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MODULATION AND DYNAMICS OF PHASE PROPERTIES IN PHOSPHOLIPID MIXTURES DETECTED BY LAURDAN FLUORESCENCE*

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Abstract—Steady-state and dynamic fluorescence properties of 6-lauroyl-2-dimethylaminonaphthalene (Laurdan) have been used to ascertain the coexistence of separate phase domains and their dynamic properties in phospholipid vesicles composed of different mole ratios of dilauroyl- and dipalmitoyl-phosphatidylcholine (DLPC and DPPC, respectively). The recently introduced generalized polarization together with time-resolved emission spectra have been utilized for detecting changes. The results indicate the coexistence of phospholipid phase domains in vesicle compositions in the range between 30 mol% and 70 mol% DPPC in DLPC. Below and above these concentrations a homogeneous phase is observed, with averaged properties. In the case of coexisting phase domains, the properties of each individual phase are largely influenced by the presence of the other phase. Implications on fluctuations between the coexisting phases and on the size and shape of domains are discussed.

INTRODUCTION

Physical properties of phospholipids in the bilayer arrangement depend on their chemical structure. Increasing gel to liquid-crystalline phase transition temperatures are observed in phospholipids with higher numbers of carbon atoms in their acyl residues. Different polar heads can also vary the phospholipid transition temperature range, generally increasing with phosphatidylcholine (PC)‡ = phosphatidylglycerol (PG) < phosphatidic acid (PA) < phosphatidylserine (PS) < phosphatidylethanolamine (PE).¹ In biological membranes, because of the complex phospholipid composition, the physical properties of the membrane, such as its phase state, can either arise from the weighted average properties of the components or from domain segregation of phospholipids displaying nonideal miscibility, each domain possessing properties peculiar to its components.

The existence of separate phase-state domains in the plane of the membrane can be of relevance for the modulation of several cell physiological functions, for the diffusion dynamics of molecules in the membrane and through the membrane and for the partition and activity of membrane-associated

enzymes. “Fluidity” variations accompany several physiological and pathological events^{2,3} and can control the mobility and expression of proteins and receptors.^{4,5} In recent years, an increasing number of reports presented evidence for the existence of domains in biological membranes,^{6,7} with different lipid and protein compositions. The variation of Ca²⁺ ion concentration can also favor the creation of domains.^{8,9} Generally, the reported evidence refers to relatively large domains, possessing specialized functions, as in the case of epithelial cells,¹⁰ or reflecting peculiar functions such as lymphocyte capping.¹¹ In the present work we are mainly concerned with the existence of lipid phase-state domains in phospholipid multilamellar vesicles, to be related to the different molecular mobility of the lipids, and with the possibility of interconversion between these phase-state domains with characteristic kinetics.

Recently, lipid domains have been observed using fluorescence digital imaging microscopy.¹² This technique may not be suitable for the observation of small phase-state domains because of the inherent limited spatial resolution of visible light microscopy. Fluorescence spectroscopy can be profitably used for the assessment of the existence of membrane domains, their quantitation and the determination of the kinetics of interconversion between them.

Fluctuations between coexisting phases have been detected by studying the rotational behavior of the two isomers of parinaric acid in phospholipid vesicles.¹³ Critical density fluctuations have been determined at temperatures slightly below the main phase transition, with an estimated lifetime of about 30 ns. The rotational behavior of a long-lived fluorophore, coronene, has been studied in mixed-phase phospholipid bilayers,¹⁴ and dramatic changes have been observed after 20–200 ns from excitation, interpreted as phase fluctuations. Ultrasound technique has also been used with the purpose of detecting phase interconversion in phospholipid bilayers.¹⁵ The ultrasound absorption has been observed to increase at temperatures close to the phase transition and

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‡Abbreviations: DLPC: dilauroyl-phosphatidylcholine; DPPC: dipalmitoyl-phosphatidylcholine; Laurdan: 6-dodecanoyl-2-dimethylaminonaphthalene; GP: generalized polarization; POPOP: 2-2'-p-phenylenebis(5-phenyl)oxazole; PBS: phosphate-buffered saline solution; PC: phosphatidylcholine; PG: phosphatidylglycerol; PA: phosphatidic acid; PS: phosphatidylserine; PE: phosphatidylethanolamine.

has been associated with dissipative density fluctuations with kinetics of 20–60 ns. The above studies imply delicate measurements, and the kinetics of the fluctuation between the two phases is often estimated to occur in a relatively wide time range.

In the present study we utilize the sensitivity of the novel fluorescent membrane probe 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) to the polarity of its environment to detect phase-state domain coexistence. Laurdan steady-state emission maximum and spectral center of gravity strongly depend on the polarity of solvents,¹⁶ with the emission maximum at 387 nm in dodecane and at 503 nm in methanol.¹⁷ In phospholipid vesicles, the Laurdan emission maximum shows a 50 nm red shift by passing from the gel to the liquid-crystalline phase. The separation of Laurdan spectral components and the possibility of selectively exciting probe molecules in different environments have been used to develop the concept of generalized polarization (GP),^{16,17} expressed as:

$$GP = (B - R)/(B + R) \quad (1)$$

where B and R are the fluorescence intensities measured at the maximum emission characteristic of the probe in gel and liquid-crystalline phospholipids and typically at 440 nm and 490 nm, respectively. Analogous to the fluorescence polarization, after selectively exciting Laurdan molecules surrounded by lipids in the gel or in the liquid-crystalline phase, the GP value will reveal if the initial photoselection has been maintained or lost.

The physical origin of the large spectral shift in the Laurdan emission is generally referred to as solvent dipolar relaxation,¹⁸ indicating a solvent dipole reorientation around the fluorophore excited-state dipole, when the solvent molecular kinetics and the fluorophore lifetime are of the same order. The loss of energy required for the reorientation of solvent dipoles is reflected in the fluorophore emission red shift. By following the time evolution of the emission maximum or of the center of mass of the emission spectrum, detailed information on the dynamics of the surrounding dipoles can be obtained.

In previous work we reported time-resolved spectra of Laurdan in a 50 mol% mixture of dilauroyl- and dipalmitoyl-phosphatidylcholine (DLPC and DPPC, respectively) at 20°C. The emission spectrum was shifted toward higher wavelengths as the time after excitation increased. After about 30 ns from excitation, an inversion in the direction of the emission spectrum shift was observed and was interpreted as due to interconversion between the coexisting gel and liquid-crystalline phases.

In this work we present a more extensive study of the dipolar relaxation phenomenon in multilamellar vesicles composed of different concentrations of the two phospholipids. Our results show that phase domains exist in a wide range of phospholipid compositions, although the relaxation properties of Laurdan in one phase are influenced by the presence of the other phase. The recently introduced GP¹⁶ has been used to estimate the domain size by its value dependence on excitation and emission wavelength. Finally, a method to determine phase-state domain coexistence and to quantitate them is given by simple steady-state GP measurements at different excitation and emission wavelengths.

MATERIALS AND METHODS

Dilauroyl- (DLPC) and dipalmitoyl phosphatidylcholine (DPPC) from Avanti Polar Lipids, Inc. (Alabaster, AL) were used without further purification. Laurdan was from Molecular Probes, Inc. (Eugene, OR). Phosphate-buffered saline solution (PBS) was from Flow Laboratories (UK). Solvents were spectroscopic grade.

Multilamellar phospholipid vesicles were prepared by evaporating the appropriate amount of phospholipids and Laurdan solutions in chloroform under a nitrogen stream. The remaining film was then resuspended in PBS, capped under nitrogen, warmed above the phospholipid transition temperature and vortexed. All operations were performed in the dark or under red light. Samples were measured immediately after preparation. The final concentration of the total phospholipids was 0.3 mM and of the probe was 0.7 μM in the samples used for time-resolved experiments, 0.5 μM in samples used for lifetime measurements and 0.3 μM in samples used for steady-state measurements.

Fluorescence measurements. Fluorescence steady-state measurements were performed using a photon counting fluorometer (model GREG PC, ISS Inc., Champaign, IL). The sample compartment was kept at 20°C with a circulating water bath.

Fluorescence lifetime measurements were performed using an automated phase fluorometer equipped with an He-Cd laser (325 nm), and the temperature in the sample compartment was kept at 20°C by a circulating water bath. The emission wavelengths were changed from 410 nm to 550 nm in 10 nm steps by an automated monochromator with 16 nm bandwidth. 2-2'-p-Phenylenebis(5-phenyl)oxazole (POPOP) in ethanol was used as the reference ($\tau = 1.35$ ns). Lifetime measurements integrated over all the Laurdan emission spectra were performed using an ISS K2 fluorometer, equipped with a xenon arc lamp. The excitation was 340 nm, and the emission was collected after a Janos 375 cutoff filter. An additional bandpass filter (Corning 754) was used after the excitation monochromator. POPOP in ethanol was used as the reference. Data analysis was performed using the Globals Unlimited software (University of Illinois at Urbana-Champaign).¹⁹ Time-resolved spectra were generated using the software provided by ISS Inc.

RESULTS

Laurdan steady-state emission spectra obtained using an excitation wavelength of 340 nm, at 20°C, in pure DLPC and DPPC multilamellar vesicles and in mixtures of different composition of the two phospholipids are reported in Fig. 1A. As the concentration of DPPC in DLPC increases, a blue shift of the Laurdan emission spectrum can be observed. In Fig. 1B the steady-state excitation spectra for the same samples, at 20°C, are reported. By increasing the DPPC concentration in the vesicles, an increase in the intensity of the excitation red band can be observed, together with a slight decrease in the blue band.

Laurdan steady-state emission spectra have also been obtained at 20°C and in the same samples but using an excitation wavelength of 410 nm. The ratios between the two emission spectra, the one obtained using 340 nm excitation and the one obtained using 410 nm excitation, for each sample, are reported in Fig. 2. This figure clearly indicates that only spectral relaxation is taking place in the homogeneous liquid-crystalline phase, whereas phase coexistence is also present in the mixtures in the range between 30 mol% and 70 mol% DPPC in DLPC.

In Fig. 3 we report the Laurdan GP values obtained at 20°C in vesicles of various concentrations of DPPC in DLPC using 340 nm or 410 nm excitation and emission wavelengths of 440 nm and 490 nm. The GP was calculated by Eq. (1).

In Fig. 4 the time-resolved spectra of pure DLPC (A), of 30 mol% (B), 40 mol% (C), 70 mol% (D), 80 mol% (E) DPPC

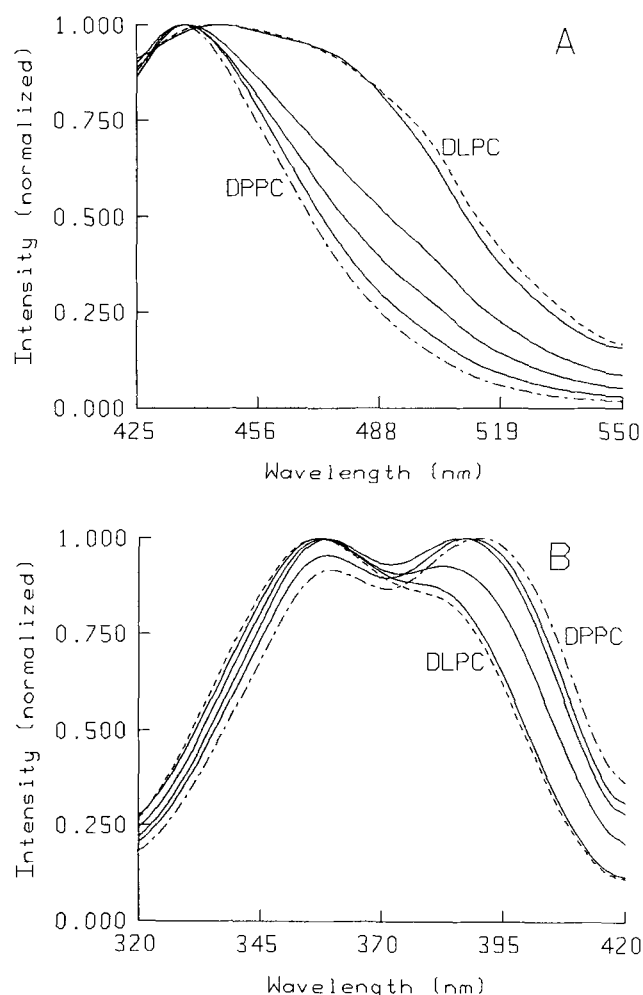


Figure 1. Laurdan steady-state emission (A) and excitation (B) spectra in multilamellar vesicles composed of various mol% of DPPC in DLPC, at 20°C. A. From left to right: DPPC, 80 mol%, 60 mol%, 40 mol%, 20 mol% DPPC in DLPC, DLPC; B. From left to right: DLPC, 20 mol%, 40 mol%, 60 mol%, 80 mol% DPPC in DLPC, DPPC. For excitation spectra the emission was 440 nm. For emission spectra the excitation was 340 nm. Bandwidths were 8 nm.

in DLPC and of pure DPPC (F) are reported. A progressive red shift of the emission maximum with time after excitation can be observed in samples of DLPC alone or where the DPPC concentration is relatively low. At high DPPC concentrations the Laurdan emission shows essentially the same shape at all times after excitation, with a very small shift toward higher wavelengths. In the sample with 40 mol% DPPC in DLPC we can distinguish two different directions of the emission shift, depending on the time after excitation. In the first 40 ns a red shift can be observed, whereas after this time the spectrum shifts slightly to the blue.

In Fig. 5 the center of gravity of the Laurdan emission spectrum as a function of time after excitation is reported for the different samples. For samples with a low DPPC concentration, a large shift of the center of mass is observed. In samples with high DPPC concentrations (70–100 mol%) the center of gravity shows a relatively small change with time. Simulated data, obtained by adding the proportional

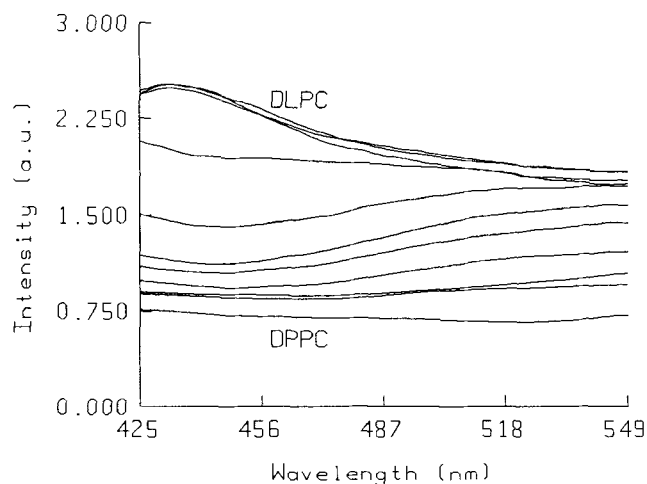


Figure 2. Ratio spectra between Laurdan steady-state emission spectra obtained using excitation at 340 nm and 410 nm, at 20°C. Bandwidths were 8 nm. From the top to the bottom spectra, the mol% of DPPC in DLPC was 0%, 10%, 15%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%.

contribution of each component and taking into account the difference of Laurdan lifetime value in the pure components, are also reported in Fig. 5.

DISCUSSION

Steady-state and time-resolved results clearly indicate that mixtures of various DLPC and DPPC concentrations have different spectroscopic properties than those expected from the simple addition of two separate phases, each independently contributing to the total signal, as shown by the sigmoidal curve of Fig. 3 and by the shift of the spectral center of mass in Fig. 5. The question arises whether the mixtures of different compositions behave as a homogeneous phase with intermediate properties or whether we can describe spectroscopic properties of the mixture using two separate

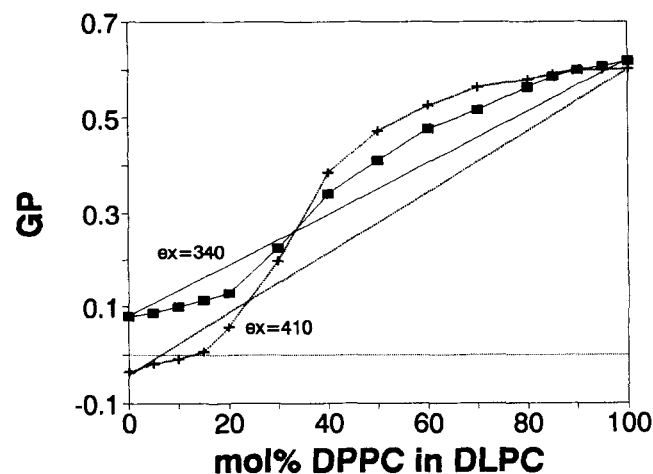


Figure 3. Laurdan GP values obtained using excitation of 340 nm (continuous line) and 410 nm (dotted line), at 20°C, in multilamellar vesicles as a function of mol% DPPC in DLPC. The straight lines represent the simulation of the simple superposition of the GP values of the pure DLPC and DPPC.

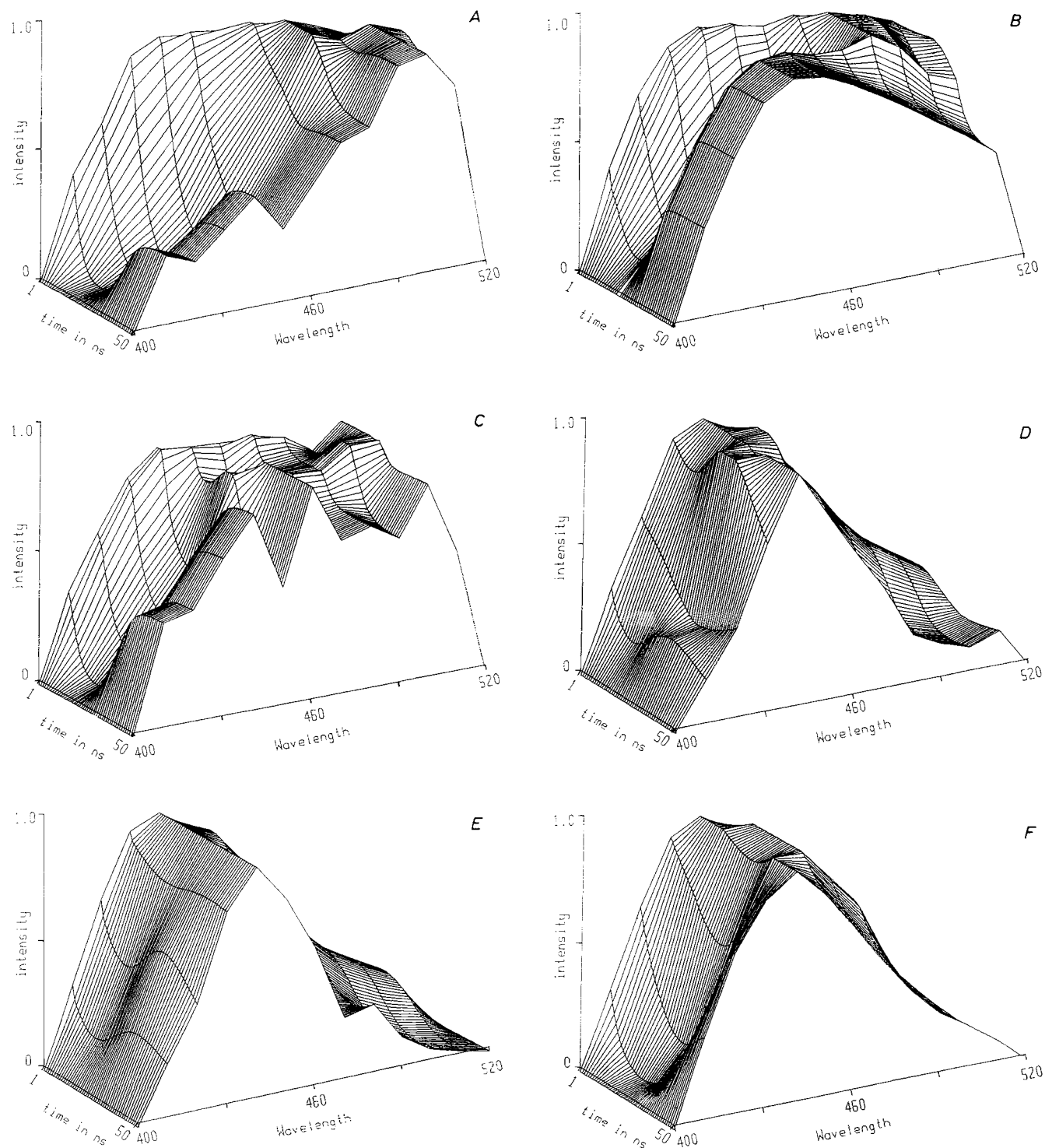


Figure 4. Normalized Laurdan time-resolved emission spectra in multilamellar vesicles of different concentrations of DPPC in DLPC, at 20°C. A. DLPC; B. 30 mol% DPPC; C. 40 mol% DPPC; D. 70 mol% DPPC; E. 80 mol% DPPC; F. DPPC. Time from 1 ns to 50 ns. Wavelengths from 400 nm to 530 nm. Intensity from 0 to 1.0.

sets of parameters that are only slightly different from those of the gel and of the liquid-crystalline phase, *i.e.* the gel phase is slightly modified by the presence of the liquid-crystalline phase, and *vice versa*.

First, we address the question concerning the existence of

a homogeneous phase with intermediate properties. We have observed that the emission spectrum depends on the wavelength of excitation (Fig. 3). There are two different reasons for the emission spectrum to depend on the excitation wavelength. One possibility is that there are at least two distinct

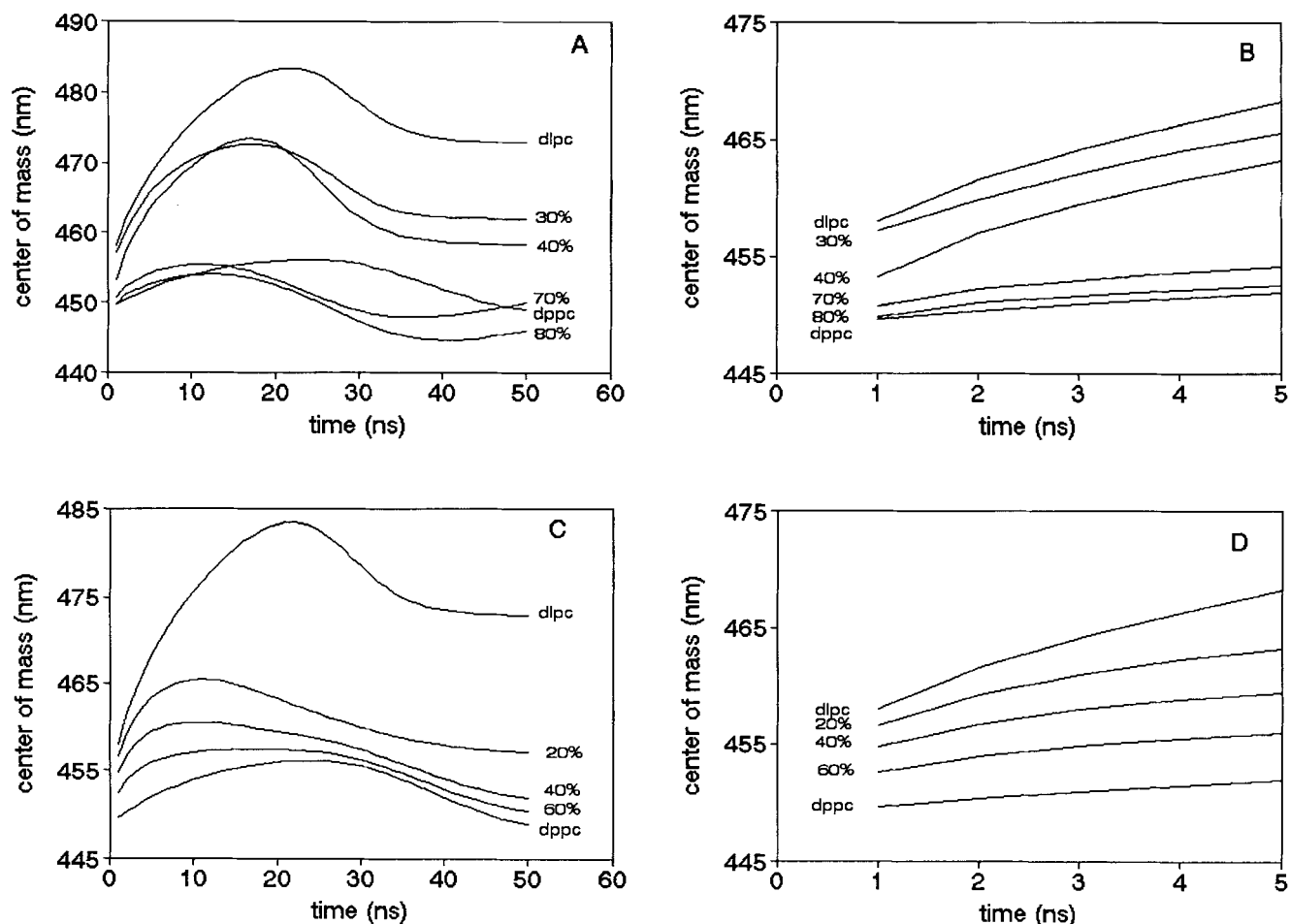


Figure 5. Center of mass of Laurdan emission spectra as a function of time after excitation in multilamellar vesicles of different mol% concentrations of DPPC in DLPC. A. and B.: experimental data. C. and D. data simulated by the superposition of the fractional contribution of the data from the pure components, taking into account the difference of Laurdan lifetime value in the two pure components. In the simulated plots the pure DPPC and the pure DLPC curve represent experimental data, reproduced for convenience.

species each one with its own excitation wavelength and emission spectrum. This would be the case for coexisting phase domains. A different reason for the emission spectrum to depend on the excitation wavelength is due to the process of dipolar relaxation. When excitation occurs in the blue part of the absorption spectrum there is a photoselection of molecules with energetically unfavorable orientation of the surrounding dipoles (Fig. 2, DLPC spectrum). These configurations can relax during the excited-state lifetime causing a time-dependent shift of the emission spectrum toward lower energies as the relaxation process proceeds. When excitation occurs in the red part of the absorption spectrum, more configurations with an energetically favorable orientation of the surrounding dipoles are selected. The resulting emission spectrum is already relaxed. Therefore the emission spectrum obtained with blue excitation will contain more intensity in the blue part of the emission spectrum as compared to the emission spectrum obtained using excitation in the red edge of the absorption spectrum. The ratio of the blue excited spectrum to the red excited spectrum has a characteristic wavelength dependence as shown in Fig. 2. In the case of two different molecular species, the wavelength dependence of the ratio between the emission spectrum excited in the

blue and that excited in the red will have a sigmoidal character, the details of which depend on the spectral characteristics of the two emitting species. In particular, Laurdan molecules surrounded by phospholipid in the gel phase are preferentially excited in the red part of the spectrum (above 390 nm, Fig. 1B), while Laurdan molecules surrounded by phospholipids in the liquid-crystalline phase are predominantly excited in the blue part of the excitation spectrum (below 350 nm, Fig. 1B). Therefore, the ratio spectra should have a sigmoidal pattern, with the red part larger than the blue part.

Of course, if one of the two species also relaxes, such as Laurdan in the liquid-crystalline phase, a more complicated pattern may result, as shown in Fig. 2. Using the Laurdan probe, it is particularly simple to distinguish between photoselection and relaxation because the two processes have opposite behaviors. Our results show that the photoselection capability persists at least in the range between 30 mol% and 70 mol% DPPC in DLPC. As Fig. 2 shows, in this composition range of the mixture, excitation at 410 nm results in a preferential selection of the gel spectrum. This result *per se* implies the existence of at least two classes of Laurdan molecules, one with properties similar to Laurdan in the gel

phase and the other with properties similar to Laurdan molecules in the liquid-crystalline phase. However, this qualitative observation does not imply that the spectroscopic properties are exactly those of the pure phases.

The above discussion excludes that there is a homogeneous phase in the mixture composition range between 30 mol% and 70 mol% DPPC, in agreement with a number of calorimetric and spectroscopic measurements on phospholipid mixtures.^{1,20} We can now proceed to estimate how much the properties of one phase are modified by the presence of the other phase, to give the observed signals at all mixture concentrations. Instead we will assume that below 30 mol% and above 70 mol% the results are compatible with the existence of a homogeneous phase.

Consider the 50 mol% mixture. To determine how much one phase influences the other, we first calculated the simple linear combination of the two phases. We then modified the values used for the linear combination to reproduce the experimental data. If there is no modification of the properties of one phase due to the presence of the other, then the GP of the mixture should be a linear composition of the GP of the pure gel and of the pure liquid-crystalline phase. Instead, Fig. 3 shows that in the 50 mol% mixture the GP value is higher than that expected by a simple addition. We have conducted simulations to quantify the modification of the GP values of the gel and of the liquid-crystalline phase necessary to reproduce the experimental value. The simulations have been performed using the addition rule of the GP given in Parasassi *et al.*¹⁷:

$$GP = \frac{xGP_g + (1-x)GP_lS_l}{xS_g + (1-x)S_l} \quad (2)$$

where x is the fraction of gel phase, GP_g and GP_l are the GP values in the gel and in the liquid-crystalline phase, respectively, and S_g and S_l represent the sum of the intensities at 440 nm and at 490 nm for the gel and for the liquid-crystalline phase, respectively.

To determine the fractional intensity for each phase necessary for the simulation, we separately measured the lifetime of the pure phases using excitation wavelength of 340 nm, and we found the values of 4.0 ns and 5.9 ns for the pure DLPC and DPPC, respectively. These measurements were obtained by integrating the emission band over all wavelengths in the respective emission spectra of the pure phospholipids. Furthermore, we have shown that there is no preferential partitioning of Laurdan between the two phases (in preparation). To reproduce the experimental values of the GP of the 50 mol% mixture, the simulation was performed using a GP value of 0.58 instead of 0.61 for pure DPPC and of 0.275 instead of 0.08 for pure DLPC. These are the best combinations of GP that fit the experimental curve at the 50 mol% point. We note that the GP values at small concentrations of DPPC in DLPC, up to about 20 mol% DPPC, closely correspond to the GP value of pure DLPC, and the GP values of the mixtures between 80 mol% and 95 mol% DPPC are close to the GP value of the pure DPPC. Therefore, if in the mixtures at low DPPC concentration, up to 20 mol%, there is a homogeneous phase in which DPPC is uniformly mixed with the DLPC, then the GP value of that hypothetical mixture still should be below 0.12. It is incompatible with the demonstrated photoselection capability that in the 50

mol% mixture there is so much DPPC in DLPC-rich domains to justify the large change in GP necessary to reproduce the experimental values.

Next we consider time-resolved properties. The spectroscopic parameter that we analyze is the time shift of the spectral center of mass (Fig. 5). Here we also want to determine how much the dynamic properties of one phase are modified by the presence of the other phase. To this purpose we first simulated the ideal mixture and then we modified the dynamic properties to reproduce the data of the hypothetical pure phases. Because the gel emission spectrum is blue and nonrelaxing, the gel component should decay with its own lifetime without changing the position of the emission maximum with time after excitation (Fig. 5A,B, plot of pure DPPC). Instead, the liquid-crystalline component will start in the blue and will shift to the red (Fig. 5A,B, plot of pure DLPC) as time progresses. The sum of the two time behaviors should be intermediate. We simulated data in which we superimposed two separate time-resolved spectral components, using the two measured behaviors of the spectral center of mass for the pure gel and the pure liquid-crystalline phase, with a contribution proportional to the lifetimes reported above for the pure components. The simulations are shown in Fig. 5C,D. Although, as expected, the simulated data reproduce some of the experimentally determined features, such as the red shift of the center of mass, the simple linear combination fails to give a quantitative agreement. To understand the time-resolved behavior in terms of the previous discussion about the GP value of the 50 mol% sample, we must consider that the GP is based on average intensities, *i.e.* only the initial decay up to the value of the lifetime contributes to the GP, whereas the time-resolved spectra are reconstructed up to several times the value of the lifetime. In fact, the initial part, up to about 3–5 ns of the decay, shows that the mixtures up to 30 mol% DPPC in DLPC behave similarly to the pure DLPC, and in the mixtures above 70 mol%, DPPC in DLPC behaves approximately as pure DPPC. Instead, in the mixtures of intermediate composition the behavior is quite different. As an example for our discussion consider the mixture composed of 40 mol% DPPC in DLPC. During the first 3–5 ns this mixture behaves according to that expected from the GP values, when we account for the differences due to the influence of one phase on the other. Instead, at longer lifetimes, the mixture appears to relax much more than expected from the simple addition of the two phases. This can only be due to the more fluid character of the gel phase, when in the presence of a large amount of DLPC, that causes a substantial shortening of the average lifetime of Laurdan in the gel phase. As a consequence, the influence of the gel phase emission is reduced with respect to that expected from the simple addition of the properties of the pure phases. When we extrapolate this effect to relatively long time after excitation, up to 50 ns, which is what we do in constructing time-resolved spectra, the enhanced relaxation in the gel phase has a major effect on the long time behavior.

The crucial observation resulting from the above discussion is that either the properties of the gel and of the liquid-crystalline phase are dramatically modified by the presence of the other phase in the range of mixture composition be-

tween 30 mol% and 70 mol%, or we must propose an appropriate model to explain the fluorescence measurements.

We present two different models that can qualitatively explain the results, without assuming a very strong influence of one phase on the other.

1) The possibility exists that instead of forming a homogeneous phase, as we have assumed, a small amount of DPPC in DLPC forms a phase that has a particular topology such as not to display the fluorescence properties of the gel phase, for example filaments of a gel in a liquid-crystalline phase. The same kind of topology should occur when there is a small concentration of DLPC in DPPC. Because the properties of the mixture at 50 mol% are more gel-like, using the same kind of reasoning, we must assume that there is an interdigitation of the liquid-crystalline phase in the gel phase. Of course, we recognize that this is an *ad hoc* hypothesis, which requires further investigation.

2) Fast interconversion between phases. This model assumes that a Laurdan molecule can diffuse a few Ångströms during its excited-state lifetime. The diffusion coefficients of pyrene in phospholipid vesicles have been recently reported by Vauhkonen *et al.*²¹ and by Sassaroli *et al.*²² to be different in the two phases, being on the order of 4×10^{-8} cm²/s and of 0.8×10^{-8} cm²/s in the liquid-crystalline and gel phases, respectively. Using the Smolukowsky–Einstein relationship and assuming a similar diffusion coefficient for Laurdan in the two dimensions, a motion of about 6–10 Å in the membrane plane is possible during approximately 10 ns in the liquid-crystalline phase, whereas no motion should occur in the gel phase. When excitation occurs at wavelengths where there is a preferential absorption of Laurdan molecules in the liquid-crystalline phase, there is a re-equilibration between the phases during the excited-state lifetime. This diffusion process can bring excited Laurdan molecules from the liquid-crystalline phase to the gel. This transient migration can result in an apparent increase in the fraction of molecules fluorescing from the gel phase. If excitation occurs at wavelengths where Laurdan molecules in the gel phase are selected, the reverse process should in principle occur, but the resulting effect is smaller, due to the difference in diffusion coefficients between the two phases. This diffusion process can explain qualitatively the different behaviors at the two excitation wavelengths of Fig. 3 in which excitation at 410 nm selects Laurdan molecules in the gel phase, and this results in an apparent preferential partitioning. For this mechanism to be efficient, the individual domains must be very small. The area-to-perimeter ratio must be as small as possible. Again, either we have a filament type of topology or a distribution of patches with some of them very small, of about 20–50 Å, to allow efficient transfer to the other phase. This estimation is based on the approximate values of diffusion coefficients of similar molecules and on the observation that a relatively large fraction of Laurdan molecules migrate to the other phase during their excited-state lifetime. This picture is in line with molecular dynamics calculations performed in homogeneous phase at the phase transition temperature,²³ which predicts the existence of very small patches of one phase into the other.

In conclusion, among the two above models the one concerning the peculiar topology of the mixing of the different phases requires a specific hypothesis to explain the data. On

the other hand, the fast interconversion model is based on the physical process of diffusion between coexisting domains with sharp boundaries. In both models domain coexistence must be assumed. The hypothesis that there is an averaged phase that displays properties that are neither of the gel nor of the liquid-crystalline phase is not compatible with the photoselection experiments. Finally, the photoselection results strongly suggest the coexistence of separate gel and liquid-crystalline domains in the same vesicles, in a wide range of phospholipid compositions (from 30 mol% to 70 mol% DPPC in DLPC). The domains must be relatively small to explain the data (from 20 Å to 50 Å). In addition, the properties of the gel and liquid-crystalline phases in the presence of the other phase are substantially different from the properties of the pure phases.

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REFERENCES

1. Lee, A. G. (1977) Lipid phase transitions and phase diagrams I. Lipid phase transitions. *Biochim. Biophys. Acta* **472**, 237–281.
2. Gasser, K. W., A. Goldsmith and U. Hopfer (1990) Regulation of chloride transport in parotid secretory granules by membrane fluidity. *Biochemistry* **29**, 7282–7288.
3. Klein, A., L. Mercure, P. Gordon, B. Bruser, S. Ramcharitar, A. Malkin and M. A. Wainberg (1990) The effect of HIV-1 infection on the lipid fatty acid content in the membrane of cultured lymphocytes. *AIDS* **4**, 865–867.
4. Conforti, G., A. Zanetti, I. Pasquali-Ronchetti, D. Quaglino, Jr., P. Neyroz and E. Dejana (1990) Modulation of vitronectin receptor binding by membrane lipid composition. *J. Biol. Chem.* **265**, 4011–4019.
5. Kuo, P., M. Weinfeld and J. Loscalzo (1990) Effect of membrane fatty acyl composition on LDL metabolism in Hep G2 hepatocytes. *Biochemistry* **29**, 6626–6632.
6. Orci, L., B. Thorens, M. Ravazzola and H. F. Lodish (1989) Localization of the pancreatic beta cell glucose transporter to specific plasma membrane domains. *Science* **245**, 295–297.
7. Finzi, L., C. Bustamante, G. Garab and C. B. Juang (1989) Direct observation of large chiral domains in chloroplast thylakoid membranes by differential polarization microscopy. *Proc. Natl. Acad. Sci. USA* **86**, 8748–8752.
8. Haverstick, D. and M. Glaser (1987) Visualization of Ca²⁺-induced phospholipid domains. *Proc. Natl. Acad. Sci. USA* **84**, 4475–4479.
9. Metcalf, T. N., III, J. L. Wang and M. Schindler (1986) Lateral diffusion of phospholipids in the plasma membrane of soybean protoplasts: evidence for membrane lipid domains. *Proc. Natl. Acad. Sci. USA* **83**, 95–99.
10. Simons, K. and G. V. Meer (1988) Lipid sorting in epithelial cells. *Biochemistry* **27**, 6197–6202.
11. Tomita-Yamaguchi, M., C. Rubio and T. J. Santoro (1991) Regional influences on the physical properties of T cell membranes. *Life Sci.* **48**, 433–438.
12. Rodgers, W. and M. Glaser (1991) Characterization of lipid domains in erythrocyte membranes. *Proc. Natl. Acad. Sci. USA* **88**, 1364–1368.
13. Ruggiero, A. and B. Hudson (1989) Critical density fluctuations in lipid bilayers detected by fluorescence lifetime heterogeneity. *Biophys. J.* **55**, 1111–1124.
14. Davenport, L., J. R. Knutson and L. Brand (1988) Time-resolved fluorescence anisotropy of membrane probes: rotations gated by packing fluctuations. In *Time-Resolved Laser Spectroscopy in Biochemistry* (Edited by J. R. Lakowicz), pp. 263–270. Proc. SPIE 909.
15. Mitaku, S., T. Jippo and R. Kataoka (1983) Thermodynamic

- properties of the lipid bilayer transition. *Biophys. J.* **42**, 137–144.
16. Parasassi, T., G. De Stasio, A. d'Ubaldo and E. Gratton (1990) Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophys. J.* **57**, 1179–1186.
 17. Parasassi, T., G. De Stasio, G. Ravagnan, R. M. Rusch and E. Gratton (1991) Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. *Biophys. J.* **60**, 179–189.
 18. Macgregor, R. B. and G. Weber (1981) Fluorophores in polar media: spectral effects of the Langevin distribution of electrostatic interactions. *Ann. N.Y. Acad. Sci.* **366**, 140–154.
 19. Beechem, J. M. and E. Gratton (1988) Fluorescence spectroscopy data analysis environment: a second generation global analysis program. *Proc. Soc. Photo-Optical Instrument. Eng.* **909**, 70–82.
 20. Shimshick, E. J. and H. M. McConnell (1973) Lateral phase separation in membranes. *Biochemistry* **15**, 4529–4537.
 21. Vauhkonen, M., M. Sassaroli, P. Somerharju, and J. Eisinger (1990) Dipyranylphosphatidylcholines as membrane fluidity probes. Relationship between intramolecular and intermolecular excimer formation rates. *Biophys. J.* **57**, 291–300.
 22. Sassaroli, M., M. Vauhkonen, D. Perry and J. Eisinger (1990) Lateral diffusivity of lipid analogue excimer probes in dimyristoylphosphatidylcholine bilayers. *Biophys. J.* **57**, 281–290.
 23. Ipsen, J. H., K. Jørgensen and O. G. Mouritsen (1990) Density fluctuations in saturated phospholipid bilayers increase as the acyl-chain length decreases. *Biophys. J.* **58**, 1099–1107.