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Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence

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ABSTRACT The organization of lipids surrounding membrane proteins can influence their properties. We have used 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) to study phase coexistence and phase interconversion in membrane model systems. The fluorescence properties of Laurdan provide a unique possibility to study lipid domains because of the different excitation and emission spectra of this probe in the gel and in the liquid-crystalline phase. The difference in excitation spectra allows photoselec-

tion of Laurdan molecules in one of the two phases. Using the difference in emission spectra it is then possible to observe interconversion between the two phases. We have performed experiments in dipalmitoyl-phosphatidylcholine (DPPC) vesicles at different temperatures, in particular in the region of the phase transition, where phase coexistence and interconversion between phases is likely to be maximal. We have also studied vesicles of different lipids and mixtures dilauroyl-phosphatidylcholine (DLPC), DPPC,

and 50% DLPC in DPPC. Both steady-state fluorescence intensity and polarization data have been collected. To quantitate phase coexistence and interconversion we have introduced the concept of "generalized polarization." We have also performed time-resolved experiments to directly prove the interconversion process. We have found that in DLPC-DPPC mixtures, at 20°C, phase interconversion occurs in ~30–40 ns.

INTRODUCTION

Phospholipids constitute the fundamental matrix of natural membranes and represent the environment in which many proteins and enzymes display their activity. Although only a few, highly specific enzymatic activities require lipids, the phase state of the membrane can be of relevance for the modulation of the function of the inserted proteins (1). In the physiological temperature range, phospholipids can be in the gel or in the liquid-crystalline phase, depending upon the length of their acyl chains and the type of polar residues. In natural membranes, there is a large compositional heterogeneity of the constituent lipids. Different phospholipids display nonideal miscibility (2–3), which gives rise to the possibility of domain coexistence. In the last decade, many efforts have been devoted to the investigation of the properties of natural membrane components, in particular with respect to the detection of different coexisting phases, generally referred to as lipid domains (4–6). Among the different spectroscopic techniques employed for the study of phase coexistence and dynamics, fluorescence spectroscopy

offers several advantages. The time-scale of the fluorescence emission, in the range of nanoseconds, is appropriate to detect the dynamics of the membrane components. A variety of fluorophores has been utilized to label membranes (7). Averaged variations of the physical state of membranes have been determined from the study of emission spectra, intensity decay, steady-state, and time-resolved anisotropy (8–10).

For the specific problem of detecting lipid domains, the fluorescence parameters must be analyzed for at least two components which must have different spectroscopic properties. Phase coexistence must correspond to the detection of at least two sets of spectroscopic properties. Instead, phase mixing can be detected by the appearance of new spectroscopic components. For example, in an interconverting system, the measured lifetime values are different from the lifetime value of the same system without interconversion. A common probe for the study of membrane properties is 1,6-diphenyl-1,3,5-hexatriene (DPH). Klausner et al. (6) investigated the lifetime behavior of DPH in phospholipid vesicles and they concluded that a mixed phase can be detected using DPH. Further studies (10) questioned their results on the basis of the small variations of DPH spectroscopic properties. It was shown that the emission properties of DPH in vesicles can be equally described using the linear superposition of the properties of the two phases (10). Other studies have

Abbreviations used in this paper: DLPC, DPPC is never used, DPPC, Dilauroyl-, dimyristoyl-, dipalmitoyl-phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; PBS, phosphate buffered saline; POPOP, 2,2'-p-phenylenebis(5-phenyl)oxazole.

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focused on membrane probes with larger spectroscopic differences between the two phases. Ruggiero and Hudson (11) have used the different rotational behavior of parinaric acid isomers in the two different phases, coupled with the large change of their lifetime values in the two phases. By using *cis*- and *trans*-parinaric acids, critical density fluctuations were determined in various phospholipid bilayers for temperatures slightly below the main transition, with an estimated lifetime of the order of 30 ns (11). Because structural phase fluctuations are thought to occur in a relatively long time scale, Davenport et al. (12) used coronene, a long-lived fluorophore. In mixed phospholipid phases, the rotational behavior of coronene shows dramatic changes after 20–200 ns. They interpreted their results as due to phase fluctuations. Phase fluctuations have been also studied using ultrasound techniques by Mitaku et al. (13). The ultrasound absorption increased dramatically at temperatures near the transition temperature in dipalmitoyl-phosphatidylcholine (DPPC) vesicles. The interpretation of these studies was that dissipative density fluctuations occur in a relatively wide temperature range around the transition temperature. From the frequency dependence of the ultrasound absorption they obtained a relaxation time for density fluctuations ranging from 20 to 60 ns.

All of these studies are based on quite delicate measurements and interpretations. In particular, the use of *cis*- and *trans*-parinaric acids is complicated by the intrinsic heterogeneity of their decay and the difficulty of accurately establishing the spectroscopic properties of the probes in the two phases (14). Furthermore, those studies necessitate time-resolved anisotropy data, which are generally more difficult to obtain, with respect to steady-state measurements. Also, the coronene approach requires accurate time-resolved anisotropy measurements. It would be desirable to have a probe that can be used in steady-state fluorescence methods, i.e., a probe that shows spectral sensitivity to the two phases.

In the present work we utilized the sensitivity of the excitation and emission spectra of Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) to the properties of the environment to study phase coexistence and fluctuations in phospholipid vesicles. Laurdan is known to be sensitive to the polarity of the environment, displaying a large red shift of the emission in polar solvents, with respect to nonpolar solvents (15). The phenomenon is generally referred to as solvent relaxation, indicating the reorientation of the solvent dipoles around the excited state dipole (15). In phospholipid vesicles, Laurdan can detect the dynamics of its environment by observing the time evolution of the spectral shape and center of gravity. In single-phase phospholipid vesicles the dynamics of the surroundings detected by Laurdan is very different in the case of the gel or of the liquid-crystalline phase. In

previous work, the values of $0.66 \times 10^9 \text{ s}^{-1}$ and $1.26 \times 10^9 \text{ s}^{-1}$ have been reported for the spectral relaxation rate in DPPC at the phase transition temperature and in the pure liquid-crystalline phase, respectively (15).

We are now presenting steady-state and time-resolved fluorescence data of Laurdan in DPPC and in dilauroylphosphatidylcholine (DLPC) in a wide temperature range and in a 50% mixture of DLPC/DPPC. We have analyzed the data using a two-state model, corresponding to the gel and to the liquid-crystalline phase of the phospholipids, and using a generalized definition of polarization. Evidences for interconversion between the two phases have been obtained using time-resolved spectral changes.

MATERIALS AND SAMPLE PREPARATION

DLPC, DPPC were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Laurdan was purchased from Molecular Probes (Eugene, OR). Solvents were spectroscopic grade, deoxygenated by extensive nitrogen bubbling before use. Multilamellar phospholipid vesicles were prepared after the procedure described in Parasassi et al. (10). The proper amount of chloroform stock solutions of the phospholipids were mixed with the probe. The solvent was evaporated by nitrogen flux. The dry samples were resuspended in phosphate buffered saline (PBS), previously deoxygenated by vigorous nitrogen bubbling for 1 h. The samples were then heated above the transition temperature of the phospholipids, vortexed, and used immediately after preparation. All preparations were carried out in red light and in nitrogen atmosphere. The final concentration of the probe and the phospholipids was $0.15 \mu\text{M}$ and 0.10 mM , respectively, for steady-state measurements, and $0.75 \mu\text{M}$ and 0.30 mM , respectively, for lifetime measurements. The final results were largely independent of the probe/lipid concentration in the range 1:1000–1:400.

FLUORESCENCE MEASUREMENTS

Steady-state emission spectra, polarization, and lifetime measurements were performed on a phase fluorometer (model GREG 200; ISS Inc., Champaign, IL), equipped with a He-Cd laser ($\lambda = 325 \text{ nm}$) or with a Xenon-arc lamp, using excitation at 400-nm and 16-nm bandwidth. Some of the lifetime measurements were also performed using the phase fluorometer described by Gratton and Limkeman (17). Excitation spectra were acquired on a photon counting spectrofluorometer (model GREG PC; ISS Inc.). Temperature was controlled by a water-

circulating bath and the actual temperature was measured in the sample cuvette. The wavelength resolved data were acquired using interference filters (Corion Corp., Holliston, MA), with 10-nm bandwidth. For lifetime measurements 2,2'-p-phenylenebis(5-phenyl)oxazole (POPOP) in ethanol was used in the reference cuvette ($\tau = 1.35$ ns). Phase and modulation data were analyzed for a double and triple exponential decay using the Globals Unlimited software (Urbana, IL). Time-resolved emission spectra were obtained using the same software.

DEFINITION OF GENERALIZED POLARIZATION

To quantitate intensity data collected at different excitation and emission wavelengths, we define the "generalized polarization" to be

$$G = \frac{I_B - I_R}{I_B + I_R}, \quad (1)$$

where I_B and I_R are the fluorescence intensity measured under conditions in which a wavelength B and R (or a band of wavelengths) is observed, respectively. This definition corresponds to the classical fluorescence polarization if B and R represent two different orientations of the observation polarizers. The "generalized polarization," as well as the classical polarization, depends on the excitation conditions. The advantage of defining the "generalized polarization" for the analysis of the spectral properties of Laurdan is related to the well-known properties of the polarization which is a quantity that contains information on the dynamics of interconversion between different "states". In the classical definition the "states" correspond to different orientations of the emitting dipole with respect to the laboratory axis. The "generalized polarization" values are limited between $+1$ and -1 . Because we are specifically interested in deriving the properties of the interconversion between two states, in the definition of "generalized polarization," we introduce the expression for the decay of a two-state system. In Results and Discussion, these two states will be identified with the gel and the liquid-crystalline phase of phospholipid vesicles. The general expression for the decay of a two-state system can be written in the following way:

$$a(t) = a_1 e^{-m_1 t} - a_2 e^{-m_2 t} \quad (2)$$

$$b(t) = b_1 e^{-m_1 t} - b_2 e^{-m_2 t} \quad (3)$$

$$a_1 = \frac{a_0(k_a - m_2) - b_0 k_{ab}}{m_1 - m_2} \quad (4)$$

$$a_2 = \frac{a_0(k_a - m_1) - b_0 k_{ab}}{m_1 - m_2} \quad (5)$$

$$b_1 = \frac{b_0(k_b - m_2) - a_0 k_{ba}}{m_1 - m_2} \quad (6)$$

$$b_2 = \frac{b_0(k_b - m_1) - a_0 k_{ba}}{m_1 - m_2} \quad (7)$$

$$m_1, m_2 = 1/2[k_a + k_b \pm \sqrt{(k_a - k_b)^2 + 4k_{ab}k_{ba}}], \quad (8)$$

where $a(t)$ and $b(t)$ represent the fluorescence emission from state a and b , respectively; k_a and k_b are the decay rates of a and b , k_{ab} and k_{ba} the interconversion rate from a to b and vice-versa, and a_0 and b_0 the initial excitation of state a and b , respectively. What we observe is the total fluorescence emission which, at any given observation condition, is the linear combination of the decay of $a(t)$ and $b(t)$, given the superposition of the two characteristic spectra. Let $B(t)$ and $R(t)$ be the total intensity decay observed under the observation conditions B and R , respectively. For the Laurdan case, these two conditions correspond to observation in the blue (B) and red (R) part of the emission spectrum. In the classical definition of polarization the two observation conditions correspond to the use of vertical and horizontal polarizers; here we use two emission filters.

$$B(t) = Ba(t) + (1 - B)b(t) \quad (9)$$

$$R(t) = Ra(t) + (1 - R)b(t) \quad (10)$$

B and R represent the relative observation of state $a(t)$ in the blue and red part of the emission spectrum, respectively. B and R have values between 0 and 1 because they represent relative fractional intensities. We can define three different generalized polarizations: the steady state, the time resolved, and the time zero generalized polarization.

$$\text{steady state} \quad \langle G \rangle = \frac{\langle B \rangle - \langle R \rangle}{\langle B \rangle + \langle R \rangle} \quad (11)$$

$$\text{time resolved} \quad G(t) = \frac{B(t) - R(t)}{B(t) + R(t)} \quad (12)$$

$$\text{time zero} \quad G_0 = \frac{B(0) - R(0)}{B(0) + R(0)}, \quad (13)$$

where $B(t)$ and $R(t)$ are defined by Eqs. 9 and 10, respectively, and the brackets indicate time average quantities. After substitution of Eqs. 9 and 10 in the definition of G , we obtain

$$G = \frac{(B - R)[a(t) - b(t)]}{(B + R)[a(t) - b(t)] + 2b(t)}. \quad (14)$$

It is convenient to separate the observation conditions contained in the values of B and R , from the physical description of the model, contained in the values of $a(t)$

and $b(t)$. After some manipulation, we obtain

$$\frac{B - R}{G} - (B + R) = \frac{2b(t)}{a(t) - b(t)}. \quad (15)$$

In this relationship the behavior of the physical system is in the right side of the equation and the details of how the system is observed is contained in the left side.

As an example, it can be shown that if B and R represent the parallel and perpendicular observation conditions, $a(t)$ represents the intensity decay in the parallel direction and $b(t)$ in the perpendicular direction, then we obtain the classical polarization equation. In the classical polarization case

$$\begin{aligned} B &= 1 \\ R &= 0 \\ a(t) &= [1 + 2r(t)]e^{-kt} \\ b(t) &= [1 - r(t)]e^{-kt} \\ P &= \frac{3r(t)}{2 + r(t)}, \end{aligned}$$

where $r(t)$ is the time-resolved anisotropy, P is the polarization, and k is the decay rate. Using the specific expression for the two-state system (Eqs. 3–8) we can obtain expressions for G directly in terms of the physical constant of the system, i.e., the initial excitation conditions, the decay rates, and the interconversion rates. The expression for the steady-state generalized polarization is the following:

$$\frac{B - R}{\langle G \rangle} - (B + R) = 2 \frac{b_0 + \frac{k_{ab}}{k_a}}{a_0 \frac{k_b}{k_a} - b_0 + \frac{k_{ba}}{k_a} - \frac{k_{ab}}{k_a}}. \quad (16)$$

In this expression, a_0 and b_0 , the initial excitation of states a and b , respectively, can be normalized such as $a_0 + b_0 = 1$. If we consider a system at equilibrium, then $k_{ba} = k_{ab}$, and Eq. 16 becomes

$$\frac{B - R}{\langle G \rangle} - (B + R) = 2 \frac{b_0 + \frac{k_{ab}}{k_a}}{a_0 \frac{k_b}{k_a} - b_0}. \quad (17)$$

The generalized polarization satisfies several important properties of the classical polarization, in particular Weber's law of addition of anisotropies and the Perrin equation.

RESULTS

The absorption spectrum of Laurdan in various solvents shows a single maximum. The sensitivity of Laurdan to the polar properties of the solvents can be illustrated by the Lippert plot reported in Fig. 1, obtained by measuring the difference between the emission maximum and the absorption maximum in solvents of different polarity.

Steady-state emission spectra of Laurdan in DPPC vesicles (data not shown) at various temperatures, using excitation wavelengths of 340 nm and 410 nm, show the shift of the emission maximum from 440 nm to 490 nm as the temperature increases (15). The spectrum in the gel phase is a factor of three more intense than the spectrum in the liquid-crystalline phase. The fluorescence intensity ratio for observation at 440 nm and 490 nm vs. temperature (Fig. 2) changes abruptly at the DPPC phase transition. The large change in the emission maximum is clearly visible to the naked eye: the sample is deep blue in the gel phase and greenish in the liquid-crystalline phase.

Excitation spectra of Laurdan in gel and in liquid-crystalline phase phospholipids have been obtained at 20°C (Fig. 3). This temperature corresponds to the gel phase for DPPC vesicles, to the liquid-crystalline phase for DLPC vesicles, and to a mixture of both coexisting phases for DLPC/DPPC vesicles. In DPPC vesicles at 20°C the excitation spectrum is characterized by two maxima at 360 nm and 390 nm, at all emission wavelengths. In the liquid-crystalline phase (DLPC vesicles at 20°C) the excitation spectrum has two maxima, at 350 nm and 380 nm, at all emission wavelengths. In the mixed

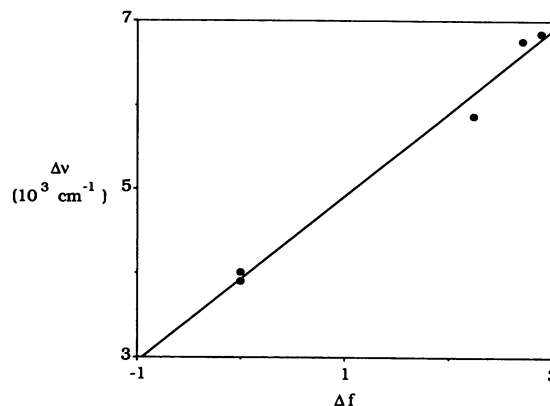


FIGURE 1 Lippert Plot for Laurdan in different solvents. From left to right: cyclohexane, dodecane, cyclohexanol, propanol, ethanol. $\Delta f = [(\epsilon - 1)/(2\epsilon + 1) - (n^2 - 1)/(2n^2 + 1)]$, where ϵ is the dielectric constant and n is the refractive index of the solvent, $\Delta\nu$ is the center of mass spectral shift in cm^{-1} .

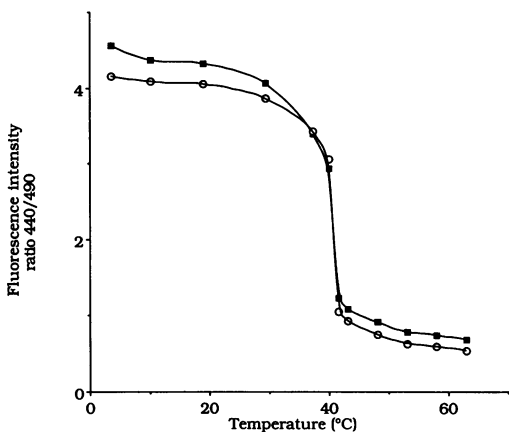


FIGURE 2 Laurdan fluorescence intensity ratio at 440 nm/490 nm in DPPC vesicles vs. temperature, by using excitation of 410 nm (○) and 340 nm (■).

phase (DLPC/DPPC vesicles) the two maxima are observed at 354 nm and 384 nm.

Emission spectra of Laurdan in single- and in mixed-phase phospholipids, at 20°C, have been obtained at two different excitation wavelengths, at the edges of the excitation spectrum, 325 nm and 400 nm, respectively (Fig. 4). In single-phase phospholipids, Laurdan emission was largely insensitive to the excitation wavelength, whereas in the mixed phase, the emission obtained by exciting the sample at 325 nm was red shifted with respect to that obtained with 400 nm excitation.

Although in principle fluorescence polarization should be independent of the excitation wavelength, for systems containing two different species, the polarization can be dependent upon the excitation wavelength. Laurdan

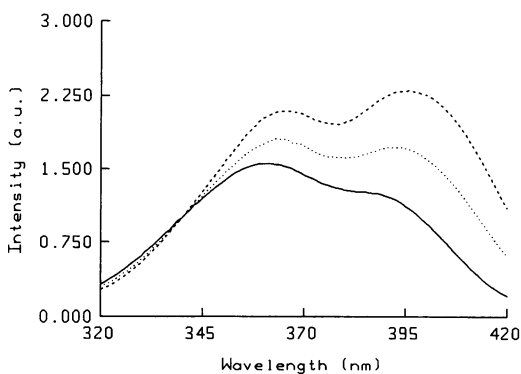


FIGURE 3 Excitation spectra of Laurdan in DPPC (---), in DLPC (—), and in 1:1 DLPC/DPPC (· · · · ·) at 20°C, emission at 450 nm. Emission and excitation bandwidth was 4 nm. Spectra are normalized at 340 nm.

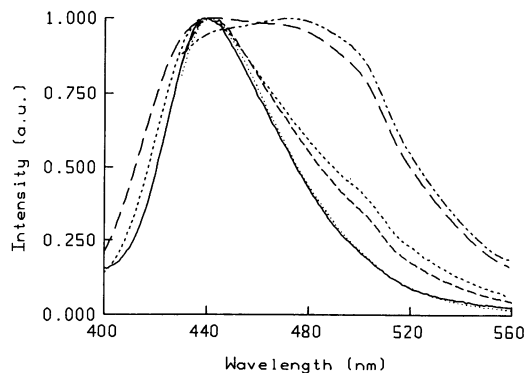


FIGURE 4 Normalized emission spectra of Laurdan (uncorrected) obtained at 20°C for DLPC (---; ·····), DPPC (—; ·····), and 1:1 DLPC/DPPC (-·-·-; -·-·-) using excitation at 325 nm and 400 nm, respectively. Emission and excitation bandwidth was 4 nm.

polarization emission spectra have been acquired in simple and mixed phase phospholipids, using different excitation wavelengths (Fig. 5). By excitation at the edge of the spectrum (325 nm and 400 nm), different polarization emission spectra were obtained for the different phases. In single-phase vesicles, the polarization was largely insensitive to the excitation wavelength (spectra ○, □, +, and △ in Fig. 5), whereas in mixed phase vesicles a 400-nm excitation wavelength selected populations with higher polarization, with respect to the 325-nm excitation (spectra ● and ▲ in Fig. 5).

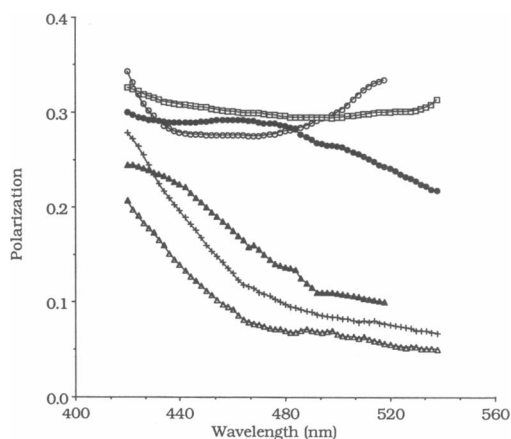


FIGURE 5 Laurdan polarization emission spectra obtained in (○) DPPC at 1°C, using 325-nm excitation; (□) DPPC at 1°C, using 400-nm excitation; (●) DLPC/DPPC = 1:1 at 20°C, using 400-nm excitation; (▲) DLPC/DPPC = 1:1 at 20°C, using 325-nm excitation; (+) DLPC at 20°C, using 400-nm excitation; and (△) DLPC at 20°C, using 325-nm excitation.

We have shown that the Laurdan excitation spectrum obtained in gel-phase vesicles shows a greater intensity in the red edge, with respect to the excitation spectrum obtained in the liquid-crystalline phase. This peculiar characteristic of Laurdan in the pure phases allows, in the mixed phase, preferential excitation of molecules in the gel phase environment, by excitation at 400 nm. This property is of interest in the case of vesicles composed of coexisting gel and liquid-crystalline domains such as 50% mixture of DLPC and DPPC and in the case of the transition temperature range of vesicles composed of a single phospholipid. By exciting at wavelengths corresponding to the maximum excitation of the gel phase and by observing the emission in the spectral region that preferentially selects the liquid-crystalline phase, we should be able to detect if interconversion between the two phases occurred during the Laurdan excited-state lifetime. With this purpose in mind, we acquired Laurdan emission spectra for the mixed phospholipid phase (DLPC-DPPC). If interconversion between phases does not occur during the Laurdan excited state, the emission spectra due to the linear superposition of the two phases should be observed when exciting at 400 nm. The emission spectrum obtained is shown in Fig. 4 and clearly contains contributions of two emitting species, corresponding to the two phospholipid phases. The contribution from the two phases cannot be accounted for by considering that we always excite a part of the spectra in both phases. To better detect the interconversion effect, we first consider the two pure phases. By measuring the emission at 440 nm and 490 nm for excitations at 340 nm and 410 nm, we can determine the characteristic generalized polarization G for the pure phase. Fig. 6 *a* shows that in DPPC in the gel phase, at 3.6°C, and excitation at 340 nm, for emissions at 440 nm and 490 nm G is 0.64, whereas exciting at 410 nm G is 0.61. In the DPPC liquid-crystalline phase, at 53.1°C, excitation at 340 nm and 410 nm gives G of -0.12 and -0.23 , respectively. In this way we can determine the characteristic G 's for each individual phase, at two different excitation wavelengths. If no interconversion between the phases occurs, G at each temperature should be the composition (using the law of addition of anisotropy) of the G 's for the two phases. However, inspection of Fig. 6 *b* reveals that there is a temperature dependence of G in the pure phases. This effect, which tends to decrease the G value as the temperature increases, can be attributed to the characteristic behavior of the Laurdan probe which is also sensitive to dipolar relaxations. To account for this temperature effect, we fit the G in the pure gel and in the pure liquid-crystalline phase to a straight line, with a slope of $\sim 0.002/C$ in the gel phase and $0.01/C$ in the liquid-crystalline phase. When the temperature variation is subtracted, the characteristic G in the two phases can be

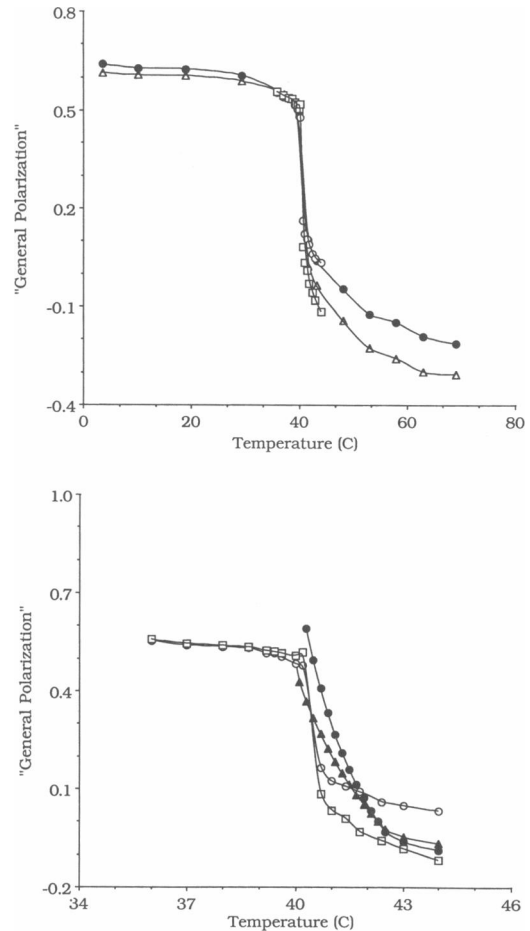


FIGURE 6 (a) Values of the "general polarization" obtained using Laurdan in DPPC vesicles using excitation at 340 nm (●, experiment 1 and ○, experiment 2) and using excitation at 410 nm (▲, experiment 1 and □, experiment 2). (b) Expanded plot for Laurdan in DPPC vesicles using excitation at 340 nm (○) and at 410 nm (□). Also reported are the simulated values of the general polarization where no interconversion (●) or an interconversion with a rate of 1/30 ns occurs (▲).

calculated and then we can compare the two values of G in the region of phase coexistence.

The value of G if no interconversion between phases occurs is given by Eq. 17 for $k_{ab} = 0$. This is shown in Fig. 6 *b* (●). When the interconversion is allowed, $k_{ab} = 0.03/\text{ns}$ (▲ in Fig. 6 *b*), then a different behavior is observed closer to the experimental points.

Experiments were performed to directly determine the characteristic interconversion time between the two phases using fluorescence time-resolved techniques. Time-resolved emission spectra of Laurdan are quite different in single- and mixed-phase phospholipids. In single-phase phospholipids, the spectral time evolution is different if the lipids are in the gel or in the liquid-crystalline phase. In DPPC vesicles, a small red shift of

the emission is observed as a function of time after excitation. Instead, the emission red shift is larger if Laurdan is inserted in DLPC vesicles, at 20°C. These results are similar to those reported in a previous study of Laurdan in DPPC at various temperatures (15).

The time evolution of the Laurdan emission spectrum in an equimolar DLPC-DPPC mixture, at 20°C, as a function of the time after excitation is reported in Fig. 7. Two families of emitting molecules can be distinguished. One emitting at higher energies, with spectra relatively blue, and the second emitting at lower energies, with red-shifted spectra. The second “red” family appears 30–40 ns after the excitation, and contemporarily, the intensity of the “blue” emitting family progressively decreases.

DISCUSSION

Our results show evidence from steady-state and time-resolved measurements, of phase interconversion in DPPC vesicles at the phase transition and in the DLPC-DPPC mixture. The fluorescence experiments are based on the different excitation and emission spectra of Laurdan in the gel and in the liquid-crystalline phase of phospholipid vesicles. Due to this peculiar property, it is possible, by photoselection, to introduce an initial “phase polarization” in the sample. The phase polarization can then relax to equilibrium by phase interconversion. It is this relaxation or mixing of the phases during the fluorescence excited state that carries information on phase fluctuations. Our results clearly indicate that interconversion is occurring. However, there is a number of problems that must be addressed before conclusive evidence can be

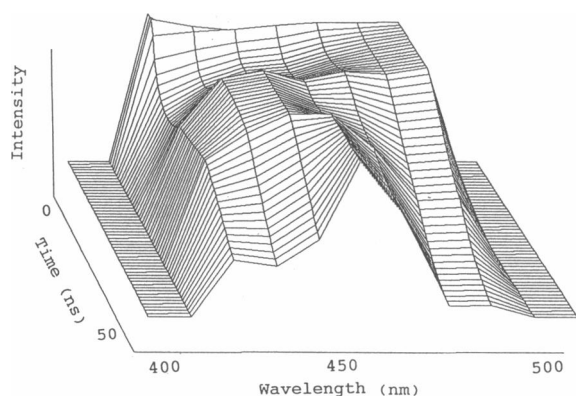


FIGURE 7 Laurdan time resolved emission spectra in the equimolar DLPC/DPPC mixture at 20°C. Excitation wavelength at 325 nm using a He Cd laser excitation.

obtained from the experiments. The first and most important one is related to the photophysical behavior of the probe. Laurdan is extremely sensitive to dipolar relaxation of the surrounding medium. Actually, the high sensitivity to different environments arises from this specific property. The problem is that dipolar relaxation processes produce time variations of the emission spectrum. The spectrum shifts as a function of time as the dipolar relaxation proceeds. The dipolar relaxation process always produces a time dependent red shift of the emission spectrum. Furthermore, due to the dipolar relaxation alone, photoselection occurs. The excitation selects those molecules which have a relaxed (red) or nonrelaxed (blue) ground state. How can we distinguish the dipolar relaxation process from the phase interconversion process? Our basic approach is to define a characteristic “gel spectrum” and a characteristic “liquid-crystalline spectrum”. The characteristic spectrum takes into account the dipolar relaxation process. The two characteristic spectra are obtained from vesicles in the pure phases. However, because dipolar relaxations are temperature dependent, the pure-phase spectra are also slightly temperature dependent. The temperature dependence of the G is clearly visible in Fig. 6 *a*, where the G values outside the transition region are not constant. The variation is not very large and we believe that, by interpolation, we can subtract this effect by measuring the G slope values above and below the transition. The most delicate part of this subtraction process is that we implicitly assume that the “slope” is the same in the transition region. This is equivalent to assuming that the characteristic properties of the gel and the liquid-crystalline phase can be extrapolated into the transition region. Then, after subtraction of the dipolar relaxation contribution, the only relaxation process left is the fluctuation between phases and this is what we have measured in this work. There is also a different check that we can perform on our data and is the photoselection of the liquid-crystalline phase. In this case, phase interconversion should produce a blue shift of the emission at longer times, due to the interconversion to the gel phase. This effect is clearly visible in Fig. 7, blue edge. With respect to the time-resolved results, there are two major difficulties in the interpretation. Firstly, dipolar relaxation processes are quite large in the liquid-crystalline phase and must be properly accounted for. Secondly, the interconversion rates that we have measured are ~ 5 – 6 probe decay rates. This is the aspect that worries us more: after 5–6 lifetimes, the total intensity has been reduced by a large amount (2 – 6×10^{-3}). However, the favorable circumstance is that we can observe at wavelengths where the time zero intensity is relatively small, and the intensity can even increase at that wavelength due to interconversion. Therefore, although the total intensity is small, the intensity ratio after 30 ns can be relatively large.

CONCLUSIONS

The peculiar spectroscopic properties of Laurdan open the possibility of studying phase fluctuations in membranes using steady-state fluorescence methods. The major complication is that the same spectroscopic property that makes Laurdan so sensitive to the environment also produces time-dependent effects that must be properly understood and subtracted from the overall time variation. We have presented an analytical method which allows us to quantitate both the relative amount of gel and liquid-crystalline phase and the fluctuations between the two phases, based on the definition of the generalized polarization. More work is required on different systems to conclusively prove the validity of our approach.

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