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Absence of lipid gel-phase domains in seven mammalian cell lines and in four primary cell types

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Fluorescence properties of 6-lauroyl-2-dimethylaminonaphthalene (Laurdan) are used to explore gel and liquid-crystalline phase domains coexistence in membranes of various cell types and in erythrocyte ghosts. Experiments and simulations were performed using liposomes composed of equimolar gel and liquid-crystalline phases in the absence and in the presence of 30 mol% cholesterol. In this model system two distinct coexisting phases can be easily recognized in the absence of cholesterol. When cholesterol is added to this phospholipid mixture, Laurdan parameters characteristic of the gel and of the liquid-crystalline phase are no longer resolvable. Coexisting domains of gel and liquid-crystalline phase were not detected in any of the examined cell membranes as judged by Laurdan excitation and emission Generalized Polarization (GP) spectra. Both in liposomes and in cell membranes, the behaviour of GP values as a function of excitation and emission wavelength corresponds to a homogeneous liquid-crystalline phase, despite the absolute GP values being relatively high, closer to the values observed in gel phase phospholipids. The presence of cholesterol appears to be the major cause for the homogeneity of phospholipids' dynamical properties in natural membranes, properties that appear close to the liquid-ordered phase state, defined to describe model systems with cholesterol concentration ≥ 30 mol%.

Introduction

Dynamical properties of lipids in cell membranes received large attention since the *fluid mosaic* model was proposed in 1972 [1]. Following this model, the membrane bilayer has homogeneous physical properties, namely a homogeneously fluid phase state. The possibility of modulating membrane fluidity by separated domains of different phase state became of relevance since the dynamic properties of the bilayer may affect the function of inserted proteins and the diffusion of molecules across membrane compartments.

Dynamical properties of phospholipids in the bilayer aggregation form are usually modeled using vesicles of synthetic components [2]. Phospholipids undergo a main phase transition between the gel and the liquid-crystalline phase at different temperature depending on the length of the acyl residues and on the type of the polar head. For example, a segregation of domains composed of the above phases within the plane of the membrane is observed [2,3] in vesicles composed of a binary mixture of phospholipids differing in the length of their acyl residues for more than four carbon atoms and at a temperature intermediate between the transition temperature of each phospholipid. Of these phospholipid phase domains, one shows dynamical properties characteristic of the low melting phospholipid, i.e., properties of the liquid-crystalline phase state slightly modified by the presence of the high melting gel-phase phospholipid, and vice versa for the other domain [4].

The occurrence of several different types of phospholipids in natural membranes could give rise, in the plane of the membrane, to a segregation of domains of different phase state. Once their presence will be ascertained, consequences on their biological relevance in the modulation of cell functions could be studied. For instance, membrane proteins can regulate their activity and diffusion by a selective partitioning between these domains. Several functions, such as diffusion dynamics of molecules and receptor exposure [5–7], can be influenced by the phase state of the sur-

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Abbreviations: Laurdan: 6-lauroyl-2-dimethylaminonaphthalene; DLPC: dilauroylphosphatidylcholine; DPPC: dipalmitoylphosphatidylcholine; DPH: 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH: 1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene; PBS: phosphate-buffered saline solution.

rounding lipids. Nevertheless, in vesicles composed of binary mixtures of dilauroyl- and dipalmitoyl-phosphatidylcholine coexisting phase domains can only be detected in the range between 30 and 70 mol% of one phospholipid over the other. At both edges of this range the properties of each phospholipid are modified by the presence of the other but no domain segregation could be observed [4].

In recent years a number of experiments using various techniques were performed to provide evidence of morphologically and functionally specialized areas in the cell membrane [8–11] such as apical and basolateral membranes of polarized epithelial cells [12], in membranes with highly specialized functions like chloroplast thylakoid membranes [10], of protein clustering in domains that exclude lipids [8,13] or of a selective concentration of certain lipids in distinct domains [11].

In this work we are concerned with the coexistence of separate lipid phase domains in cell membranes, characterized by a different phase state, gel and liquid-crystalline, i.e., by different molecular dynamics in the nanosecond time scale.

Some fluorescent probes show distinct spectroscopic properties in membranes of different phase states. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and parinaric acids are good examples, showing different lifetime values in the gel and in the liquid-crystalline phase [3,14]. In the case of DPH, changes of the average lifetime value are also associated to changes in the width of the lifetime distribution [15]. However, using DPH and parinaric acids in phospholipid vesicles composed of a mixture of the two phase states the resolution and the quantitation of coexisting domains are partially prevented by the complex photophysical behaviour of these probes [14,16]. Using DPH it has been very difficult to distinguish between the linear combination of a set of different spectroscopic properties and a homogeneous set of intermediate properties. For example, the fluorescence lifetime value of DPH changes from about 10 ns to 7 ns during the phase transition. A measured value of 8.5 ns can be equally interpreted as arising from a composition of the two phases each contributing in an equal amount, or from a homogeneous membrane of intermediate properties [16]. This is due to the limited lifetime resolution of today's instrumentation. On the other hand, the value of steady-state fluorescence anisotropy of DPH shows quite different values in the gel and in the liquid-crystalline phase of phospholipids. Unfortunately, the anisotropy value is also dependent on temperature so that the mixture of two coexisting phases can only be resolved by measuring the anisotropy decay and lifetime values. However, in this last case the number of parameters is large and data should be globally analyzed for measurements at several temperatures.

In the present work we utilize the spectral sensitivity to the lipid phase state of the fluorescent probe 6-lauroyl-2-dimethylaminonaphthalene (Laurdan). The physical origin of Laurdan spectral properties resides on its extreme sensitivity to the polarity and to the molecular dynamics of dipoles in its environment due to the effect of dipolar relaxation processes [17,18]. Laurdan shows relevant spectral shifts in solvents of different polarity. In phospholipid bilayers the polarity and the dynamics of the dipoles surrounding the fluorescent moiety of Laurdan is dramatically different in the two phases. A 50 nm red shift of the emission maximum is observed by passing from the gel to the liquid-crystalline phase. The relative intensity of the two bands of Laurdan excitation spectrum also depends on phospholipid phase state, which gives the possibility of a selective excitation of Laurdan molecules in different environments.

Laurdan spectroscopic properties have been described using the Generalized Polarization (GP) [17,18]:

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

where B (blue) and R (red) are the fluorescence intensities measured at the maximum emission characteristic of the gel and of the liquid-crystalline phase. After selective excitation of Laurdan molecules in the gel or in the liquid-crystalline phase, a measurement of the GP value can detect if the initial photoselection has been maintained or if interconversion between coexisting phases occurred in the time scale of the fluorescence lifetime. In addition, a GP value can also be obtained by measuring the relative intensities of the two excitation bands associated with the two phases, when observed at one of the two emission wavelengths characteristic of each phase. For the possibility of selective excitation of different populations of the probe and for the possibility of utilizing the properties of fluorescence polarization, Laurdan GP measurement is of extreme potential interest [18–20].

Laurdan GP characteristic values in the pure gel and in the pure liquid-crystalline phospholipid phases have been determined [18]. The additivity property of the GP can then be used to quantitate the relative fraction of the two coexisting phases in samples of unknown composition [19]. However, in this last case the observation of a GP value intermediate between the GP value of the gel and of the liquid-crystalline phase is not a final proof of the coexistence of separate phospholipid phase domains. Such an intermediate GP value can arise from averaged properties of the components, homogeneously mixed. Instead, GP measurements at more than one excitation or emission wavelength can determine if domains of different composition and phase properties coexist in the plane of the membrane [4].

The spectroscopic origin of the behaviour of Laurdan excitation and emission GP spectra in the two phases and in the presence of coexisting domains of the two phases has been discussed elsewhere [4] and will be only briefly summarized here. Laurdan excitation spectrum is characterized by two bands. The red one (centered at about 390 nm) is associated to blue emitting Laurdan molecules, i.e., to Laurdan molecules surrounded by gel phase phospholipids. The blue excitation band (centered at about 355 nm) is equally populated by blue and red emitting molecules, i.e., by Laurdan molecules surrounded by gel and by liquid-crystalline phospholipids. In the phospholipid liquid-crystalline phase, where a strong relaxation process occurs, excitation in the red part of the absorption band photoselects molecules with already relaxed surroundings. In this case, the emission spectrum will be more intense in the red. By exciting in the blue part of the absorption spectrum, Laurdan molecules relax during their excited-state lifetime, giving a large initial intensity in the blue part of the emission, i.e., higher average GP values. Then in the case of the pure liquid-crystalline phase by moving the excitation wavelength from the blue to the red lower GP values will be obtained. A different situation occurs when different phospholipid phases coexist, the red band of excitation spectrum will be populated by Laurdan molecules surrounded by gel phase phospholipids that are blue emitting, with higher GP values. In this case, by moving the excitation wavelength from the blue to the red, higher GP values will be obtained. A similar reasoning can be made for GP values obtained at different emission wavelengths. This general behaviour of Laurdan GP value as a function of excitation and emission wavelength is not affected by the length of the phospholipid acyl residues, by their polar head or by the pH value from 4 to 10 [18]. Actually, all these factors only modify the temperature range of the phase transition of phospholipids but not the characteristic GP values of the gel and of the liquid-crystalline phase and the behaviour of GP value vs. wavelength.

Cholesterol is ubiquitous in mammalian cells, being present at variable and relatively high concentrations, typically of about 30 mol% and above with respect to phospholipids. Cholesterol is known to be a major modifier of the structural organization and dynamical properties of the phospholipid bilayer. In general, cholesterol has different effects depending on the bilayer phase state. Cholesterol increases both the lateral diffusion rate and the axial molecular motion of phospholipids in the gel phase while decreases lateral mobility and increases the order of the liquid-crystalline phase [21–23]. Phase diagrams of systems composed of pure phosphatidylcholines and cholesterol have been constructed [22–24] showing that at about the physiological concentration of cholesterol the difference between dynamic properties of the gel and of the liquid-crystalline phase disappear. The liquid-ordered phase has thus been defined, showing liquid-like properties as for lateral diffusion but solid-like properties with respect to the acyl chain order [21].

Materials and Methods

Laurdan-labeled liposomes

Multilamellar phospholipid vesicles were prepared by mixing the appropriate amounts of solutions in chloroform (spectroscopic grade) of phospholipids (Avanti Polar Lipids, Alabaster, AL) with or without cholesterol (Sigma, St. Louis, MO) and Laurdan (Molecular Probes, Eugene, OR), then evaporating the solvent by nitrogen flow. The dried samples were resuspended in Dulbecco's phosphate-buffered saline solution, pH 7.4 (PBS, Flow Laboratories, UK), 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.

Cell preparation and labeling

Human proerythroblastoid K562 cells, human limphoblastoid T Molt4 cells, human limphoblastoid B Raji cells, Friend leukemia cells and human histiocytic lymphoma U937 cells were routinely subcultured in RPMI 1640 with 10% fetal calf serum (FCS). Mouse myeloma P3U and NS0 cells were subcultured in DMEM with 10% FCS. Splenocytes and thymocytes were prepared from spleen and thymus, respectively, of Balb/C inbred mice of 6 weeks of age, following [25]. Postnatal cerebellar granules (Granule Cells) were prepared from 8-day-old rats following Ref. 26. Rabbit erythrocyte ghosts were prepared following Ref. 27.

Laurdan cell labeling procedure is slightly modified with respect to the method already reported [19]. Depending on the cell size, $3 \cdot 10^6$ to $6 \cdot 10^6$ cells of each type were washed three times with PBS, then resuspended in 2.5 ml of PBS. 0.5 μl of a 0.25 mM solution of Laurdan in DMSO (Sigma, St. Louis, MO) were added to the cell suspension under mild magnetic stirring. Incubation was carried out for 1 h in the dark or under red light and under mild stirring. Cells were then pelleted and washed with PBS, resuspended in 2.5 ml of PBS, equilibrated for 10 min in the fluorometer cuvette at 20°C, then measured. Temperature equilibration and measurement were carried out under continuous mild stirring. Final concentrations of DMSO and Laurdan were 0.02% (vol%) and 0.05 μM, respectively. These labeling conditions were determined after dilution experiments where the GP value was reported as a function of the ratio (millions of cells per ml)/(final micromolar Laurdan concentration) (C/L) (see below, Fig. 4). For cells of a given size, the C/L region

where the GP value does not vary depends on the cell number and on the Laurdan concentration. The Laurdan stock solution was renewed every 3–4 weeks. For some cell types (K562, Raji, U937, granule cells) a blank was prepared with the same number of cells, treated in the same way as the labeled cells. The blank spectra were then subtracted from the spectra of labeled cells.

Fluorescence measurements

Laurdan excitation and emission spectra in cells were obtained using a GREG 200 fluorometer using a xenon arc lamp as the light source and the accompanying software (ISS, Champaign, IL). The fluorometer was equipped with photon counting electronics PX01 (ISS). Monochromators bandpass were 8 nm. Temperature was controlled to ± 0.1 C° with a water circulating bath. The spectra were only corrected for the lamp intensity variations. The emission GP spectra were constructed by calculating the GP value for each emission wavelength as follows:

$$\text{em GP} = (I_{410} - I_{340})/(I_{410} + I_{340}) \quad (2)$$

where I_{410} and I_{340} are the intensities at each emission wavelength, from 425 nm to 550 nm, obtained using fixed excitation wavelength of 410 nm and 340 nm, respectively. The excitation GP spectra were constructed in the same way but from the excitation spectra using:

$$\text{ex GP} = (I_{435} - I_{490})/(I_{435} + I_{490}) \quad (3)$$

where I_{435} and I_{490} are the intensities at each excitation wavelength, from 320 nm to 410 nm, obtained using fixed emission wavelength of 435 nm 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 [17,18]. The excitation wavelengths were chosen as to obtain the maximum separation of Laurdan excitation bands in the gel (390 nm band) and in both phospholipid phases (355 nm band).

Results

First we show how the coexistence of separate phase domains can be distinguished from a homogeneous phase with intermediate properties using Laurdan excitation and emission GP spectra. Laurdan excitation and emission spectra and GP spectra in synthetic phospholipid vesicles in the pure gel and in the liquid-crystalline phases, and in the presence of the two coexisting phases are reported in Fig. 1. In gel phase phospholipids (DPPC at 5°C) Laurdan excitation spectrum shows a maximum at 390 nm and its emission maximum is at about 440 nm (Fig. 1A). In the liquid-crystalline phase (DLPC at 40°C) Laurdan excitation maximum is at 355 nm and its emission maximum is at 485 nm (Fig. 1A). Spectra with intermediate characteristics are obtained using vesicles composed of an equimolar mixture of gel and liquid-crystalline phase (DLPC/DPPC, 1:1 at 20°C) (Fig. 1A). In pure gel phase phospholipids, both excitation and emission GP spectra show very little variation with the excitation and emission wavelength, respectively (Fig. 1B). In pure liquid-crystalline phase the excitation GP spectrum shows decreasing values with increasing excitation wavelength, while the emission GP spectrum shows

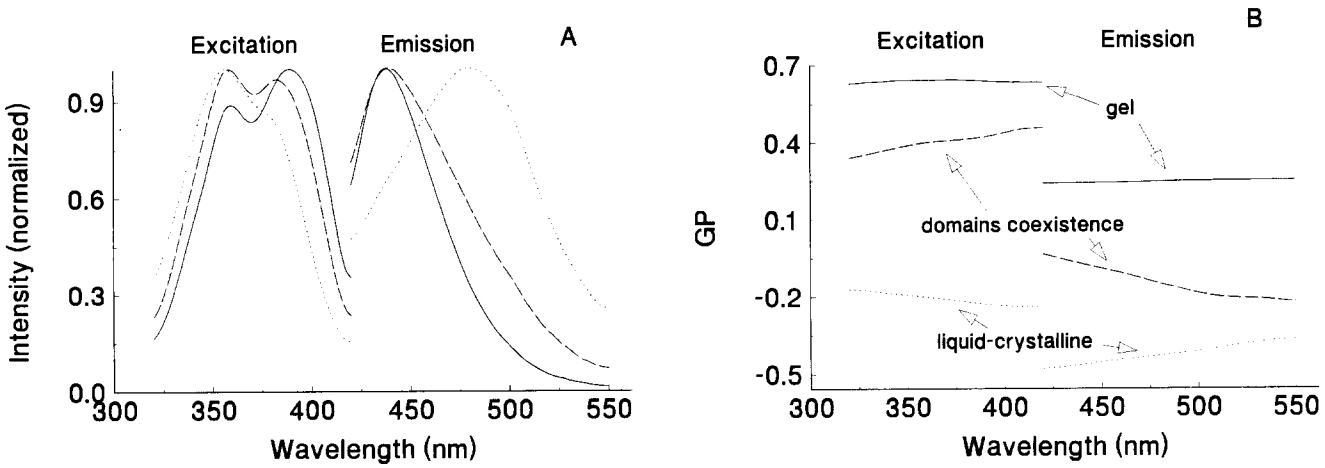

Fig. 1. (A) Normalized Laurdan excitation and emission spectra in multilamellar vesicles composed of gel phase (continuous line), liquid-crystalline phase (dotted line) and coexisting phases (– – –). (B) Laurdan excitation and emission GP spectra in phospholipids of the two phase state, as in (A), obtained as reported in Materials and Methods. DPPC at 5°C (continuous line); 50 mol% DLPC in DPPC at 20°C (– – –), DLPC at 40°C (dotted line).

increasing values as the emission wavelength increases (Fig. 1B). This is the characteristic behaviour due to dipolar relaxation process, observed in the liquid-crystalline phase of vesicles composed of phospholipid with different polar heads and various length of acyl residues, at pH values variable from 4 to 10 [4,18]. Instead, the GP spectra obtained in a 50% mixture of phospholipids in the two phases show an opposite behaviour. The excitation GP spectrum shows increasing values and the emission GP spectrum shows decreasing values with the wavelength increase (Fig. 1B). The explanation of this qualitatively different behaviour of the mixture as compared to the pure phospholipids has been summarized in the Introduction and extensively discussed in previous articles [4,18].

By adding 30 mol% cholesterol to an equimolar mixture of DLPC and DPPC, Laurdan excitation and emission spectra and GP values are modified with respect to the spectra obtained in the same mixture without cholesterol. At 20°C the excitation spectrum shows a decrease in intensity of the red band, centered at about 390 nm, and a small blue shift (Fig. 2A). The emission spectrum shows a blue shift and a decrease of intensity at about 490 nm (Fig. 2A). The excitation GP spectrum in the presence of cholesterol shows higher values and, rather than the increasing values with increasing wavelength observed in the DLPC/DPPC mixture, in the presence of cholesterol the GP value decreases with increasing excitation wavelength (experimental spectrum in Fig. 2B). Laurdan emission GP spectrum in the equimolar DLPC/DPPC mixture and in the presence of 30 mol% cholesterol shows increasing values with the increase of emission wavelength, while in the absence of cholesterol the GP value has an opposite behaviour (experimental spectrum in Fig. 2B).

Simulations have been performed to further clarify the modifications due to cholesterol on the behaviour of Laurdan GP values as a function of the excitation and of the emission wavelength. Laurdan excitation and emission spectra in vesicles composed of pure DLPC with 30 mol% cholesterol and of pure DPPC with 30 mol% cholesterol were measured at 20°C. The spectra obtained from the two samples were then linearly combined. Ideally, this situation should correspond to a mixture of the two phases of pure DLPC and of pure DPPC without mutual interaction. The linear combination has been performed taking into account that the contribution to the total fluorescence of Laurdan in DPPC is 60% of the total fluorescence of the mixture. This higher contribution is due to the difference of Laurdan lifetime values in the two pure components [4]. Excitation and emission GP spectra were then calculated. The simulated GP spectra show a behaviour qualitatively similar to the experimental GP spectra (Fig. 2B). In the simulations, the GP values decrease as the excitation wavelength increases and slightly increase with the increase of the emission wavelength. However, the opposite behaviour of the GP values vs. wavelength, observed in the equimolar DLPC/DPPC mixture at 20°C and in the absence of cholesterol, where phase domains coexistence has been demonstrated [3,4,17], is not reproduced in the simulated GP spectra.

With the aim of investigating if there is preferential partitioning of Laurdan in possible domains of pure cholesterol, GP values have been measured in DLPC

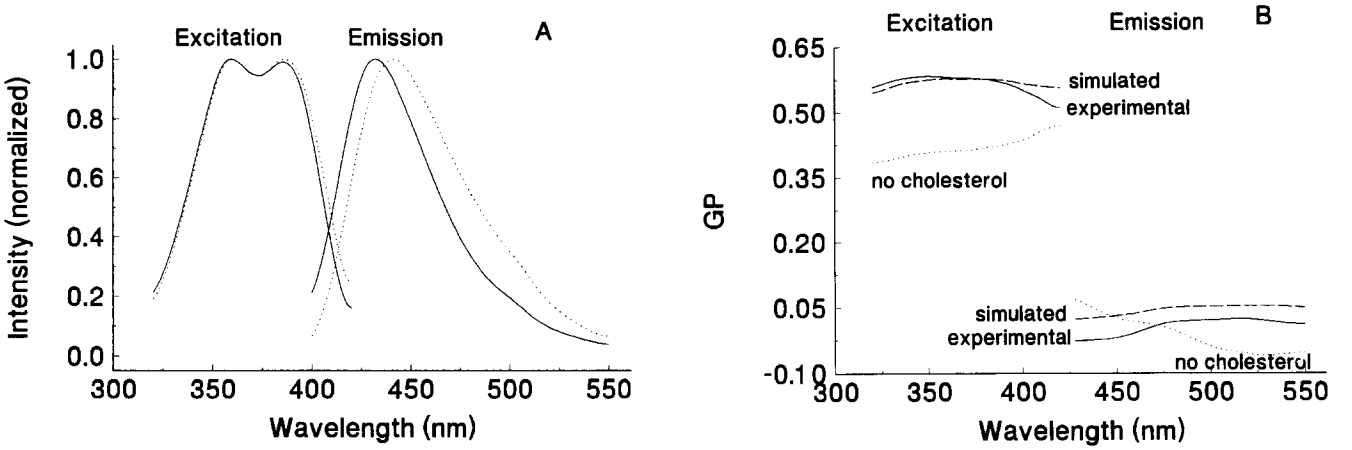

Fig. 2. (A) Normalized Laurdan excitation and emission spectra in multilamellar vesicles composed of an equimolar mixture of DLPC and DPPC, at 20°C, in the presence (continuous line) and in the absence (dotted line) of 30 mol% cholesterol with respect to phospholipids. (B) Laurdan excitation and emission GP spectra obtained at 20°C in multilamellar vesicles composed of an equimolar mixture of DLPC and DPPC (---) and in the presence of 30 mol% cholesterol (– – –, ———). Experimental data (———) and data simulated as reported in Results (– – –).

vesicles at 20°C as a function of cholesterol concentration (Fig. 3). A small deviation from linearity can be observed at cholesterol concentrations up to about 30 mol%. Above this concentration a plateau is reached, in agreement with previous results reporting the absence of further modifications of bilayer properties above 30–35 mol% cholesterol [21–24,28]. The small deviation from linearity must be explained by the occurrence of phase fluctuations and interconversion [4] rather than by a small preferential partitioning of Laurdan in cholesterol domains. From preliminary experiments, not reported here, on Laurdan time-resolved emission spectra performed using DLPC vesicles with cholesterol concentration varying from 5 to 55 mol%, no evidence of Laurdan preferential partitioning in cholesterol-rich domains has been achieved.

In summary, the experiments on the effect of cholesterol on an equimolar mixture of DLPC and DPPC, where phase coexistence has been experimentally proven, show that cholesterol strongly modifies the dynamical behaviour of the mixture. The perturbation is so profound that the characteristic properties of the two separate phases can no longer be detected.

Cell labeling conditions were modified with respect to Ref. 19, after the observation that by adding to PBS a few microliters of Laurdan solution in DMSO, Laurdan forms some kind of aggregates with time, of the order of several minutes, and these aggregates are fluorescent. The formation of Laurdan aggregates can then be followed by monitoring changes of the shape of emission spectrum and of the GP value with time of the probe in PBS (not shown). The emission spectrum of Laurdan aggregates is bluer and the GP value increases with time. The probe is then incorporated in its aggregated form in cell membranes. The recovery of the usual Laurdan spectra observed in phospholipids [17,18], corresponding to unaggregated probe molecules, takes hours. These observations come from parallel experiments using cells and phospholipid vesicles labeled with the procedure used for cells. The emission spectra and the GP values have been compared to those obtained using phospholipid vesicles prepared by evaporating together Laurdan and phospholipid stock solutions in chloroform, then resuspending the dried film in PBS and vortexing [17,18]. The same comparison has been used to establish that cells and vesicles must be washed after incubation, not only pelleted and resuspended, to eliminate from the total fluorescence the small contribution of Laurdan fluorescence arising from the buffer.

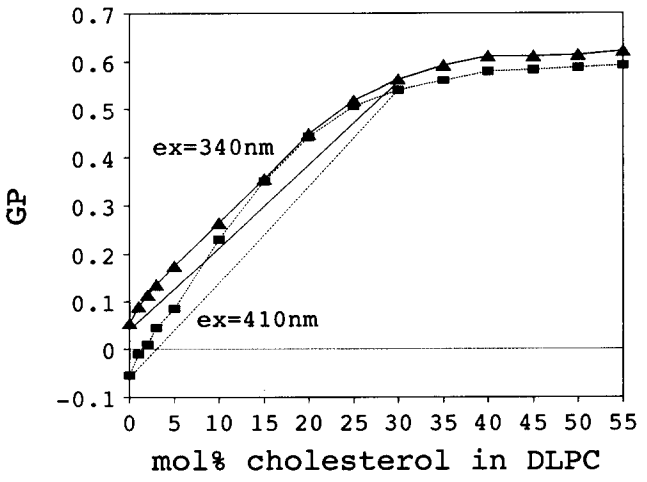

Fig. 3. Laurdan GP values obtained using excitation wavelength of 340 nm and 410 nm in DLPC vesicles, at 20°C, as a function of cholesterol concentration.

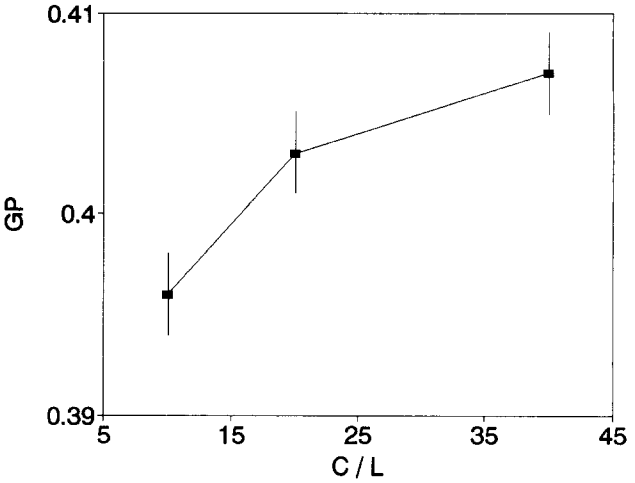

Fig. 4. Dependence of the excitation GP on the ratio between K562 cell concentration per ml and the final Laurdan concentration in the buffer. Excitation wavelength of 360 nm C/L (millions of cells per ml)/(Laurdan micromolar concentration). Experiments have been performed by varying both the cell concentration and the Laurdan concentration in the stock solution of DMSO to give the same C/L ratio in the figure. The final DMSO concentration in the buffer is kept constant at 0.02% (vol%).

The relative concentration of Laurdan in cell membranes affects the GP value and depends on the membrane area, i.e., on the molar ratio between membrane lipids and Laurdan. This ratio is related to cell size. As an example, in Fig. 4 a plot of the GP value vs. the relative concentration of K562 cells and Laurdan is reported. The experiments have been performed by varying both the number of cells and the Laurdan concentration. After these dilution experiments, our measurements were always performed in the C/L range between 25 and 40, as reported in Materials and Methods. The final DMSO concentration in PBS was kept constant at 0.02% (vol%). Small cells such as splenocytes, thymocytes and granule cells were used at approximately twice the concentration used for other cells, as established after the above dilution experiments, at C/L values between 35 and 50.

Both the incubation time of 60 min and the concentration of Laurdan stock solution in DMSO of 0.25

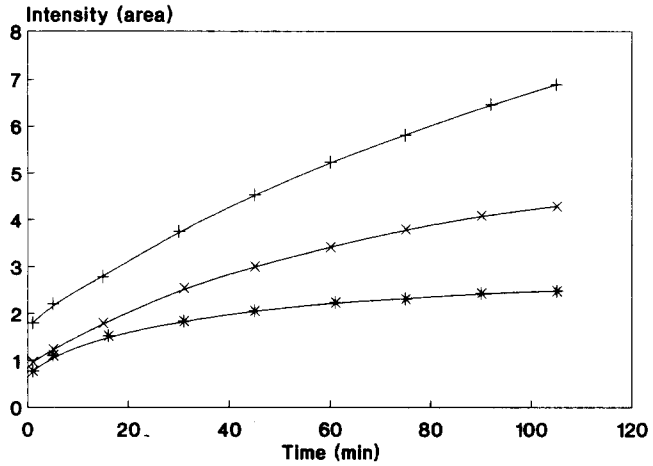

Fig. 5. Time evolution of Laurdan fluorescence intensity integrated over all the emission spectrum, from 400 nm to 550 nm, vs. time of incubation with K562 cells. Excitation was at 360 nm. Excitation and emission bandpass of 8 nm. Final Laurdan concentrations in the buffer were 1.6 μM (+), 0.4 μM (×) and 0.2 μM (*). Measurements at 20°C.

mM were determined following several observations. (i) Laurdan incorporation in cell membranes from the buffer is slow, as can be judged by the increase of fluorescence intensity with time (Fig. 5). Our maximum time of incubation was 105 min and this time was taken as the 100% fluorescence intensity. After 60 min the incorporation is more than 80%. (ii) Labeling experiments performed using phospholipid vesicles showed that, for a given Laurdan concentration, the incorporation is faster if the vesicles are in the liquid-crystalline with respect to the gel phase (Fig. 6A). For a given phospholipid, the incorporation is faster if Laurdan concentration is lower (Figs. 5 and 6B). These differences tend to disappear at incubation times longer than about 40 min. As previously reported [19], for this cell labeling procedure Laurdan lateral and transbilayer partitioning must be considered uniform, similar to what was reported using TMA-DPH [29,30].

Laurdan excitation and emission spectra in K562 cells, 24 and 96 h after the culture medium renewal, are reported in Fig. 7A. Bluer emission spectra and higher intensity of the excitation red band are obtained in cells harvested after 96 h from the medium renewal, as discussed in a previous work [19]. Laurdan spectra in cells show some differences with respect to pure phospholipid vesicles [19]. The emission spectrum with its maximum at 435 nm is similar to the gel phase spectrum. However, the excitation spectrum is similar to the liquid-crystalline spectrum as judged from the low intensity of the red band at 390 nm. This is the typical behaviour of synthetic membranes with a relatively high concentration of cholesterol [19]. From the excitation spectra, taken using fixed emission wavelengths of 435 nm and 490 nm, the excitation GP spectrum can be calculated using the Eqn. 3. From the emission spectra, taken using fixed excitation wavelengths of 410 nm and 340 nm, the emission GP spectrum can be calculated using Eqn. 2. The resulting GP spectra, for the two samples of K562 cells, 24 and 96 h after the medium renewal, are reported in Fig. 7B. The excitation GP spectra show relatively high values, decreasing with increasing excitation wavelength. The emission GP spectra show low values, increasing with increasing emission wavelength. This behaviour is typical of cholesterol-rich membranes. As

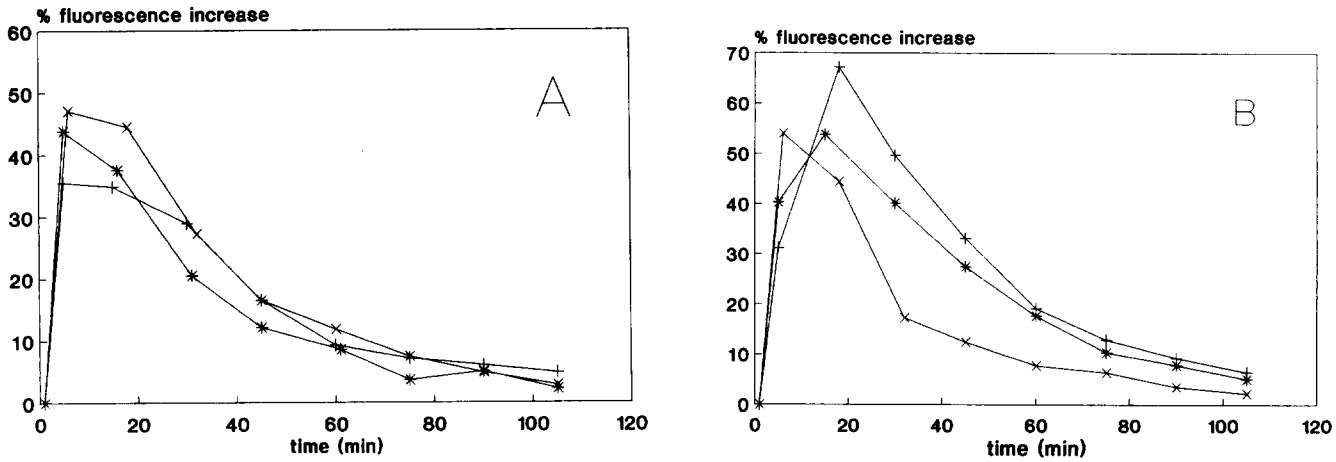

Fig. 6. Percent increase of Laurdan fluorescence intensity, integrated over all the emission spectrum from 400 nm to 550 nm, vs. time, as a function of the phospholipid phase state (A) and as a function of the final Laurdan concentration in the buffer (B). (A) liquid-crystalline phase (DLPC vesicles, ×); gel phase (DPPC vesicles, +); equimolar mixture of two coexisting phases (50 mol% of DLPC in DPPC, *). Laurdan concentration of 0.2 μM. (B) experiments using DLPC vesicles and final Laurdan concentration in PBS of 1.6 μM (+), 0.4 μM (*) and 0.2 μM (×). Excitation at 360 nm. Excitation and emission bandpass of 8 nm. Measurements at 20°C.

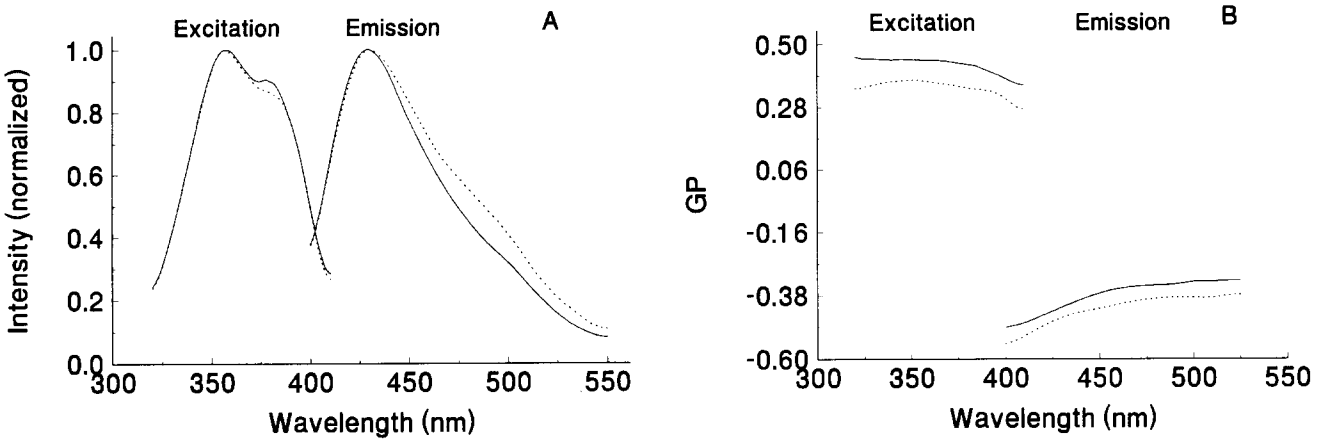

Fig. 7. Normalized Laurdan excitation and emission spectra (A) and excitation and emission GP spectra (B) in K562 cells, at 20°C, after 24 h (dotted line) and after 96 h (continuous line) from the medium renewal. For the emission spectra excitation was 340 nm, and for excitation spectra emission was 435 nm. Excitation and emission bandpass of 8 nm.

previously reported [19], cells harvested 96 h after the medium renewal show higher emission and excitation GP values with respect to 24-h-old cells.

Laurdan GP spectra have been calculated from emission and excitation spectra using all the cell types reported above (Fig. 8). The absolute GP values vary with the cell type, but the same trend of the excitation and emission GP spectra can be observed as a function of wavelength. Both excitation and emission GP spectra are characteristic of a homogeneous phase rather than displaying the characteristic behaviour of a mixture of gel and liquid-crystalline phases (Fig. 8).

For K562 cells, a plot of the excitation GP value, obtained using excitation wavelengths of 340 nm and 410 nm, vs. temperature, from 5°C to 45°C, is reported in Fig. 9. The absolute GP values decrease with increasing temperature, but no abrupt changes of the GP values, indicative of a phase transition, are observed. At all temperatures, GP values obtained using excitation at 340 nm are higher that GP values obtained using excitation at 410 nm, in agreement with an excitation GP spectrum characteristic of a homogeneous liquid-crystalline phase.

Discussion

Besides the capability of allowing easy, fast and precise measurements, Laurdan GP value offers the unique advantage of the quantitative resolution of coexisting phospholipid phase domains and of the assessment of their coexistence. When cholesterol is present in phospholipid vesicles, its combined and opposite influence on the two phases has the final effect of preventing the formation of separate coexisting do-

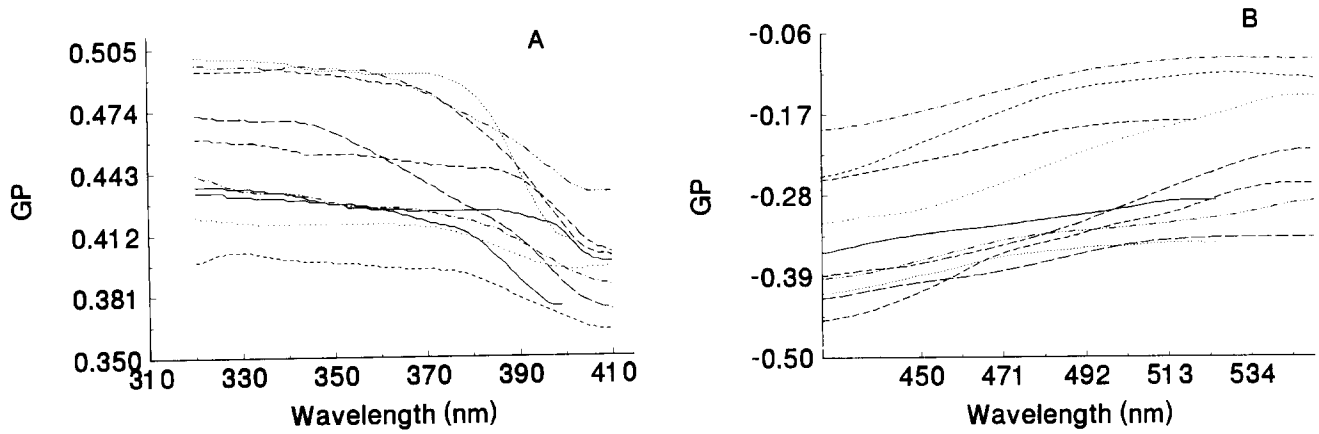

Fig. 8. Excitation (A) and emission (B) GP spectra of Laurdan in various cell types, at 20°C, obtained as reported in Materials and Methods. (A) From the top to the bottom, at wavelength of 320 nm: erythrocyte ghosts, FLC, Raji, Molt4, U937, splenocytes, P3U, granule cells, NS0, thymocytes. (B) From the top to the bottom, at wavelength of 425 nm: P3U, NS0, erythrocyte ghosts, FLC, granule cells, Molt4, thymocytes, U937, splenocytes, Raji.

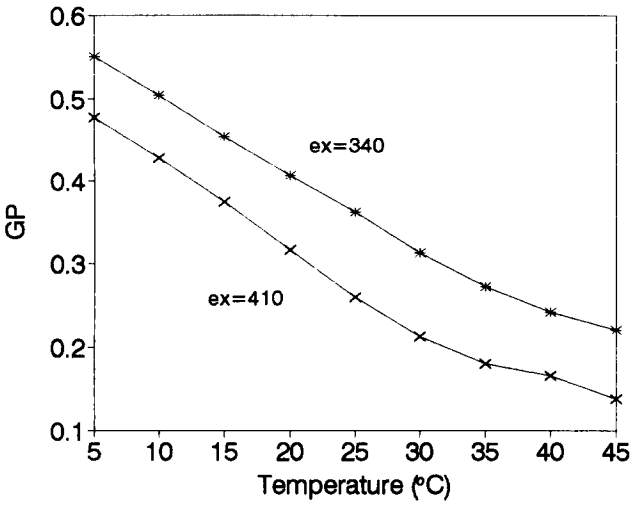

Fig. 9. Laurdan excitation GP values, using excitation at 340 nm (*) and at 410 nm (x) in K562 cells as a function of temperature.

mains. The comparison between experimental and simulated excitation and emission GP spectra performed using phospholipid vesicles showed that 30 mol% cholesterol modify Laurdan properties in such a way that the dynamical properties of the two phases are similar. From experimental and simulated Laurdan excitation GP spectra obtained in the equimolar mixture of DLPC and DPPC in the presence of 30 mol% cholesterol, the decreasing GP values as the excitation wavelength increases indicate that dipolar relaxation can occur and that there is no selective excitation of Laurdan populations in different environments. This is a typical behaviour of the homogeneous liquid-crystalline phase. The absolute GP values are higher in the presence of cholesterol, indicating that cholesterol has the average effect of decreasing both the polarity and the molecular motion of Laurdan environment [17]. The effect of cholesterol on reducing the hydration of phospholipid membranes has been known for several years [31]. A similar reasoning holds for the emission GP spectra, obtained from the same samples. The behaviour vs. wavelength is qualitatively similar to that observed in the liquid-crystalline phase and quite different from that observed in the presence of coexisting domains of the two phases.

Several recent articles reported on the progressive removal of phospholipid phase transition by cholesterol addition [32–34]. Many groups are concerned with the occurrence of phospholipid domains containing different cholesterol concentrations in model systems [21–24,35,36]. In the liquid-crystalline phase of phospholipids, the presence of cholesterol modifies the bilayer structure, giving rise to arrangements indicated as liquid-disordered and liquid-ordered, for low – up to about 10–15 mol% – and for high cholesterol concentrations. Phase diagrams showing the temperature and concentration intervals for the coexistence of these liquid-ordered and liquid-disordered phases have been constructed for vesicles composed of a single phospholipid [21–23,28]. Interestingly, 'classical' gel and liquid-crystalline domain coexistence can only be detected at cholesterol concentrations up to about 7 mol%. Above that concentration only the coexistence of gel with liquid-ordered or of liquid-disordered with liquid-ordered domains, for low and high temperatures, respectively, have been detected. Moreover, above 30 mol% cholesterol only liquid-ordered phase can be detected.

In all seven different cell lines studied and in the four primary cell types, the Laurdan excitation GP spectrum shows decreasing values with the increase of excitation wavelength and the emission GP spectrum shows increasing values with the increase of the emission wavelength (Fig. 8). In particular, the faster decrease of the GP value at excitation wavelengths > 380 nm, indicative of liquid-crystalline dipolar relaxation [4], is also visible. The absolute GP values are different in the various cell types, being probably characteristic of the membrane composition, of the cell physiological state, or depending on several factors such as the passage number [19]. In all cell types the behaviour of excitation and emission GP spectra vs. wavelength is indicative of an homogeneous liquid-crystalline phase, with no evidence of coexisting phase domains.

A plot of the excitation GP value at 340 nm and at 410 nm vs. temperature in K562 cells is shown in Fig. 9. At all temperatures the GP value obtained using excitation of 340 nm is higher than the GP value obtained exciting at 410 nm. Moreover, no abrupt changes of both values are observed in the temperature range from 5°C to 45°C, showing the lack of a phase transition. The GP values are relatively high at low temperatures, decreasing with temperature increase, but they remain relatively high also at 45°C.

Analogous to the above quoted studies performed using various fluorescent probes such as DPH, parinaric acids, TMA-DPH [29,30], the results obtained in the present work can be attributed to all cell membranes, with no distinction between plasma membranes and membranes of the various organelles or between bilayer leaflets [19].

The reported results cannot be attributed to a preferential partitioning of Laurdan in selected membrane domains. Laurdan equally partitions between different phospholipids [4]. In DLPC vesicles and in the presence of high cholesterol concentration, Laurdan GP value monitors the dynamical properties of the vesicles with no evidences of a preferential partitioning in cholesterol-rich domains (Fig. 3). Preliminary time-resolved measurements, not reported here, using DLPC vesicles and various cholesterol concentrations show

the modification of the kinetics of Laurdan relaxation as a function of cholesterol addition. Nevertheless, no specific relaxation kinetics due to the presence of cholesterol and indicative of a preferential Laurdan partitioning can be observed.

Apparently, the results reported here are in contrast with a number of observations regarding evidences of coexisting membrane domains, obtained using various techniques [8–10] and, recently, also using fluorescence microscopy [11]. Generally, the reported evidences refer to relatively large domains, functionally specialized, as in the case of epithelial cells [12], or observed during activities such as lymphocyte capping [37]. None of the reported evidence clearly concerns lipid gel and liquid-crystalline phase domains. In some cases lipid domains are formed because of their exclusion from protein domains [8] or because of a preferential exclusion/concentration of certain labeled lipids in selected membrane areas [11]. Recently reported results [38] performed on epithelial cells using Laurdan technique show the excitation GP spectra with decreasing values with the increase of excitation wavelength, in agreement with our results. Actually, the only observation of the occurrence of separate phase domains in natural membranes has been obtained on ram sperm plasma membranes by the detection of two endothermic transitions separated by a 35°C difference [39]. We must point out that Laurdan can detect the coexistence of phase domains regardless of their dimension and of their lifetime, at least in scales relevant for biological functions. Domains as small as 20 $Å^2$ with a lifetime as short as a few nanoseconds can be detected [4].

The same biological material of fluorescence microscopy observations reported in Ref. 11 has been also utilized for excitation and emission GP measurements. Rabbit erythrocyte ghosts behave similarly to all other cell types, with decreasing values of the excitation GP and increasing values of the emission GP with the increase of wavelength, indicating a homogeneous liquid-crystalline phase. The concentration of fluorescently-labeled lipids in large membrane areas detected by fluorescence microscopy in [11] must be different from phospholipid phase domains, i.e., such a selective concentration does not yield a domain with characteristic resolvable dynamics.

From experiments performed using time-resolved techniques on Laurdan in mixtures of synthetic phospholipids such as the equimolar DLPC and DPPC that originate phase domains segregation [4], the possible dimension of domains has been calculated and resulted of about 20–50 Å, too small to be detected by microscopy. Similar dimensions were obtained by ESR measurements on dimyristoyl- and dipalmitoyl-phosphatidylcholine mixtures [40]. This same phospholipid mixture was used for fluorescence microscopy observations but no domains were visualized [41].

The effect of cholesterol on membrane properties requires some additional comments. Apparently, cholesterol acts as the major modifier of lipid phase properties, averaging the differences of lipids dynamics [23]. The importance of the complex lipid composition of natural membranes seems not to be related to the availability of coexisting environments with very different dynamic properties as those of the gel and of the liquid-crystalline phase. On the contrary, the presence of cholesterol with its 'averaging' effect appears to function as a buffer of the various dynamic properties of membrane lipids. The modulation of membrane dynamic properties or of membrane 'fluidity' may occur by the modulation of cholesterol concentration. Moreover, some results obtained using model membranes composed of binary mixtures of phospholipids must be evaluated. The coexistence of separate domains, i.e., the detection of different resolvable fluorescence parameters arising from separate environments can only be obtained when the ratio between the two phospholipids is in the range between 30 and 70 mol% [4]. Above and below this range, modifications of the properties of each phospholipid by the presence of the other can be observed, but not separate domains are detected. Given the complex lipid composition of natural membranes, the probability of observing separate phase domains must be very low.

The results of our measurements of Laurdan fluorescence on several cell types appear to exclude the existence of phospholipid gel phase in membranes of these cells. The relatively high excitation GP value and the relatively low emission GP value could be indicative either of the presence of an homogeneous liquid-crystalline phase or of the coexistence of liquid-crystalline phases with different cholesterol concentrations. However, in the equimolar DLPC/DPPC mixture, it is unlikely that there is coexistence of two separate liquid-crystalline domains at high cholesterol concentration. By comparing the experimental results with the simulated ones (Fig. 2B) we can observe that the properties of each phospholipid are profoundly modified by the presence of cholesterol. Actually, the simulated results show some differences from the experimental ones. However, these results are obtained in synthetic phospholipid vesicles and we cannot completely exclude that in natural membranes liquid domains with different dynamic properties coexist. The study of the extent of modifications of Laurdan excitation and emission spectra and GP values produced by various cholesterol concentrations will be the aim of further studies intended to ascertain if this probe shows resolvable parameters in the case of coexisting liquid-crystalline domains with different degree of order, i.e., the cholesterol-rich and the cholesterol-poor phases, described as liquid-ordered and liquid-disordered phases.

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