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Abrupt Modifications of Phospholipid Bilayer Properties at Critical Cholesterol Concentrations

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ABSTRACT The fluorescence generalized polarization (GP) of 2-dimethylamino-6-lauroylnaphthalene (Laurdan) reveals different effects of cholesterol on the phase behavior of phospholipid bilayers. Phospholipid vesicles composed of gel, liquid-crystalline, and coexisting domains of the two phases have been studied at temperatures from 1 to 65°C, without cholesterol and with cholesterol concentrations of 3–50 mol %. Laurdan GP measurements show the general effect of cholesterol of increasing the molecular dynamics of the gel and of decreasing the molecular dynamics of the liquid-crystalline phase. In the liquid-crystalline phase, the increased order yields Laurdan GP values close to those obtained in the gel phase. At cholesterol concentrations >15 mol % a phase transition cannot be detected. Using the wavelength dependence of the excitation and emission GP spectra we determine that differences between the two phospholipid phases cannot be detected. In particular, in vesicles composed of coexisting gel and liquid-crystalline phases the GP wavelength dependence characteristic of coexisting domains cannot be observed at cholesterol concentrations ≥15 mol %. Cholesterol causes the decrease in both the polarity and the dipolar relaxation effects on the neighborhood of the fluorescent naphthalene moiety of Laurdan. Probably because of a cholesterol-induced increase in the bilayer packing, these effects do not occur continuously with the increase of cholesterol concentration in the bilayer. Cholesterol concentrations inducing higher Laurdan GP values have been determined at about 5, 10, 15, 30, and 45 mol % with respect to phospholipids. We propose that the formation of ordered molecular microdomains at critical cholesterol concentrations can explain the occurrence of the observed discontinuities.

INTRODUCTION

Cholesterol has a profound influence on the phase properties of phospholipid bilayers. Variations of several structural and dynamical parameters of the bilayer have been observed in the presence of cholesterol. From phase diagrams of phospholipids and cholesterol, relevant modifications of the phase state have been observed at specific cholesterol concentrations (Tampé et al., 1991; Vist and Davis, 1990; Sankaram and Thompson, 1990; Almeida et al., 1993). These variations have been interpreted as arising from specific complexes between phospholipids and cholesterol in the molar ratios 3:1, 2:1, and 1:1 (Mabrey et al., 1978; Melchior et al., 1980). Ipsen et al. (1987 and 1989) proposed an alternative model that does not require specific complexes between cholesterol and phospholipids. The essential aspect of this model accounts for the different interactions between cholesterol and both the conformational and crystalline degrees of freedom of the lipid molecules. Phase diagrams of cholesterol in dimyristoyl phosphatidylcholine (DMPC) vesicles (Tampé et al., 1991), in deuterated dipalmitoyl PC (DPPC) vesicles (Vist and Davis, 1990), partial phase diagrams of cholesterol in DPPC and sphingomyelin mixed vesicles (Sankaram and Thompson, 1990), and ternary-phase diagrams of DMPC, disaturated PC, and cholesterol (Almeida et al., 1993), constructed on the basis of nuclear magnetic resonance, electron paramagnetic resonance, differential scanning calorimetry, and fluorescence data have been reinterpreted by a thermodynamic and a microscopic interaction model (Ipsen et al., 1987 and 1989; Mouritsen, 1991). To a first approximation, for cholesterol concentrations up to about 6 mol %, there is an almost ideal mixing between cholesterol and phospholipids. Above 6 mol %, cholesterol has a disordering effect on the gel phase and an ordering effect on the liquid-crystalline phase. Specifically, in the gel phase, lateral diffusion and axial rotation are increased by cholesterol. In the liquid-crystalline phase, axial rotation is decreased. Above a cholesterol concentration of 30 mol % in the liquid-crystalline phase, lateral diffusion is also decreased. The basic concept in this interpretation of cholesterol effect on phospholipid dynamical properties can be summarized as the property of decoupling the lateral diffusion from the axial rotation. The liquid-ordered phase, occurring at cholesterol concentrations >25 mol %, namely, at concentrations considered to be well within the limits of physiological cholesterol concentrations (Yeagle, 1985), has been described as "liquid" from the point of view of the lateral diffusion and as "ordered" from the point of view of the axial rotation of acyl chains.

Following the above phase diagrams, the coexistence of the gel and of the liquid-crystalline phase can only be observed at a cholesterol concentration <6 mol % in a narrow temperature interval, about 1°C, corresponding to the transition temperature of the phospholipid (Tampé et al., 1991; Vist and Davis, 1990). Regarding Laurdan (2-dimethylamino-6-lauroylnaphthalene) generalized polarization (GP) data in an equimolar nonideal mixture of two phospholipids,

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evidence of phase separation can only be obtained for cholesterol concentrations ≤15 mol % (Parasassi et al., 1994a). In biological membranes, once the method for a rapid and facile detection of phase domains was established by means of Laurdan GP measurements, no evidence was obtained for the presence of gel-phase domains (Parasassi et al., 1993a). Instead, the fluorescence parameters arising from the membranes of whole cells resemble those obtained in phospholipids in the presence of cholesterol concentrations ≥15 mol %, with homogeneous properties that are fluid, i.e., liquid-crystalline-like, and for other properties also relatively rigid (Parasassi et al., 1994a). Qualitatively, the results obtained using Laurdan fluorescence are in agreement with the above reported description of the liquid-ordered phase.

Studies performed using Laurdan fluorescence properties in binary and ternary systems composed of vesicles of phospholipids and cholesterol (Parasassi et al., 1994a,b) showed some general features of the modifications introduced by cholesterol on the phospholipid phase behavior. 1) In vesicles displaying coexistence of gel and liquid-crystalline phases, the differences between these two phases are abolished by cholesterol concentrations ≥15 mol %. 2) The dynamic properties of the bilayer depend in a nonlinear fashion upon cholesterol concentration. 3) Specific cholesterol concentrations inducing abrupt dynamical changes in the membrane bilayer can be identified.

Other studies, using different guest molecules in the phospholipid bilayer, but at relative concentrations similar to those we have used for cholesterol, have shown that there is a regular pattern relating the total cholesterol concentration to the observed effects. Recent results from Tang and Chong (1992) and Somerharju et al. (1985) indicated a peculiar behavior of the excimer-to-monomer ratio when pyrene-PC is added to a model membrane in the liquid-crystalline state. In particular, at some specific concentrations of pyrene-PC the amount of excimer drastically decreased. These results were interpreted in terms of the formation of an ordered microstructure in which diffusion of pyrene-PC is inhibited, at least during the excited-state lifetime of the probe, i.e., ~200 ns. A theory has been proposed by Virtanen et al. (1988) that predicts the critical pyrene-PC concentrations at which the changes in excimer-to-monomer ratio should occur. The theory is based on the regular distribution of pyrene-PC into a hexagonal superlattice. Following this theory, the steric hindrance due to the large pyrene moiety causes the guest molecules to separate maximally to minimize the total free energy. A surprising agreement has been found between the particular concentrations of pyrene-PC that yield the dips of the excimer/monomer ratio and the cholesterol concentrations resulting in abrupt modification of Laurdan spectral features (Parasassi et al., 1994b).

Laurdan is a membrane probe that displays spectral sensitivity to the polarity of the environment (Parasassi et al., 1990). In polar solvents Laurdan displays a very large red spectral shift of the emission maximum and a high ratio of the red to the blue excitation maximum. When inserted into the membrane, Laurdan aligns its lauroyl tail with the lipid moiety and locates its naphthalene ring at the phospholipid glycerol backbone. Contrary to other similar probes such as Prodan (which has a propionic acid group in place of the lauric acid group of Laurdan) that can also locate at the membrane surface (Chong, 1988), Laurdan is only found in the membrane interior (Parasassi et al., 1994c). Laurdan is virtually non-fluorescent in aqueous environments, with a fluorescence lifetime of <100 ps in water. In organic solvents and in membranes, Laurdan displays a strong fluorescence with an average lifetime of about 4–8 ns, depending on the solvent. One of the advantages of using Laurdan is that steady-state fluorescence intensity measurements suffice to obtain information about the polarity and the dynamics of its environment (Parasassi et al., 1992, 1993). Of course, these results were achieved after extensive characterization of this probe using time-resolved studies, but now we can confidently assign dynamic effects, in many cases in a quantitative manner, using only steady-state measurements. In each of the phospholipid phases, namely, the gel and the liquid-crystalline, both excitation and emission spectra of Laurdan show typical features. In the gel phase the excitation maximum is at 390 nm, and the emission maximum at 440 nm. In the liquid-crystalline phase the excitation maximum is at 355 nm, and the emission maximum at 490 nm. In phospholipid vesicles composed of two nonmiscible phospholipids such as DPPC and dilauraylPC (DLPC) DPC, we can observe the signals produced from two separate phases, hence demonstrating phase coexistence in those well-known binary systems (Parasassi et al., 1990).

Laurdan spectral features have been described by the GP:

$$GP=(I_g - I_{lc})/(I_g + I_{lc}) \quad (1)$$

where I_g is the fluorescence intensity at the excitation or emission maximum in the gel phase, and I_{lc} is the fluorescence intensity at the excitation or emission maximum in the liquid-crystalline phase (Parasassi et al., 1990). Dynamical and steady-state information similar to that contained in the fluorescence polarization is also obtainable from the GP, as, e.g., the dipolar relaxation rate of the probe and time-dependent modifications of the spectral shape. The property of additivity of the GP can be used to quantitate different coexisting phases in samples of unknown composition (Parasassi et al., 1991). Moreover, it has been shown that the wavelength dependence of the GP value can be used to determine the coexistence of domains of different phase states in the membrane. An opposite wavelength dependence of the GP value is observed in pure and in mixed-phase phospholipids, respectively (Parasassi et al., 1993).

The major focus in the description of membrane structure performed using Laurdan and also other fluorescent probes and spectroscopic techniques has been on ordered and disordered structures, gel-like and liquid-crystalline-like. Although this description may be adequate when only one type of phospholipid is present in the membrane, for more complex systems including mixtures with cholesterol and different types of phospholipids, additional structures can be formed, i.e., phases with new properties and microscopic

heterogeneous structures. The focus of this work is on the identification and characterization of these microstructures using Laurdan GP values. Experiments have been performed in binary and ternary systems composed of phospholipids and increasing cholesterol concentrations, by 5-mol % steps, from 5 to 50 mol %. Discontinuities between the increase of cholesterol concentration and the modifications introduced in the properties of the bilayer have been detected.

MATERIALS AND METHODS

Multilamellar phospholipid vesicles were prepared by mixing the appropriate amounts of solutions in chloroform (spectroscopic grade) of phospholipids (Avanti Polar Lipids, Inc., Alabaster, AL) with or without cholesterol (Sigma Chemical Co., St. Louis, MO) and Laurdan (Molecular Probes Inc., Eugene, OR), then evaporating the solvent by nitrogen flow. The dried samples were resuspended in Dulbecco's phosphate-buffered saline (PBS) solution, pH 7.4 (Sigma Chemical Co.), heated above the transition temperature, and vortexed. All samples were prepared in red light and used immediately after preparation. The final lipids and probe concentrations were 0.3 mM and 0.3 μM, respectively.

Laurdan excitation and emission spectra in cells were obtained by using a GREG 200 fluorometer with a xenon arc lamp as the light source and the accompanying software (ISS Inc., Champaign, IL). The fluorometer was equipped with photon-counting electronics PX01 (ISS Inc.). Bandpasses of the monochromators were 4 nm. Temperature was controlled to ± 0.1°C with a water-circulating bath. The spectra were only corrected for the xenon lamp-intensity fluctuations. The excitation GP value was calculated using:

$$\text{Excitation GP} = (I_{440} - I_{490})/(I_{440} + I_{490}) \quad (2)$$

(2) where I_{440} and I_{490} are the excitation intensities at 370 nm, measured using emission wavelength of 440 and 490 nm, respectively. The choice of the emission wavelengths for the calculation of GP values was based on the characteristic emission wavelengths of pure gel and liquid-crystalline phases (Parasassi et al., 1990). The emission GP value was calculated using:

$$\text{Emission GP} = (I_{410} - I_{340})/(I_{410} + I_{340}) \quad (3)$$

(3) where I_{410} and I_{340} are the emission intensities at 490 nm, measured using excitation wavelength of 410 nm and 340 nm, respectively. The excitation wavelengths were chosen so as to obtain the maximum separation of Laurdan excitation bands in the gel (390-nm band) and in both phospholipid phases (355-nm band). Excitation and emission GP spectra were obtained by the same procedure, calculating the GP values for each excitation and emission wavelength, respectively.

Surface plots have been obtained using the software Axum from TriMetrix Inc. The underlying grid has been obtained from the same data of the plots, using the 3D spline option of the Axum software. Some of the experiments were repeated in two different laboratories (those of E. G. and T. P.) with identical results.

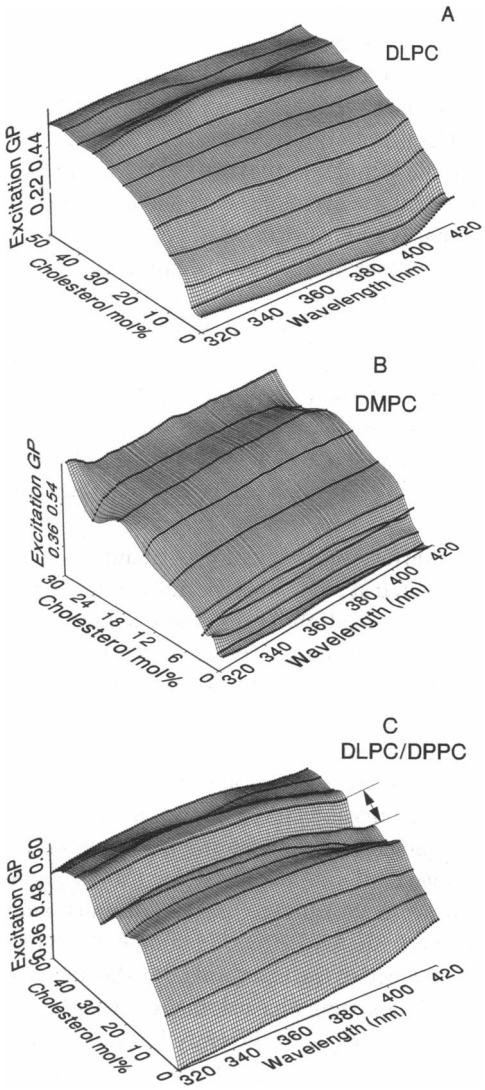

FIGURE 1 Surface representation of Laurdan excitation GP spectra obtained at 25°C as a function cholesterol concentration in phospholipid vesicles composed of DLPC (*A*), DMPC (*B*), and an equimolar mixture of DLPC and DPPC (*C*). The excitation GP spectra have been calculated using Eq. 2. The surface has been generated using the 3D spline surface option of the Axum software. The individual GP spectra of samples at each cholesterol concentration are superimposed. Cholesterol concentrations were: 0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mol % for DLPC; 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 mol % for the DMPC sample; and 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mol % for the DLPC-DPPC sample. *Arrows* (*C*) represent the cholesterol region that has been expanded by 1 mol % cholesterol increase (shown in Fig. 7 *A*, below).

RESULTS

The effect of various cholesterol concentrations in phase transition of phospholipid bilayers has been studied in multilamellar vesicles composed of three kinds of phospholipids: 1) pure liquid-crystalline phase (DLPC vesicles); 2) a phospholipid that, in the temperature range investigated, 1–65°C, displays both phases and the transition between them (DMPC vesicles); and 3) an equimolar mixture of phospholipids where the coexistence of the two phases occurs in the temperature range between 15 and 35°C (DLPC and DPPC mixture).

In Fig. 1, surface representations of Laurdan excitation GP spectra, measured at 25°C, are shown for multilamellar vesicles composed of DLPC at cholesterol concentrations of 0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mol % (Fig. 1 *A*), of DMPC at cholesterol concentrations of 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 mol % (Fig. 1 *B*) and of an equimolar mixture of DLPC and DPPC at cholesterol concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mol % (Fig. 1 *C*). The increase of cholesterol concentration in all the three samples causes the increase of the excitation GP value in the spectra. Notably, the increase of GP values is not monotonic with the increase of cholesterol concentration. A plateau can

be identified at cholesterol concentrations ≥20 mol % cholesterol. Further addition of cholesterol corresponds to a lesser increase of the excitation GP values. Moreover, some peculiar discontinuities can be observed, corresponding to cholesterol concentrations between 25 and 35 mol %, depending on the sample. In the DLPC/DPPC sample, the disappearance of the wavelength dependence of the excitation GP value that is typical of a coexistence of different phase domains (Parasassi et al., 1993a) can also be observed at cholesterol concentrations ≥15 mol % (Fig. 1 *C*). In the absence of cholesterol, the excitation GP value increases with excitation wavelength. This behavior can be observed in the presence of cholesterol up to the concentration ≤15 mol %, and then no appreciable wavelength dependence of the GP value can be detected. The absence of a wavelength dependence of the GP value can be only observed in the pure gel phase.

In Fig. 2, surface representations of Laurdan emission GP spectra, measured at 25°C, are reported for the same samples. The results described above for the excitation spectra are more evident in the emission GP spectra. In general, the emission GP spectrum was shown to be more sensitive to the effect of cholesterol than the excitation GP spectrum. Following previous observations, 1) the emission GP values increase with the increase of cholesterol in the samples; 2) the increase of the emission GP values is not monotonic with the increase of cholesterol concentration; 3) peculiar concentrations of cholesterol can be clearly identified, giving rise to discontinuities in the surface, peaks, plateaus, and valleys; and 4) finally, in the DLPC/DPPC sample, the typical wavelength dependence of the emission GP value that is observed in the case of coexisting phase domains, showing decreasing values with the increase of the emission wavelength, can be observed only at low cholesterol concentrations, up to about 15 mol % (Fig. 2 *C*).

The excitation GP value, at 370 nm, of the three samples, has been plotted as a function of temperature, from 1 to 65°C, and as a function of cholesterol concentration (Fig. 3). The variation of the emission GP value at 490 nm, as a function of temperature and of cholesterol concentration, is reported in Fig. 4. The wavelengths for the measurement of excitation and emission GP values, of 370 nm and 490 nm, respectively, were chosen so as to minimize the contribution of the scattering to the total fluorescence, following previous determinations (Parasassi et al., 1994a). Also from these plots we can observe 1) a non-monotonic increase of the GP values with the increase of cholesterol concentration; 2) points of discontinuities at peculiar concentrations of cholesterol showing an abrupt modification of the GP value; and 3) at high cholesterol concentrations, a more homogeneous behavior of the GP value as a function of temperature. In particular, the difference between the GP value observed at low and at high temperature is small for cholesterol concentrations ≥15 mol % in all three samples. In agreement with the observations made about the surfaces of Figs. 1 and 2, obtained using excitation and emission GP spectra, larger irregularities can be observed in the surfaces reporting the emission GP (Fig. 4) than in the surfaces reporting the excitation GP (Fig. 3). Especially the surfaces obtained using the data of the DLPC and DPPC mixture show irregularities (Figs. 3 *C* and 4 *C*).

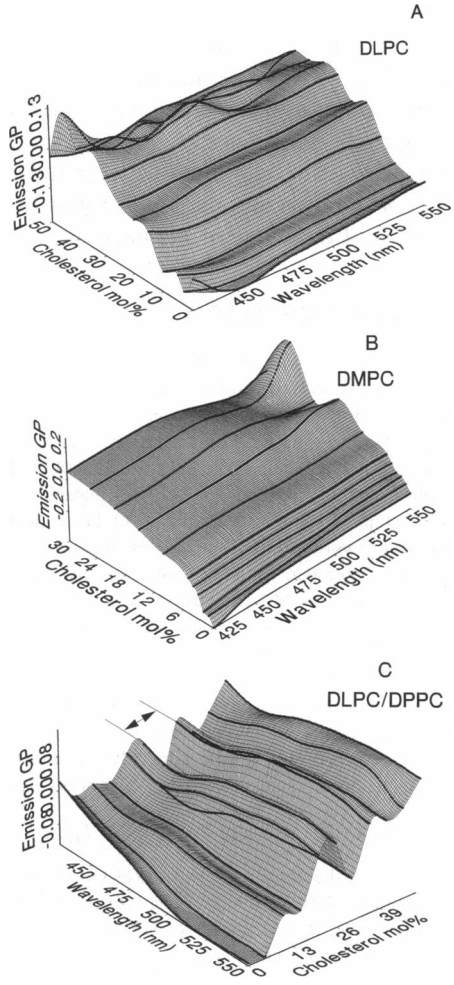

FIGURE 2 Surface representation of the variation of Laurdan emission GP spectra obtained at 25°C as a function cholesterol concentration in phospholipid vesicles composed of DLPC (*A*), DMPC (*B*), and an equimolar mixture of DLPC and DPPC (*C*). The spectra and the surface have been obtained as reported for Fig. 1. The sample composition is the same as reported for Fig. 1. The emission GP spectra have been calculated using Eq. 3. *Arrows* (*C*) represent the cholesterol region that has been expanded by 1 mol % cholesterol increase (shown in Fig. 7 *B*, below).

The surface discontinuities are better evidenced when reported as derivatives with respect to cholesterol concentration. Derivatives are calculated as the difference between two successive values of GP divided by the change in cholesterol concentration. Fig. 5 reports the excitation GP discrete differential versus temperature and versus cholesterol concentration, for the three samples. With the increment in cholesterol concentration being constant (5 mol %) and the plots being rather smooth, the differential is proportional to the derivative of GP value. This quantity will be referred to as "derivative." In Fig. 6 the corresponding surface plots have been built using the emission GP derivatives. In these two figures, both the nonlinear variation of the GP value with the increase of cholesterol and several points of discontinuity are

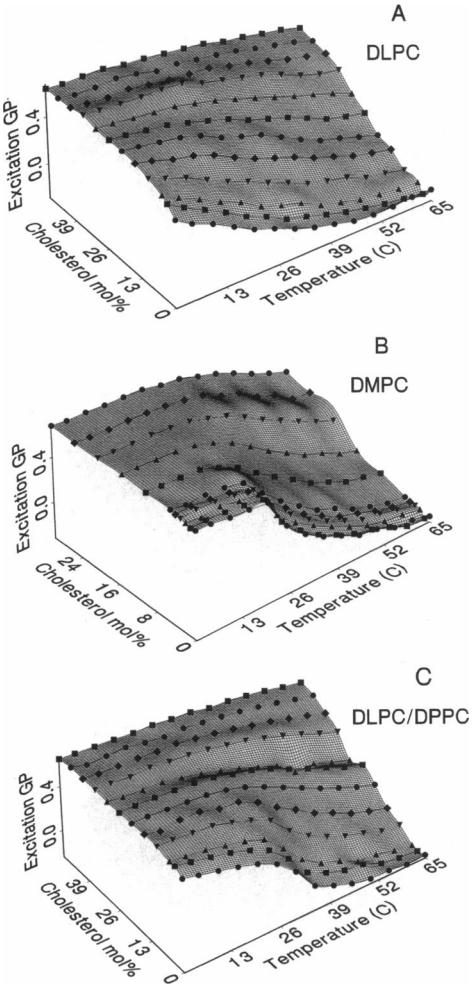

FIGURE 3 Laurdan excitation GP, at 370 nm, calculated using Eq. 2, reported as a function of temperature and of the concentration of cholesterol, for the three samples composed of DLPC (*A*), DMPC (*B*), and equimolar DLPC and DPPC (*C*). The underlying surface has been built as reported in Materials and Methods. Experimental points are superimposed.

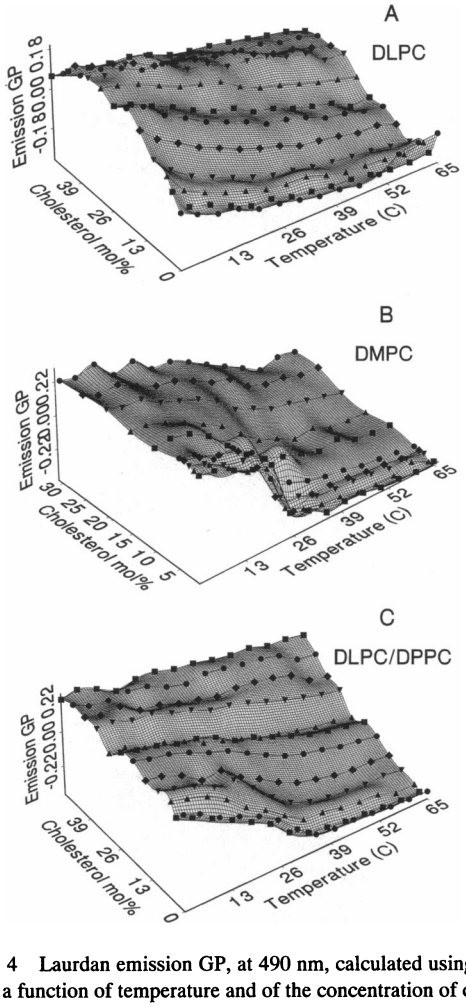

FIGURE 4 Laurdan emission GP, at 490 nm, calculated using Eq. 3, reported as a function of temperature and of the concentration of cholesterol, for the three samples composed of DLPC (*A*), DMPC (*B*), and equimolar DLPC and DPPC (*C*). The underlying surface has been built as reported in Materials and Methods. Experimental points are superimposed.

dramatically evident. The surfaces underlying the experimental points are quite irregular. Some general considerations can be made. Larger irregularities are observed at high temperatures, corresponding to the liquid-crystalline phase of phospholipids. Abrupt variations of the GP values can be observed at 10, 30, and 50 mol % cholesterol for the DLPC sample (Figs. 5 *A* and 6 *A*); 10 and 20 mol % cholesterol for the DMPC sample (Figs. 5 *B* and 6 *B*); 5, 20, 35; and 45 mol % cholesterol for the DLPC/DPPC sample (Figs. 5 *C* and 6 *C*).

DISCUSSION

A fundamental quantity that is frequently used to monitor membrane ordering and dynamics is the fluorescence polarization (or anisotropy). The relevance of this quantity is that the value of the polarization can convey information on the dynamics responsible for the depolarization motions (Weber, 1952). Schematically, the steady-state value of the polarization indicates how fast the intensity emitted in the vertical polarized direction is converted into the intensity emitted in the horizontal polarized direction (directions are defined with respect to the lab reference frame and for excitation in the vertical polarized direction). In other words, the polarization contains information on the rate of rotational relaxation. If we consider the emission of Laurdan in the blue part of the spectrum due to those molecular species that are not relaxed and the emission from the red part of the spectrum as arising from those molecular species that have relaxed, we can formally define a quantity called GP that conveys the information about the rate of the relaxation process (Parasassi et al., 1990). For this reason, Laurdan steady-state spectral properties have been described by the GP (Eq. 1). Other useful quantities defined by analogy with the conventional polarization are 1) the excitation GP spectrum and 2) the emission GP spectrum. The analogy with the conventional polarization can be extended further, and it is possible to derive a generalized Perrin equation that can be used to obtain values of the dipolar relaxation rate (instead of the rotational relaxation rate) (Parasassi et al., 1992). In contrast

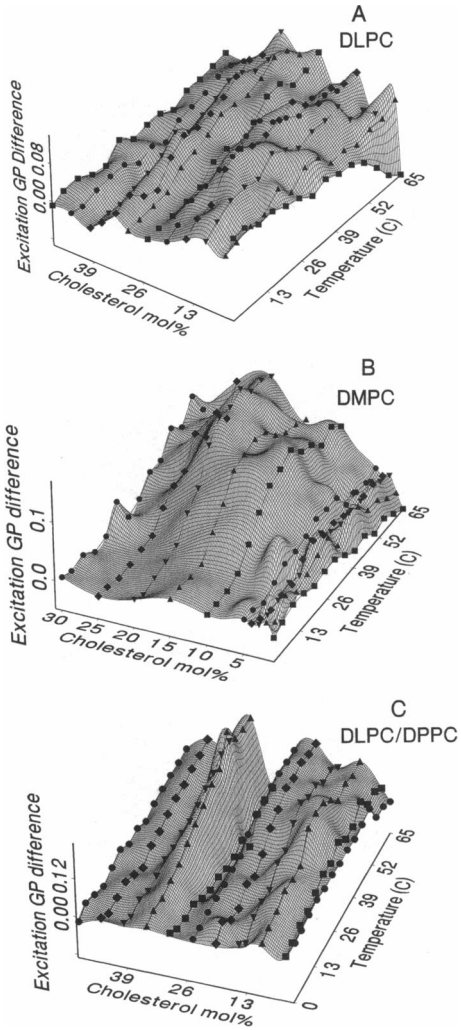

FIGURE 5 Derivative of excitation GP as a function of cholesterol concentration and temperature for the samples composed of DLPC (*A*), DMPC (*B*), and equimolar DLPC and DPPC (*C*). The graph has been obtained from the data reported in Fig. 3 and by computing the difference in GP values at every two successive cholesterol concentrations at constant temperature. The underlying surface has been built as reported in Materials and Methods. Experimental points are superimposed.

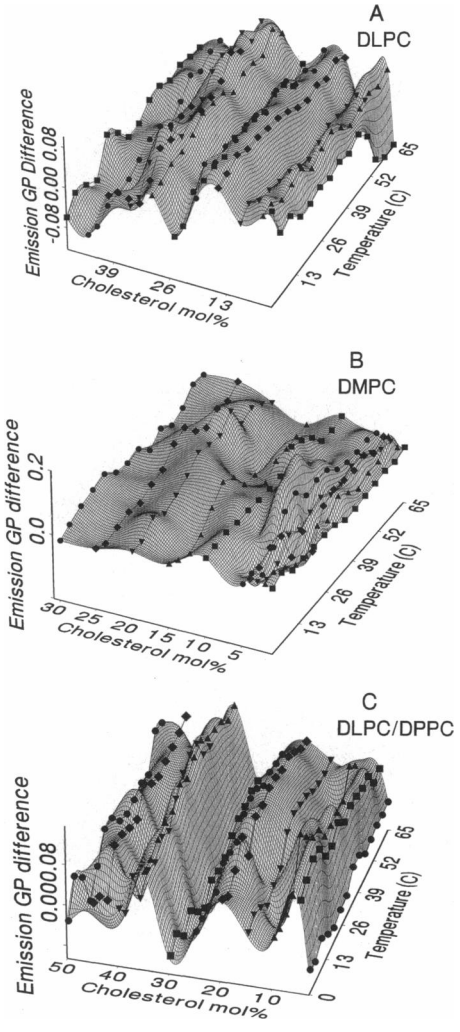

FIGURE 6 Derivative of emission GP as a function of cholesterol concentration and temperature for the samples composed of DLPC (*A*), DMPC (*B*), and equimolar DLPC and DPPC (*C*). The graph has been obtained from the data reported in Fig. 4 and by computing the difference in GP values at every two successive cholesterol concentrations at constant temperature. The underlying surface has been built as reported in Materials and Methods. Experimental points are superimposed.

to common ratiometric methods used for probes displaying spectral sensitivity to their environment, the GP allows comparison between different samples without requiring a calibration curve. We have shown that the phospholipid phase transition can be easily detected from a plot of the GP as a function of temperature. The GP is additive, a useful property for the quantitation of the fraction of each coexisting phase. Also, asymptotic values have been determined for the GP of Laurdan in the gel phase (0.6) and for the liquid-crystalline phase (–0.3). These values are not dependent on the kind of phospholipid polar head and are constant between pH 4 and 10 (Parasassi et al., 1991). Laurdan spectroscopic properties originate from the sensitivity of the probe to the polarity of its local environment and to the dipolar relaxation process. In solvents of low polarity, both excitation and emission spectra are blue-shifted. If the time scale characterizing the dynamics of solvent dipoles surrounding the Laurdan fluorescence moiety is on the same order of magnitude as the fluorescence lifetime, then solvent dipoles can orient around the excited dipole of the probe during the emission process. The energy required for solvent reorientation is reflected in the red shift of Laurdan emission. The rate of dipolar relaxation of Laurdan in phospholipid vesicles is influenced by the phase state of the phospholipids. This rate has been measured using the generalized Perrin equation, being about $1.5 \times 10^9\ s^{-1}$ in the liquid-crystalline state and about $4 \times 10^7\ s^{-1}$ in the gel phase (Parasassi et al., 1992).

The molecules in the probe surroundings responsible for the dipolar relaxation have been determined to be water molecules present at the hydrophobic-hydrophilic interface of the bilayer, in the close vicinity of the fluorescent naphthalene moiety of Laurdan, at the level of the glycerol backbone

(Parasassi et al., 1993b). Compared with that of the "bulk" water, these water molecules have slower dynamics, comparable to Laurdan fluorescence lifetime. In the gel phase these water molecules are slowly rotating and cannot reorient around Laurdan excited-state dipole. Also their concentration is reduced in the gel phase with respect to the liquid-crystalline phase.

Cholesterol has been reported to reduce the hydration of the bilayer (Simon et al., 1982), and a decrease in water content is clearly reflected in the fluorescence excitation and emission properties of Laurdan (Parasassi et al., 1994a). Specifically, in the gel phase, where the dipolar relaxation is virtually absent, cholesterol induces a further shift of the excitation and emission spectrum toward the blue. In the liquid-crystalline phase, the effect of cholesterol addition is more complex. 1) Cholesterol decreases water content causing a blue shift of the excitation and emission spectra. 2) The presence of cholesterol slows dipolar relaxation and, in general, decreases the rate of water diffusion. This effect is clearly visible in the change of the relaxation rate carried out by addition of cholesterol (T. Parasassi and E. Gratton, unpublished results). 3) The effect of cholesterol varies depending on the total cholesterol concentration (Parasassi et al., 1994a,b). The origin of the decreased polarity and of the decreased dipolar relaxation in the presence of cholesterol can be related to the general effect of cholesterol of increasing the bilayer packing, particularly in the liquid-crystalline phase.

The decrease in axial molecular motion induced by cholesterol has been previously mentioned (Ipsen et al., 1987 and 1989; Mouritsen, 1991). In binary mixtures of phospholipids and cholesterol, at about 25–30 mol % of cholesterol, a liquid-ordered phase has been described over a broad temperature range. In this phase, lateral diffusion is slower than in the liquid-crystalline phase, but still faster than in the gel phase. It has been suggested that this liquid-ordered phase can exist in natural membranes. The fluorescence properties of Laurdan show all the effects discussed above. In particular the rate of dipolar relaxation around Laurdan decreases as cholesterol is added, as shown by the blue shift of the emission spectrum. Note that this effect is dynamic; i.e., not only does the spectrum shift because of the change of the average membrane polarity, but also the rate of spectral shift decreases. What is pertinent to this work is that the effects of cholesterol are not smooth. Some incremental additions of cholesterol cause a much larger (or smaller) effect than the equivalent incremental addition when the absolute cholesterol concentration is different. These observations show that there is a richer microscopic behavior than previously thought. Our hypothesis is that the fluorescence properties of Laurdan reveal the formation of microdomains at some specific cholesterol concentrations. These microdomains must have definite local order and dynamical properties. In these microdomains, water content is changed and the diffusional properties are altered.

The larger effects due to particular concentrations of cholesterol on the phospholipid phase state can be better determined by observing the surface obtained by the GP derivatives, for both excitation and emission GP values (Figs. 5 and 6). In the DLPC sample, peaks can be seen at 5, 15, 30, and 45 mol % cholesterol; in the DMPC sample, at 5, 10, 20, and 30 mol % cholesterol; and in the equimolar mixture of DLPC and DPPC, at 5 for emission GP and 10 for excitation GP and 20, 35, and 45 mol % cholesterol. These concentrations are in agreement with some of the concentrations that have been found to originate dips of the excimer/monomer ratio of pyrene-PC in phospholipids (Tang and Chong, 1992; Somerharju et al., 1985). Moreover, these effects are mainly observable in the liquid-crystalline phase of phospholipid, as already found by Tang and Chong (1992). In turn, these concentrations are in agreement with theoretical calculations of a hexagonal superlattice structure formed by phospholipids and a host molecule (Virtanen et al., 1988). Based on these calculations, if cholesterol behaves as a two-chain phospholipid, regular arrays must be formed at 28, 33, and 50 mol %, whereas if cholesterol behaves as a single-chain phospholipid, regular arrays must be found at 25, 40, and 50 mol %. Our data seem to favor the two-chain behavior. However, more detailed measurements are needed to discriminate between these two possibilities.

Relatively large steps, of 5 mol %, have been used to vary the concentration of cholesterol in the used phospholipids. The exact concentration of cholesterol at which abrupt variation of the membrane properties can be observed may be determined with higher precision by a finer variation of cholesterol concentration. To explore this possibility, the equimolar DLPC and DPPC sample has been used to measure the effect of sequential 1-mol % increments of cholesterol, in the range between 25 and 35 mol %. The results are reported in Fig. 7. In Figs. 1 *C* and 2 *C*, the cholesterol concentration range explored in Fig. 7 is indicated by arrows. Using both excitation (Fig. 7 *A*) and emission (Fig. 7 *B*) GP spectra, a relative minimum has been obtained at about 33–34 mol % cholesterol.

The properties of Laurdan GP have been previously used to investigate the coexistence of separate phase domains in natural membranes (Parasassi et al., 1993a). No evidence has been found of the coexistence of gel-like and liquid-crystalline-like domains in several mammalian cell types. Moreover, by measuring the time-evolution of Laurdan emission spectrum in vesicles of non-miscible phospholipids we calculated the average dimension of the domains to be of about 50 A^2. The relative concentration range of the two phospholipids at which a phase segregation can be observed is between 0.3 and 0.7. The above observations provide quite strict limitations for the occurrence of phase domains in natural membranes. Given both the complex effect of cholesterol on phospholipid lateral and axial motion and the nonmonotonic behavior of the modifications introduced by the linear increase in its concentration, our hypothesis is that of the occurrence of ordered microdomains in the hydrophobic matrix.

Additional experiments are required for a more precise determination of the critical concentration of cholesterol in

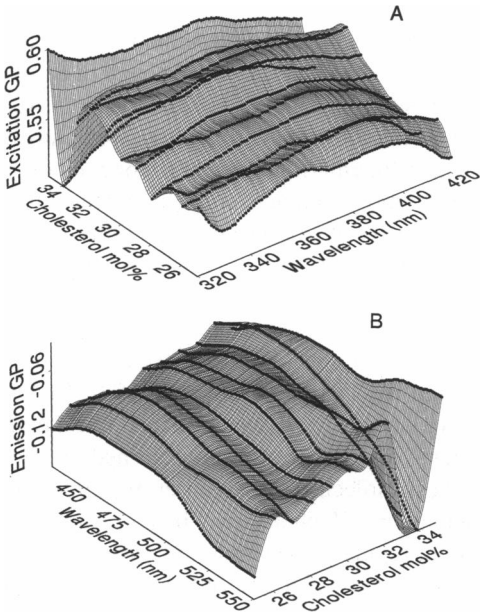

FIGURE 7 Surface representation of Laurdan excitation GP spectra (*A*) and emission GP spectra (*B*) obtained at 25°C in phospholipid vesicles composed of an equimolar mixture of DLPC and DPPC as a function cholesterol concentration varying from 25 to 35 mol % by 1-mol % steps.

phospholipids able to create stable, ordered microdomains with low polarity. More complex, ternary systems give rise to more complicated situations, as shown here by the surfaces obtained with the mixture of DLPC and DPPC. Moreover, the binary mixture of two phospholipids, without cholesterol, can give rise to a local organization in stable microdomains at specific relative concentrations. Once verified and generalized to multicomponent systems, these findings can provide a powerful description of the membrane lipid matrix, with a relevant potential in the understanding of the biological regulation of membrane activity.

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