a 1-mm aperture on the side of a teflon cup that separates two aqueous compartments (8). We experienced difficulty in observing fluorescence from this planar membrane because the incident beam was strongly scattered by the teflon support (9). This difficulty was obviated by use of a spherical form of the bilayer (10). Fluorescence measurements could readily be made on spherical bilayer membranes suspended in a sucrose density gradient.

Membranes were formed from a solution containing a fluorescent probe and 40 mg/ml of oxidized cholesterol (11) in octane. Oxidized cholesterol was used in preference to a phospholipid because of the rapidity and ease of formation of stable bilayer membranes at room temperature, despite the probably greater biological pertinence of membranes formed from phospholipids. The fluorescent chromophores

incorporated in the membranes were *N,N'*-di(octadecyl)oxacarbocyanine (I), 12-(9-anthroyl)-stearic acid (II), and *p*-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (III, dimethyl-POPOP). I and II were synthesized as described (12, 6). III was purchased from Pilot Chemicals. The concentration of the fluorescent chromophore was chosen to give an absorbance of about 1 for a 1-cm pathlength at its long-wavelength absorp-

(Left) FIG. 1. Formation of fluorescent spherical membranes in a sucrose density gradient. The fluorescent probe was anthroyl stearic acid (II). The excitation wavelength was 365 nm, and the emission was viewed through a 3-71 Corning filter. The width of the quartz cuvet was 1 cm.

(Right) FIG. 2. (a) Fluorescent spherical membrane in the process of thinning to a bilayer. The fluorescent probe was II. The three zones are an intensely-fluorescent cap, a strongly-fluorescent upper region, and a weakly-fluorescent lower region. Octane and excess oxidized cholesterol and probe are migrating to the cap. (b) Fluorescent spherical membrane that has thinned to a bilayer, except for the intensely fluorescent cap. The fluorescent probe was II. The diameter of the sphere was 3 mm.

tion maximum. The ratio of oxidized cholesterol to fluorescent probe was, therefore, more than 1000:1 for all three probes. A platinum wire, with a 2-mm diameter loop at one end, was dipped into this membrane-forming solution. The loop was then slowly brought to the bottom of a $1 \times 1 \times 4.5$ cm quartz cuvet that contained a sucrose density gradient ranging from 1.01 to 1.11 g/ml in 0.1 M KCl. An abrupt upward motion of the loop resulted in the formation of several fluorescent spherical membranes (1–4 mm in diameter) that remained suspended at different heights in the gradient (Fig. 1).

FIG. 3. Excitation spectrum of the bilayer (—) and cap (---) regions of a spherical membrane containing II. The emission was detected through a 3-71 Corning filter.

Excitation spectra, emission spectra, and polarized fluorescence intensities were measured with a microspectrofluorimeter to be described in detail elsewhere.

RESULTS

Fluorescent spherical bilayer membranes

The newly formed fluorescent spheres could readily be observed by eye. When first formed, the spheres exhibited a bright, uniform fluorescence. An intensely fluorescent cap formed at the top of the sphere in about a minute, followed by an abrupt and marked lessening of the fluorescence intensity at the bottom of the sphere. These changes result from the spontaneous migration of octane and some of the oxidized

FIG. 4. Orientation of the bilayer membrane in the side (S), front (F), and bottom (B) regions of the sphere, as viewed by the detection system.

cholesterol and fluorescent probe to the cap region. Three clearly demarcated regions were evident at this stage (Fig. 2a): (i) an intensely fluorescent cap, (ii) a strongly fluorescent upper region, and (iii) a weakly fluorescent lower region. The weakly fluorescent zone spread until it covered the whole sphere except for the intensely fluorescent cap (Fig. 2b).

The decrease in fluorescence intensity resulted from the spontaneous thinning of the membrane, as evidenced by concurrent changes in reflectivity. The spheres were illuminated with a beam of white light and viewed against a black background. When first formed, a sphere exhibited specular reflection. After the cap appeared, interference colors were transiently observed from the rest of the sphere, which suggests that the thickness of the membrane was between 1000 and 5000 Å (13). Next, the sphere exhibited a silvery appearance, indicative of a membrane thickness of about 1000 Å. The bottom of the sphere then abruptly turned black, concomitant with the appearance of weak fluorescence in this region, denoting a loss of reflectivity characteristic of membranes less than 300 Å thick (14). When the completely thinned sphere was viewed under ordinary room light, only the caps were seen floating in the sucrose gradient. The completely thinned membrane was almost invisible, since it was virtually transparent and reflected little light.

The precise molecular arrangement of our membrane is not yet known. However, it seems likely that the oxidized cholesterol molecules are arranged in a bimolecular layer in the completely thinned region of the membrane. Electron microscopic, light reflectance, and electrical capacitance measurements have shown that completely thinned phosphatidyl choline membranes have a thickness of 60–100 Å, whereas oxidized cholesterol membranes are 40–50 Å thick (15). These dimensions correspond to the width expected for a bilayer of these molecules. The phosphatidyl choline molecules, and presumably the oxidized cholesterol molecules, are oriented in the bilayer so that their polar portions are located at the aqueous interfaces, whereas the nonpolar portions constitute the hydrocarbon interior of the membrane (see Fig. 4).

Fluorescence spectra

The fluorescence excitation spectrum of a bilayer membrane containing II (Fig. 3) agrees with the absorption spectrum of the anthroyl chromophore. The fluorescence emission spectrum of this bilayer region also was characteristic of the anthroyl chromophore. These spectra demonstrate that light absorption and fluorescence came from the fluorescent probe rather than from an impurity. Lipid bilayer membranes made from membrane-forming solutions without a fluorescent probe showed no detectable fluorescence. The fluorescence excitation spectrum of the bilayer region was distinct from that of the cap region (Fig. 3); thus, the probe has different environments in the cap and bilayer regions.

Dependence of fluorescence polarization on chromophore orientation

The orientation of the probe in the bilayer membrane can be determined from fluorescence polarization measurements. Ideally, a small patch of membrane is excited with light polarized alternately along *a*, *b*, and *c*. For each of these modes of excitation, the intensity of fluorescence polarized along and perpendicular to the direction of polarization of the incident light is measured. These measurements can readily be made with a spherical bilayer membrane. The side, front, and

bottom regions (Fig. 4) provide three mutually perpendicular orientations of the bilayer membrane. All of the polarized fluorescence intensities needed to define orientation can be measured without altering the position of the light source or detector.

In our experiments, four polarized fluorescence intensities were measured for each region of the membrane: VV (excitation and emission vertically polarized), VH (excitation vertically polarized, emission horizontally polarized), HV (excitation horizontally polarized, emission vertically polarized), and HH (excitation and emission horizontally polarized). A total of 12 polarized fluorescence intensities are thus obtained from the side, front, and bottom regions. However, some of these measurements are redundant. For a bilayer membrane that is symmetric in the *ab* plane, these 12 intensities reduce to 4 fundamental anisotropic fluorescence parameters, termed α , β , γ , and δ (Table 1). These parameters depend on the orientation of the absorption and emission transition moments of the chromophore in the bilayer and on rotational motions during the lifetime of the excited state. The expressions relating the anisotropic fluorescence parameters to orientation are simplest for the case in which the absorption and emission transition moments are coincident and rotational motions during the excited state lifetime are absent. If the distribution of transition moments in the bilayer is $\rho(\varphi)$, where φ is the angle between the transition moment and the *c* axis, then

$$\alpha_0 = \frac{6\pi}{4} \int_0^{\pi/2} \rho(\varphi) \sin^3 \varphi d\varphi \quad (1)$$

$$\beta_0 = \frac{\pi}{2} \int_0^{\pi/2} \rho(\varphi) \sin^5 \varphi d\varphi \quad (2)$$

$$\gamma_0 = 2\pi \int_0^{\pi/2} \rho(\varphi) \cos^2 \varphi \sin^3 \varphi d\varphi \quad (3)$$

$$\delta_0 = 4\pi \int_0^{\pi/2} \rho(\varphi) \cos^4 \varphi \sin \varphi d\varphi \quad (4)$$

The subscript 0 emphasizes that these equations assume that rotational motions are absent during the lifetime of the excited state.

Anisotropic fluorescence intensities for different orientations of the fluorescent probe in the bilayer membrane were calculated from Eqs. 1–4. Plots of VV, VH, HV, and HH polarized-fluorescence intensities for the side, front, and bottom regions of a spherical bilayer membrane are given in Fig. 5 for four distributions of the fluorescent probe symmetric with respect to the *ab* plane. These plots show that the ratio of α_0 to δ_0 is the most sensitive indicator of orientation. For preferential alignment in the *ab* plane, $\alpha_0 > \delta_0$. For preferential alignment about the *c* axis, $\delta_0 > \alpha_0$.

TABLE 1. Region of sphere

Excitation and Emission Component	Side	Front	Bottom
VV	α	α	δ
VH	γ	β	γ
HV	β	γ	γ
HH	γ	γ	β

FIG. 5. Calculated polarized fluorescence intensities for the side, front, and bottom regions of a spherical bilayer membrane in which (a) the transition moments are perfectly parallel to the *ab* plane of the bilayer; (b) the transition moments are partially aligned parallel to the *ab* plane of the bilayer; (c) the transition moments are partially aligned parallel to the *c* axis of the bilayer; and (d) the transition moments are perfectly aligned parallel to the *c* axis of the bilayer. The intensities in (b) and (c) were cal-

Molecular orientation in the bilayer membrane

Polarized fluorescence intensities for bilayer membranes that contain probes I, II, III are given in Fig. 6. The chromophores were excited at their longest-wavelength absorption band so that the directions of their absorption and emission transition moments are almost certainly coincident. It is evident that the probes are oriented in the bilayer membrane, since α differs from δ for all three probes. The polarization plot (Fig. 6) for I shows that this probe is highly oriented, with its transition moment in the *ab* plane of the bilayer membrane. This alignment is consistent with that expected from the structure of I and from the results of Kuhn and his colleagues (16) on dry, oriented multilayers of fatty acids.

The transition moment of II is also preferentially aligned parallel to the *ab* plane of the bilayer, since $\alpha > \delta$ (Fig. 6). However, the degree of orientation is not as high as for I, as is evident from a comparison of the polarization data for II with polarization plots of the kind shown in Fig. 5, calculated for Gaussian distributions of different widths. The emission and long-wavelength-absorption transition moments of anthracene are known to be in the aromatic plane along its short axis (17). This axis is thus aligned in the *ab* plane of the bilayer. The transition moment of the second absorption band at shorter wavelength is oriented along the long axis of the anthracene molecule. The polarized fluorescence intensity observed on excitation of II in its second absorption band indicated that the anthroyl chromophore is aligned with its long axis parallel to *c*. The less well-defined orientation of II compared to I is consistent with the results of spin-labeling studies that have shown that the hydrocarbon interior of lipid membranes is distinctly more fluid than the polar interface region (18, 19).

In contrast to I and II, the transition moment of III is preferentially aligned parallel to *c*, since $\delta > \alpha$ (Fig. 6). A comparison of the observed polarization plot for III with those calculated for different distributions (Fig. 5c and d) indicates that there is a fairly broad range of orientations for this probe. The emission transition moment of III is probably aligned along its long axis. Thus, it can be inferred that III tends to be oriented in the bilayer with its long axis parallel to *c*.

ROTATIONAL MOTIONS

Information concerning rotational mobility, as well as orientation, can be derived from the anisotropic fluorescence parameters. In the absence of rotational motion during the life of the excited state, the ratio of α to β is 3, irrespective of the orientation of the chromophore, as can be seen from Eqs. 1 and 2. On the other hand, if there are very rapid rotational motions about the *c* axis, the ratio of α to β will approach 1. The observed α to β ratios for I, II, and III in the bilayer membrane (Fig. 6) are 2.3, 1.7, and 2.0, respectively. These intermediate ratios suggest that rotational motions about the *c* axis of the bilayer occur on the same time scale as the lifetime of the excited state, i.e., nanoseconds. A definitive quantitative

culated for a Gaussian distribution, in which half of the transition moments are within 43° of the *ab* plane and *c* axis, respectively.

FIG. 6. Observed polarized fluorescence intensities for the side, front, and bottom regions of a spherical bilayer membrane containing I (top), II (middle), and III (bottom). Each chromophore was excited at its longest-wavelength absorption band.

characterization of these rotational motions can best be obtained by nanosecond fluorimetry (3). The explicit dependence of α and β on time after excitation with a light pulse will reveal rotational motions about the c axis, whereas the time dependence of α and δ will reveal rotational motions perpendicular to that axis. These measurements would also yield values of α_0 , β_0 , γ_0 , and δ_0 , making the determination of orientation more precise.

DISCUSSION

Our principal finding is that fluorescence techniques can readily be applied to lipid bilayer membranes to elucidate aspects of their structure and dynamics. The emission transition moments of I and II were found to be aligned parallel to the plane of the bilayer, whereas that of III was aligned in a perpendicular direction. A markedly higher degree of orientation was observed for the chromophore of I, which is probably located at the aqueous interface, than for II and III, which are likely to be situated in the interior of the bilayer. All three probes exhibited appreciable rotational mobility parallel to the plane of the bilayer in durations of nanoseconds.

The significance of this fluorescent model membrane is two-fold. First, its availability should facilitate the interpretation of fluorescence changes observed in a number of biological membranes as a function of their physiological state. For example, changes of this kind have been observed in nerve-axon membranes during an action potential (20) and in mitochondrial membrane particles in the course of energy transduction (21). It will be of particular interest to explore the effects of electric field and of ionic gradients and fluxes on the conformation and dynamics of spherical bilayer membranes. Electric field effects can be most readily studied by forming a planar membrane in a hole at the bottom of a teflon cup and then bulging the membrane, by hydrostatic pressure, into a fluorescent hemisphere still attached to the cup. An especially attractive feature of this model membrane is that fluorescence measurements can be made at the same time as electrical measurements and perturbations. Second, it may be feasible to incorporate functional protein assemblies into this model and to use fluorescence spectroscopy to elucidate some conformational aspects of their assembly and function. The recent studies demonstrating active transport of cations across lipid bilayer membranes after the incorporation of a Na^+ - K^+ -activated ATPase are very encouraging in this regard (22, 23).

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