Open access • Journal Article • DOI:10.1007/BF00718783

Membrane lipid domains and dynamics as detected by Laurdan fluorescence.

Tiziana Parasassi, Enrico Gratton

Published on: 01 Mar 1995 - Journal of Fluorescence (Kluwer Academic Publishers-Plenum Publishers)

Topics: Laurdan, Phase (matter), Bilayer and Membrane

Journal of Fluorescence, Vol. 5, No. 1, 1995

Membrane Lipid Domains and Dynamics as Detected by Laurdan Fluorescence

Tiziana Parasassi[1] and Enrico Gratton[1,2]

Received February 16, 1994; revised September 27, 1994; accepted September 27, 1994

2-Dimethylamino-6-lauroylnaphthalene (Laurdan) is a membrane probe of recent characterization, which shows high sensitivity to the polarity of its environment. Steady-state Laurdan excitation and emission spectra have different maxima and shape in the two phospholipid phases, due to differences in the polarity and in the amount of dipolar relaxation. In bilayers composed of a mixture of gel and liquid-crystalline phases, the properties of Laurdan excitation and emission spectra are intermediate between those obtained in the pure phases. These spectral properties are analyzed using the generalized polarization (*GP*). The *GP* value can be used for the quantitation of each phase. The wavelength dependence of the *GP* value is used to ascertain the coexistence of different phase domains in the bilayer. Moreover, by following the evolution of Laurdan emission vs. time after excitation, the kinetics of phase fluctuation in phospholipid vesicles composed of coexisting gel and liquid-crystalline phases was determined. *GP* measurements performed in several cell lines did not give indications of coexistence of phase domains in their membranes. In natural membranes, Laurdan parameters indicate a homogeneously fluid environment, with restricted molecular motion in comparison with the phospholipid liquid-crystalline phase. The influence of cholesterol on the phase properties of the two phospholipid phases is proposed to be the cause of the phase behavior observed in natural membranes. In bilayers composed of different phospholipids and various cholesterol concentrations, Laurdan response is very similar to that arising from cell membranes. In the absence of cholesterol, from the steady-state and time-resolved measurements of Laurdan in phospholipid vesicles, the condition for the occurrence of separate coexisting domains in the bilayer has been determined: the molecular ratio between the two phases must be in the range between 30% and 70%. Below and above this range, a single homogeneous phase is observed, with the properties of the more concentrated phase, slightly modified by the presence of the other. Moreover, in this concentration range, the calculated dimension of the domains is very small, between 20 and 50 Å.

KEY WORDS: Cholesterol; domains; Laurdan; generalized polarization; membrane; phospholipids.

THE FLUID MOSAIC MODEL IN 1972

It is well established that phospholipids in the bilayer aggregation can be found in two main phase states, the gel and the liquid-crystalline [1]. Instead, lipids in biological membranes were proposed, in 1972 [2], to be in a homogeneously fluid phase state. This *fluid* state was identified with the liquid-crystalline phase, in which molecules can freely diffuse in the plane of the membrane. Natural membranes show a complex lipid composition, each component having specific phase properties. For phospholipids, the lipid class present at the highest concentration in membranes, the differences

[1] Istituto di Medicina Sperimentale, CNR, Viale Marx 15, 00137 Rome, Italy.
[2] Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801.

1053-0509/95/0300-0059$07.50/0

in the nature of their polar heads, in the length and unsaturation of their acyl residues, give rise to very different temperatures of transition from the gel to the liquid-crystalline phase [3,4]. This leads to the hypothesis of a possible coexistence of domains of different phases in the plane of the membrane at the physiological temperature: gel-like domains composed of phospholipids with higher transition temperature and liquid-crystalline-like domains composed of phospholipids with lower transition temperature. These coexisting domains will possess different molecular dynamics and different kinetics of in-plane diffusion. A corollary of this hypothesis was the possibility of modulating cell functions by a preferential partition of selected enzymes between the two types of domains [5,6]. In general, phase domains could create separate compartments for the different membrane activities, each requiring peculiar local dynamical properties.

THE SEARCH FOR COEXISTING PHASE DOMAINS IN VESICLES AND IN MEMBRANES

Several spectroscopic techniques have been used to study the bilayer phase state [3,4,7–9]. Fluorescence spectroscopy offers several advantages for both the intrinsic time scale of the fluorescence, allowing observations on events occurring in the nanosecond time scale, which is typical of several biochemical events [10], and for the advantages offered by this technique of measurement. Fluorescence measurements are fast and require a small amount of sample, and the low concentration of the fluorophore makes it possible to exclude any significant perturbing effect. These are important considerations when working with "living" biological material, such as cells in culture. For studies on the lipid components of membrane, the more interesting fluorescent probes are those with a similar chemical structure so that the lipid organization is not disturbed. The probe should have a high quantum yield in hydrophobic environment and virtually nil in water, negligible affinity with other components of natural membranes, such as proteins, and high sensitivity to the membrane phase state. 1,6-Diphenyl-1,3,5-hexatriene (DPH) is one of the most popular membrane probes, widely utilized for measurements of the average fluidity by its fluorescence polarization [11–13]. DPH fluorescence decay is sensitive to the polarity of its surroundings. The fluorescence lifetime decreases with the increase of polarity [14]. In phospholipids, this sensitivity to polarity results in higher average lifetime values in the gel with respect to the liquid-crystalline phase [15,16]. In vesicles of known composition, the DPH decay has been resolved into two components, corresponding to the lifetime values measured in each pure phase, and the associated relative fractions were in a good agreement with the reported phase diagrams [16]. Nevertheless, the characteristic lifetime values are also dependent on the temperature at which measurements are performed, so that in samples of unknown composition the quantitative resolution of two coexisting phases cannot be obtained in a simple way [14]. Additional experimental problems prevented the use of DPH for the detection and the resolution of coexisting domains in membranes: (i) The difference in the average lifetime values measured in each pure phase is small. (ii) DPH decay in phospholipid vesicles as a function of temperature can be equally described by a linear superposition of the properties arising from the two phases or by a continuous variation of the properties of the probe along the phase transition. During the phase transition the intermediate lifetime value can originate from the contribution of two coexisting phases or from a homogeneous phase with intermediate properties. (iii) DPH decay is better described by a continuous distribution of lifetime values [17,18], the largest difference between the two phospholipid phases residing in the value of its width, narrow in the gel and broader in the liquid-crystalline phase. The width of DPH lifetime distribution reflects the microheterogeneity of its environment, reflecting water concentration differences along the membrane normal [14] (see also C. Stubbs in this issue). The measurement of the width of DPH lifetime distribution can be used to monitor membrane alterations that affect the water gradient, such as oxidative damage [19,20], but does not help for the quantitation of lipid phases.

The fluorescence properties of the two isomers of parinaric acid have also been studied for the detection and quantitation of coexisting domains in membranes [21]. Due to the different configuration of their unsaturations, the two isomers show a different preferential partitioning between the two phases. When measured in the same sample, the polarization of the *cis* isomer is generally lower than that of the *trans* isomer [21]. Nevertheless, these two probes show quite a complex decay of fluorescence. In isotropic solvents their emission decay has been described by three discrete exponential components [22] or by a two-component Lorentzian distribution [23,24]. Even in vesicles composed of synthetic phospholipids, the resolution of coexisting domains requires long and delicate measurements [23,24].

To ascertain the coexistence of lipid-phase domains in membranes, the ideal probe should possess all the properties of the membrane probes described above,

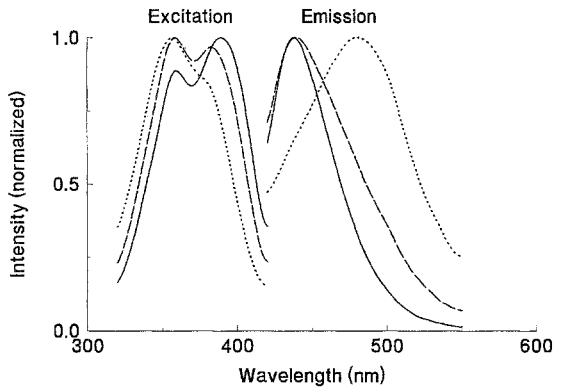

Fig. 1. Normalized Laurdan excitation and emission spectra in phospholipid multilamellar vesicles composed of gel (continuous line), liquid-crystalline (dotted line), and an equimolar mixture of the two phases (dashed line). Phospholipid composition of the vesicles and the temperature of measurements are dilauroylphosphatidylcholine at 40°C (dotted line), dipalmitoylphosphatidylcholine at 5°C (continuous line), and an equimolar mixtures of the two phospholipids at 20°C.

coupled with a high sensitivity to the phospholipid-phase state. It should display a limited set of parameters, typical for each phase, that could be easily resolved, i.e., it should display a steady-state, spectral sensitivity to the lipid phase.

LAURDAN FLUORESCENCE CAN RESOLVE COEXISTING DOMAINS IN VESICLES

Laurdan has all the properties needed for the detection and quantitation of coexisting phase domains in membranes. This probe was synthesized by G. Weber for the study of the effect of solvent polarity on the fluorescence emission [25,26]. In solvents of higher polarity, Laurdan displays a red shift of its emission spectrum [27], due to dipolar relaxation. The dipole moment of the fluorescent moiety of the Laurdan molecule increases several debyes upon absorption. If the molecular dynamics of solvent molecules is of the same time scale as that of the Laurdan fluorescence lifetime, part of the energy of the probe in the excited state can be spent for the reorientation of the solvent dipoles in the close vicinity. Laurdan emission is thus red-shifted [28]. When the solvent is constituted of phospholipids, Laurdan emission strongly depends on their phase state, being blue, nonrelaxed, in the gel phase, and red, relaxed, in the liquid-crystalline phase (Fig. 1) [29,30]. From the gel to the liquid-crystalline phase, the Laurdan emission maximum shifts by about 50 nm, from a maximum at 440 nm in the gel to a maximum at 490 nm in the liquid-crystalline phase. This behavior indicates that in the gel phase the molecular dynamics of the dipoles surrounding Laurdan is slower than the probe lifetime. During the phospholipid transition, and in vesicles composed of a mixture of phospholipids in the two phases, Laurdan emission spectra with intermediate maximum wavelength and center of mass are observed [29,30]. Also the excitation spectrum of Laurdan is modified by the polarity of solvents and by the phase state of phospholipids [27,30]. In isotropic nonpolar solvents, the Laurdan excitation spectrum shows a single blue band, with the maximum at about 340 nm. In polar solvents, the Laurdan excitation spectrum is red-shifted, with a maximum at about 370 nm, and displays a second excitation band with a maximum at about 390 nm. This second, red, excitation band increases its intensity with the increase of the polarity of the solvents and has been attributed to the stabilization of the probe ground-state $L\alpha$ conformation, due to polar solvent molecules oriented around the probe dipole, i.e., already relaxed [27]. When Laurdan is inserted in phospholipid bilayers, the red excitation band is present and its intensity depends upon the phase state of phospholipids. In the gel phase, this excitation band is particularly intense, constituting the maximum excitation (Fig. 1), while in the liquid-crystalline phase, the red band is still present, but the maximum excitation is at 355 nm, corresponding to the blue excitation band [30]. Thus the intensity of the red excitation band depends both on the polarity of the Laurdan environment and, when inserted in phospholipid vesicles, also on their phase state. Laurdan molecules that populate this red band are those molecules surrounded by oriented dipoles and, if present, by phospholipids in the gel phase.

LAURDAN GENERALIZED POLARIZATION

A method for the treatment of the differences observed in both the excitation and emission spectra of Laurdan has been developed. The generalized polarization (GP) is defined as $GP = (I_g - I_{lc}) / (I_g + I_{lc})$, where I_g and I_{lc} are the intensities observed at the wavelengths typical of the maximum excitation or, alternatively, of the maximum emission in the gel and in the liquid-crystalline phase, respectively [30]. The choice of the precise excitation and emission wavelengths for the calculation of the GP value is dictated by the wavelengths of the maximum intensity in the two phases. In our calculation of excitation GP, the intensities of the emission at wavelengths of 440 and of 490 nm have been chosen. In our calculation of emission GP, the intensities of the exci-

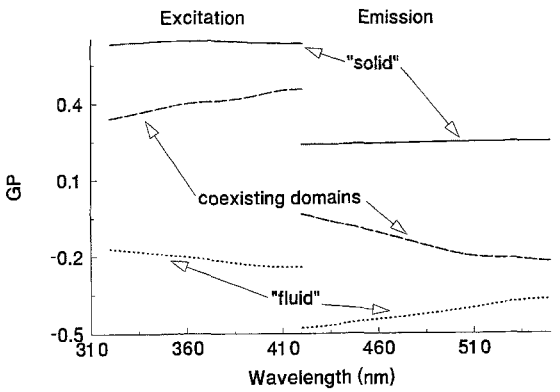

Fig. 2. Laurdan excitation and emission *GP* spectra of phospholipids in different phases, as reported in Fig. 1. Excitation *GP* spectra were calculated by $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$, using excitation wavelengths from 320 to 420 nm. Emission *GP* spectra were calculated by $GP = (I_{410} - I_{340})/(I_{410} + I_{340})$, using emission wavelengths from 420 to 550 nm.

tation at 410 and 340 nm have been chosen, at the edges of the two excitation bands. The two *GP* values were then calculated by excitation $GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$ and emission $GP = (I_{410} - I_{340}) / (I_{410} + I_{340})$. With the above definitions, high *GP* values are measured in the gel, while low *GP* values are measured in the liquid-crystalline phase [30]. The use of the *GP* offers several advantages: (i) once characteristic values for the *GP* in the gel and in the liquid-crystalline phase are determined, the property of additivity of fluorescence polarization can be used to quantitate the domains [27]; (ii) all properties of fluorescence polarization can be used, among others the possibility of determining the kinetics of dipolar relaxation by an equation equivalent to the Perrin equation [27,30,31]; (iii) in contrast to common ratiometric measurements used for probes displaying spectral sensitivity to the properties of the environment, such as pH and calcium concentration probes, the *GP* measurement does not require calibration; and (iv) the measurement is fast and easy.

GP values typical of the gel and of the liquid-crystalline phase have been determined, using vesicles composed of phospholipids differing in their acyl and in their polar head residues, at pH between 4 and 10 [27]. The determined excitation *GP* values are 0.6 and −0.2 for the gel and liquid-crystalline phases, respectively, using an excitation wavelength of 340 nm and emission wavelengths of 440 and 490 nm, while the emission *GP* values are 0.4 and −0.5 for the gel and liquid-crystalline phases, respectively, using an emission wavelength of 440 nm and excitation wavelengths of 410 and 340 nm [27]. The *GP* value has been found to depend only on the phospholipid-phase state, and not on the type and charge of their polar residue. Following these observations, the process of dipolar relaxation which determines the spectral properties must then be attributed to a few water molecules, present at the hydrophobic–hydrophilic interface of the bilayer, where the fluorescent moiety of Laurdan is located [27]. Rotational rearrangement of the Laurdan molecule itself during its excited state has been excluded as the origin of the dipolar relaxation, from the measurement of Laurdan "classical" polarization along its emission spectrum [30] and from experiments using various Laurdan derivatives (unpublished observations). With respect to the "bulk" water, whose rotational kinetics is known to be on the picosecond time scale, those water molecules in the bilayer involved in the relaxation should rotate more slowly. Moreover, the dynamics of these water molecules is of the order of nanoseconds only when phospholipids are in the liquid-crystalline state. The reason for the absence of dipolar relaxation in the gel state can be an even slower motion of water molecules.

WAVELENGTH DEPENDENCE OF THE GENERALIZED POLARIZATION TO ASCERTAIN THE COEXISTENCE OF PHASE DOMAINS

Once a *GP* value intermediate between the high value of the gel and the low value of the liquid-crystalline phase is observed, the property of additivity can be utilized to quantitate each domain [27]. An intermediate value per se is not a proof of the coexistence of different domains, since it can originate from a homogeneous environment with intermediate properties. The behavior of the *GP* value as a function of excitation and of emission wavelength can be used to distinguish between these two cases. Both excitation and emission *GP* spectra are flat in the gel phase (Fig. 2). In the liquid-crystalline phase, the excitation *GP* spectrum shows decreasing values with increasing excitation wavelength, while the emission *GP* spectrum shows increasing values with increasing emission wavelength. When domains of different phases coexist in the membrane, the behavior of the *GP* value is opposite to that observed in the liquid-crystalline phase. The excitation *GP* increases and the emission *GP* decreases with increasing excitation and emission wavelength, respectively (Fig. 2) [27]. For the favorable spectroscopic properties of Laurdan, we can easily distinguish between a homogeneous liquid-crystalline phase and a mixed phase of coexisting domains. The red band of the excitation spectrum is populated by Laurdan

molecules stabilized in the $L\alpha$ conformation by oriented solvent dipoles, and by Laurdan molecules surrounded by phospholipids in the gel phase. If phospholipids in the gel phase are not present, the red band is only populated by relaxed Laurdan molecules, with a red-shifted emission spectrum and a low *GP* value. If phospholipids in the gel phase are present, they mainly populate the red band of excitation, emitting with a blue spectrum and with a high *GP* value [32]. Thus, by moving the excitation toward the red, in the homogeneous liquid-crystalline phase the *GP* value decreases, while in bilayers composed of coexisting phases the *GP* value increases. Similar reasoning can be made for the *GP* values obtained at different emission wavelengths, as extensively reported and discussed elsewhere [32]. Note that, as reported above, the emission *GP* is calculated by the difference between the intensity of the red excitation band and that of the blue one, while the excitation *GP* is calculated by the difference between the intensity at the blue and that at the red part of the emission spectrum. Thus, the wavelength dependence of excitation and emission *GP* values is opposite.

PHASE FLUCTUATIONS DETERMINED BY LAURDAN TIME-RESOLVED EMISSION SPECTRA

Although longer and involving more sophisticated instrumentation, the determination of the coexistence of domains of different phase states can be also performed using time-resolved techniques. Due to dipolar relaxation, the Laurdan spectral shape and center of mass change with time after excitation [28–30]. Time-resolved emission spectra of Laurdan in vesicles composed of phospholipids in the liquid-crystalline phase, i.e., in a relaxing environment, show a progressive red shift of the maximum and an increase of the center of mass of the emission with time. In vesicles composed of gel-phase phospholipids, i.e., in a nonrelaxing environment, Laurdan emission does not change appreciably with the time after excitation [32]. When domains of the two phases coexist, the Laurdan maximum and center of mass increase up to about 20 ns after excitation; then the spectrum slightly shifts back toward the blue (Fig. 3) [30,32]. Such an inversion of the spectral shift has been interpreted as due to fluctuation between the different phases [30], with kinetics of about 25 ns, and imply *per se* the coexistence of separate phase domains. This result is in agreement with those obtained by the use of ultrasound techniques [33] and by the measurements of the rotational behavior of the fluorescent coronene [34], where phase fluctuations were found to occur in the range from 20 to 60 ns and from 20 to 200 ns, respectively. Laurdan lifetime measured over all the emission spectrum is 5.9 ns in the gel and 4.0 ns in the liquid-crystalline phase [32]. Although the emission intensity after 25 ns—about six lifetimes—may be small, the experimental conditions allowed the observation at selected emission wavelengths where the time-zero intensity is small [30]. Time-resolved experiments have been performed using vesicles composed of binary mixtures of phospholipids with various relative concentrations of the two phase [32]. The results showed that the coexistence of domains can be observed only in the range between 30% and 70% of one phase relative to the other. Below and above this range the phospholipids are homogeneously mixed, with the dynamical properties of the more concentrated phospholipid, modified by the presence of the other. These results pose an important limit to the existence of separate and coexisting phase domains in bilayers of more complex composition. A further limit arises when the possible dimensions of the domains are calculated, based both on time-resolved and on steady-state measurements [32]. In binary mixtures of the two phospholipid phases, and in the range between 30 and 70 mol% of one phase relative to the other, the dimension of domains calculated from the time-resolved fluorescence spectra is very small, between 20 and 50 Å. A similar size of the domains was calculated by Ipsen *et al.* [35] for a single phospholipid bilayer at the transition temperature.

ABSENCE OF COEXISTING PHASE DOMAINS IN CELL MEMBRANES

Laurdan has been used to label membranes of cells in culture and of primary cells. Seven cell lines, four primary cell types [36,37], and membranes purified from kidney cortex [38] have been studied with Laurdan. Both Laurdan spectra and *GP* values in these samples show some peculiarities: (i) the *GP* values are relatively high, similar to those obtained in phospholipids where the phase state is more than 50% gel (Fig. 4); (ii) accordingly, the excitation and emission spectra are close to the spectra obtained in gel-phase phospholipids [36]; (iii) on the contrary, excitation and emission *GP* spectra show the wavelength behavior that is typical of the liquid-crystalline phase, i.e., the excitation *GP* spectrum shows decreasing values and the emission *GP* spectrum shows increasing values as the wavelength increases (Fig. 4). These results cannot be attributed to the association of Laurdan with membrane proteins, since the

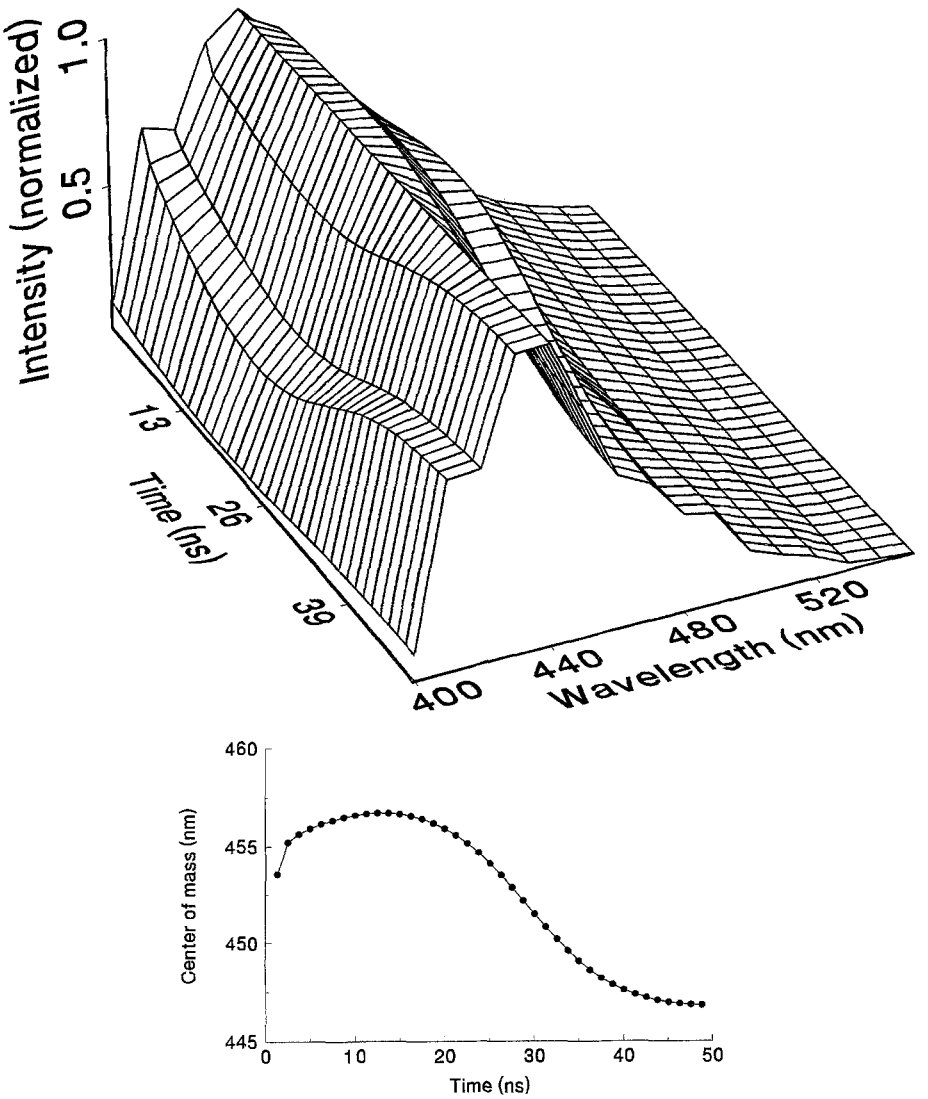

Fig. 3. (A) Normalized Laurdan time-resolved emission spectra in multilamellar vesicles composed of an equimolar mixture of dipalmitoyl- and dilauroylphosphatidylcholine at 20°C. The measurements were performed using a K2 fluorometer (ISS Inc., Champaign IL) equipped with an argon-ion laser. The excitation was 351 nm and the emission was collected after the instrument monochromator, with a 10-nm step for each measurement. An additional bandpass filter (Corning 754) was used after the excitation. POPOP in ethanol was used as the reference. The analysis of lifetime data and the generation of time-resolved spectra were performed using the Globals Unlimited software (™University of Illinois at Urbana-Champaign) [58]. (B) Shift of the emission center of mass as a function of the time after excitation, calculated from the time-resolved spectra.

probe affinity for proteins has been shown to be very low, as is its fluorescence intensity in their presence [36,37]. Also, a preferential partitioning of Laurdan in selected phospholipid domains must be excluded [32,39].

THE HOMOGENIZING EFFECT OF CHOLESTEROL

With the aim of explaining the apparent conflict between the absolute *GP* value, relatively high and indicative of a rigid environment, and its wavelength dependence, typical of a fluid environment, as measured in cell membranes, we investigated the influence of cholesterol on the phospholipid phases [37,39].

Several spectroscopic studies reported a strong modification of phase properties of phospholipids due to the presence of cholesterol [40–51]. Depending on their relative concentration and on temperature, the phase properties of vesicles composed of binary mixtures of cholesterol and phospholipids have been described by

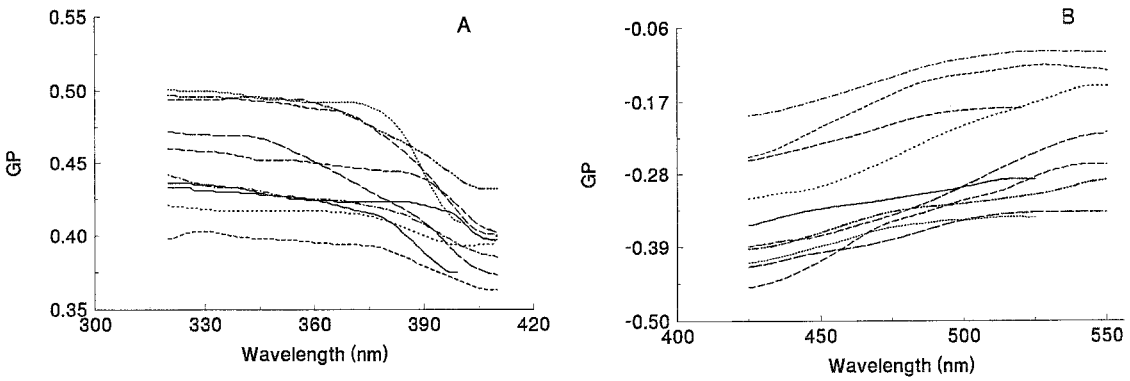

Fig. 4. Laurdan (A) excitation and (B) emission *GP* spectra in various cell types at 20°C. (A) From top to bottom, at wavelength of 320 nm: rabbit erythrocyte ghosts, Friend leukemia cells (FLC), human lymphoblastoid B Raji cells, Molt4 cells, human histiocytic lymphoma U937 cells, mouse splenocyte cells, mouse myeloma P3U cells, rat postnatal cerebellar granule cells (Granule Cells), mouse myeloma NS0 cells, mouse thymocyte cells. (B) From top to bottom, at wavelength of 425 nm: P3U, NS0, erythrocyte ghosts, FLC, Granule Cells, Molt4, Thymocytes, U937, Splenocytes, Raji [37].

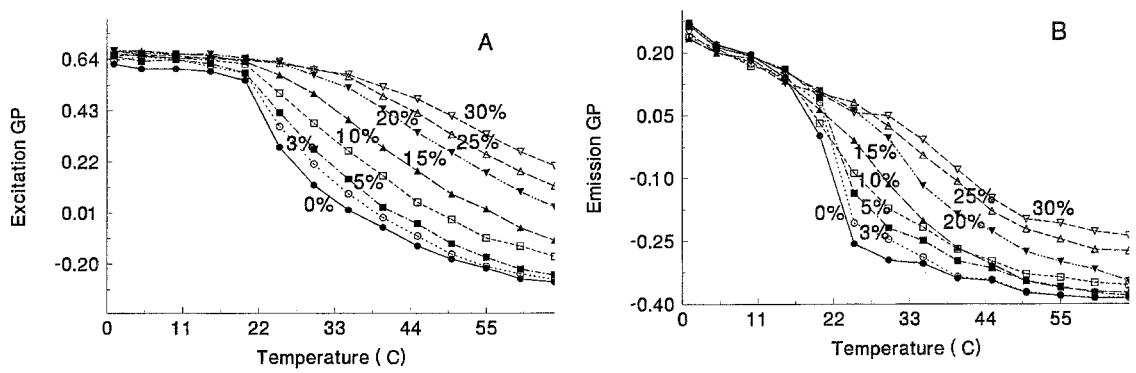

Fig. 5. Laurdan (A) excitation and (B) emission *GP* values vs. temperature obtained in dimyristoylphosphatidylcholine multilamellar vesicles at various cholesterol concentrations. Percent values represent mol% cholesterol.

the solid-ordered, liquid-disordered and liquid-ordered phases [52,53]. These newly defined phases account for the increase of translational and rotational motions of the phospholipid gel phase in the presence of cholesterol, and for their decrease in the liquid-crystalline phase [52]. In simple words, cholesterol renders more fluid the gel and more solid the liquid-crystalline phase. Noticeably, above 30 mol% cholesterol with respect to phospholipids, only a liquid-ordered phase can be observed, even at relatively low temperatures, below the phospholipid phase transition [41,51,52]. These are the average physiological cholesterol concentrations in most biological membranes [53].

The results obtained by labeling with Laurdan vesicles composed of various phospholipids, pure and mixed, and cholesterol [39] are in agreement with the observations reported above. The cholesterol concentration has been varied from 3 to 60 mol% with respect to phospholipids. The phospholipids were composed of pure and mixed phases. With respect to the gel phase, larger variations in Laurdan spectra and *GP* values are produced by cholesterol addition to the liquid-crystalline phase (Fig. 5). The emission spectra are blue-shifted and the excitation spectra show an increase of the red band. Both excitation and emission *GP* values are higher. In the gel phase, the increase of *GP* values is observed for emission spectra and excitation *GP* values, while the emission *GP* values show a slight decrease with the increase of cholesterol concentration (Fig. 5B) [39]. From the point of view of Laurdan spectroscopy, the blue shift observed in the emission spectra (higher excitation *GP* values) and the increased intensity of the red excitation band (higher emission *GP* values) indicate a decreased amount of dipolar relaxation. Also, the decreased polarity due to the presence of cholesterol is monitored by the emission blue shift, while the excitation spectrum

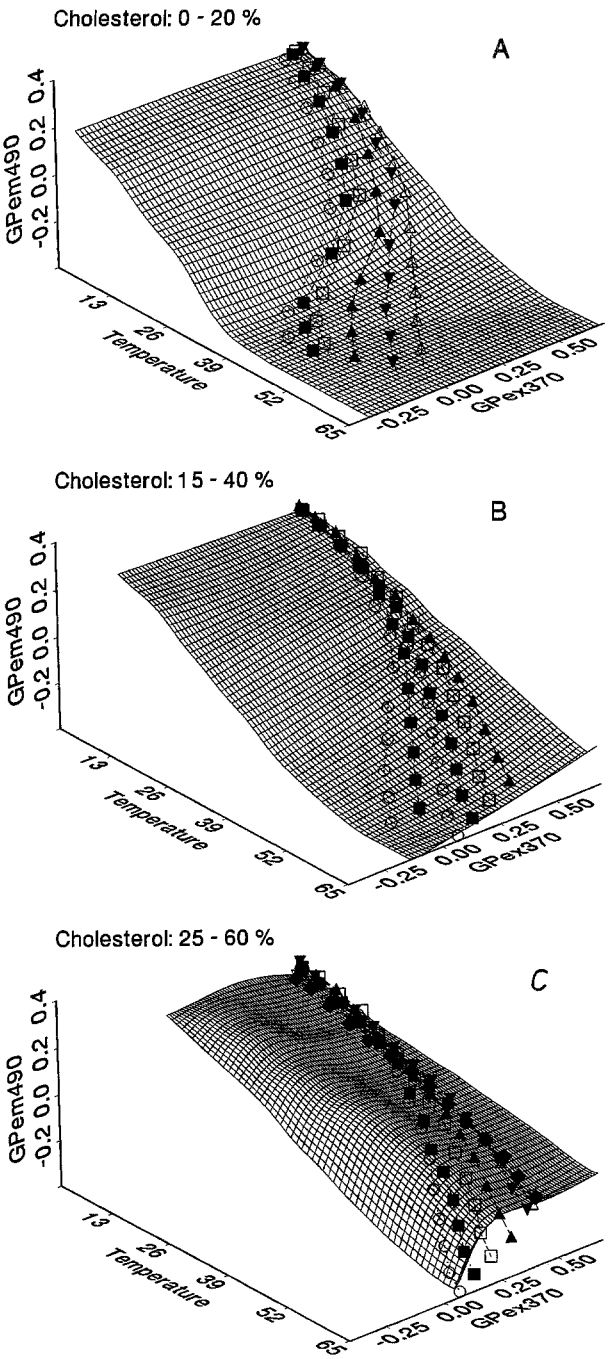

Fig. 6. Three-dimensional surface obtained from Laurdan excitation and emission *GP* values and from the temperature, in vesicles composed of an equimolar dilauroyl- and dipalmitoylphosphatidylcholine mixture, with cholesterol concentrations of (A) 0, 3, 5, 10, 15, 20 mol %, (B) 15, 20, 25, 30, 35, 40 mol%, (C) 25, 30, 35, 40, 45, 50, 55, 60 mol%. The excitation *GP* values were measured using excitation at 370 nm and the emission *GP* values using emission at 490 nm. Intensities of the blank were subtracted. In all cases the individual 3D curves of samples at each cholesterol concentration are superimposed. The underlying surface has been generated using all the data of the superimposed plots, by the 3D spline option of the Axum software (TriMetrix Inc.).

shows a slight decrease of the red band, as observed in the gel phase (Fig. 5B). Both the decreased dipolar relaxation and the decreased polarity produce a blue shift of the emission spectrum, while their effect on the red band of excitation is opposite. On this basis, variations of dipolar relaxation are better evidenced in the emission spectrum, while variations of polarity can be better observed in the excitation spectrum, especially in the gel phase, i.e., when the amount of dipolar relaxation is reduced. This implies that excitation *GP* does not vary the same amount as emission *GP* and that plots of excitation *GP* vs. emission *GP* obtained in binary mixtures of phospholipids and cholesterol at various temperatures do not overlap. Three-dimensional plots have been built and a complex surface has been obtained [39]. In Fig. 6 the surface obtained using vesicles composed of an equimolar mixture of dilauroyl- and dipalmitoylphosphatidylcholine and various cholesterol concentrations is reported. This surface shows three different regions (Fig. 6A). High values of both excitation and emission *GP* define a region of solid phase. Low *GP* values define a region of liquid phase. Intermediate excitation and emission *GP* values fall in a region where, for the pure lipids and in the absence of cholesterol, the coexistence of phase domains is observed (Fig. 6A). By increasing the concentration of cholesterol in the sample, the individual plots are confined to the region of the solid phase, and the surface appears flattened, with relatively homogeneous and high *GP* values (Figs. 6B and 6C). As a first observation, the presence of cholesterol reduces both the concentration and dynamics of water molecules present at the hydrophobic–hydrophilic interface of the bilayer. The first effect is due to the displacement of water molecules operated by cholesterol, and is in agreement with the reported data [54]. The second effect is be due to the closer packing of phospholipids with the consequence of a restriction of their molecular motions.

A further observation concerns the removal of the phospholipid phase transition when cholesterol is present at concentrations higher than 20 mol% (Fig. 5) [39], in agreement with reported observations [40,45]. By plotting excitation or emission *GP* values vs. temperature, the abrupt change observed in the absence of cholesterol and indicative of the phospholipid phase transition is progressively smoothed in samples with increasing cholesterol concentration. At 20 mol% cholesterol and above, the variation of *GP* values resembles more closely a gradual temperature effect rather than a phase transition (Fig. 5).

As for the wavelength dependence of *GP* values, the effect of cholesterol is even more dramatic. As discussed above, the characteristic behavior of the *GP* value

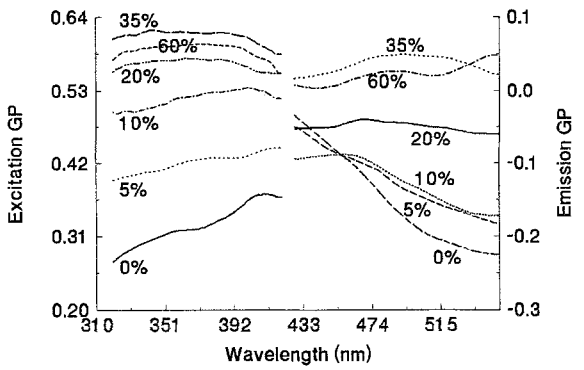

Fig. 7. Laurdan excitation and emission *GP* spectra obtained in multilamellar vesicles composed of equimolar dilauroyl- and dipalmitoylphosphatidylcholine at various cholesterol concentrations at 25°C. The percent values represent mol% of cholesterol in phospholipids.

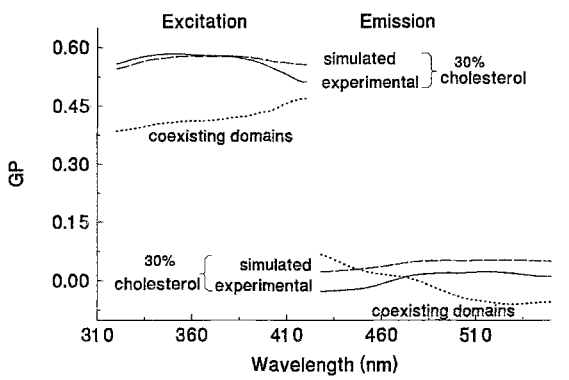

Fig. 8. Laurdan excitation and emission *GP* spectra obtained at 20°C in multilamellar vesicles composed of an equimolar mixture of dilauroyl- and dipalmitoylphosphatidylcholine (dotted line, *coexisting domains*) and in the presence of 30 mol% cholesterol (dashed and continuous lines). Experimental data (continuous line) and simulated data (dashed line). The simulation was performed by acquiring excitation and emission spectra of the individual phospholipids in the presence of 30 mol% cholesterol. The spectra were then added. To account for the difference of Laurdan lifetime between the two phospholipids, the addition was done as 0.6 gel + 0.4 liquid-crystalline. The *GP* values were calculated from the resulting spectra.

as a function of excitation and emission wavelength discriminates between bilayers composed of coexisting domains of different phases and of homogeneous, intermediate, phase properties [32]. By adding cholesterol to phospholipid vesicles composed of an equimolar concentration of the two phases, the typical behavior of *GP* values is progressively abolished, so that at cholesterol concentrations above 10–15 mol% excitation and emission *GP* spectra show the wavelength dependence typical of a homogeneous liquid-crystalline phase, although the absolute *GP* values are higher (Fig. 7) [39].

We also performed simulation experiments where the spectra of the individual phospholipids in the presence of 30 mol% cholesterol were separately acquired. Then the spectra were added and the *GP* was calculated from the resulting spectra [37]. These simulated *GP* spectra impressively reproduced the experimental ones (Fig. 8).

THE FLUID MOSAIC MODEL IN 1994

The reported observations on Laurdan fluorescence behavior in phospholipid bilayers lead to the conclusion that cholesterol has a profound and opposite influence on both phospholipid-phase states. Cholesterol renders the dynamical properties of the two phases very similar, so that they cannot be resolved. Besides several known effects of cholesterol on lipid bilayers, a new biological meaning for its presence can be outlined. The function of cholesterol can be related to the "smoothing" of the local phase differences that would otherwise arise from the heterogeneous composition of membranes. The presence of cholesterol should avoid the occurrence of extremely solid or extremely fluid environments for cell functions. No coexisting domains were detected by labeling 12 natural membrane types with Laurdan [36–38]. Instead, in natural membranes, relatively high *GP* values with a wavelength dependence typical of a homogeneously fluid environment were obtained, in agreement with the above data on Laurdan in synthetic phospholipid vesicles in the presence of cholesterol.

We must point out that even in the absence of cholesterol, limiting conditions for the occurrence of phase domain coexistence have been found. Using Laurdan fluorescence, we have been able to demonstrate that in a binary phospholipid mixture composed of the two phases, coexisting domains each with properties close to the pure phases can be observed only in the concentration range between 30 and 70 mol% of one phospholipid with respect to the other [32]. In this concentration range, the calculated dimensions of domains are small, on the order of 20–50 Å [32].

Using the sensitive Laurdan probe to label phospholipids, evidence for the coexistence of domains of different cholesterol concentrations [51] could not be inferred [39]. Further investigations are needed to characterize subtle effects of different cholesterol concentrations on the lipid bilayer. Recent observations revealed that Laurdan steady-state parameters do not change continuously with cholesterol concentration. Depending on temperature and on the composition of the bilayer, there are some cholesterol concentrations that produce abrupt changes of the Laurdan spectral shape

[55]. These concentrations are in a close agreement with critical pyrene-PC concentrations, having a local ordering effect on the phospholipid liquid-crystalline phase [56,57].

ACKNOWLEDGEMENTS

Sincere thanks are due to Dr. D. M. Jameson for the use of his instrumentation for the measurement of the time-resolved spectra reported in Fig. 3. This work was supported by NIH RR03155 (E. G.) and, in part, by a Wood/Whelan fellowship (1991) (T. P.).

REFERENCES

1. C. J. Scandella, P. Devaux, and H. M. McConnell (1972) Rapid lateral diffusion of phospholipids in rabbit sarcoplasma reticulum, *Proc. Natl. Acad. Sci. USA* **69**, 2056–2060.
2. S. J. Singer and G. L. Nicolson (1972) The fluid mosaic model of the structure of cell membranes, *Science* **175**, 720–731.
3. E. J. Shimshick and H. M. McConnel (1973) Lateral phase separation in phospholipid membranes, *Biochemistry* **12**, 2351–2360.
4. M. P. Andrich and J. M. Vanderkooi (1976) Temperature dependence of 1,6-diphenyl-1,3,5-hexatriene fluorescence in phospholipid artificial membranes, *Biochemistry* **15**, 1257–1261.
5. H. K. Kimelberg (1976) Na,K-dependent adenosine triphosphatase activity in reconstituted systems and in cultured cells, *Biochem. Soc. Trans.* **4**, 755–777.
6. P. L. Chong, P. A. G. Fortes, and D. M. Jameson (1985) Mechanisms of inhibition of (Na,K)-ATPase by hydrostatic pressure studied with fluorescent probes, *J. Biol. Chem.* **27**, 14484–14490.
7. P. M. Macdonald and J. Seelig (1987) Calcium binding to mixed phosphatidylglycerol–phosphatidylcholine bilayers as studied by deuterium nuclear magnetic resonance, *Biochemistry* **26**, 231–240.
8. V. Luzzati, A. Tardieu, and D. Taupin (1972) An approach to the phase problem in the X-ray diffraction study of biological membranes and model systems, *Chem. Phys. Lipids* **8**, 292–297.
9. M. R. Morrow and J. H. Davis (1988) Differential scanning calorimetry and ^{2}H NMR studies of the phase behaviour of gramicidin–phosphatidylcholine mixtures, *Biochemistry* **27**, 2024–2032.
10. D. M. Jameson (1984) Fluorescence: Principles, methodologies and applications, in E. Voss, Jr. (Ed.), *Fluorescein Hapten: An Immunological Probe*, CRC Press, Boca Raton, Florida, pp. 23–48.
11. B. W. van der Meer (1988) Biomembranes structure and dynamics viewed by fluorescence, in H. J. Hilderson and J. R. Harris (Eds.), *Subcellular Biochemistry*, Vol. 3, Plenum Press, New York, pp. 1–53.
12. B. R. Lentz (1989) Membrane "fluidity" as detected by diphenylhexatriene probes, *Chem. Phys. Lipids* **50**, 171–190.
13. M. Shinitzky, A. C. Dianoux, C. Gitler, and G. Weber (1971) Microviscosity and order in the hydrocarbon region of micelles and membranes determined with fluorescent probes. I. Synthetic micelles, *Biochemistry* **10**, 2106–2113.
14. T. Parasassi, G. De Stasio, R. M. Rusch, and E. Gratton (1991) A photophysical model for diphenylhexatriene fluorescence decay in solvents and in phospholipid vesicles, *Biophys. J.* **59**, 466–475.
15. R. D. Klausner, A. M. Kleinfeld, R. L. Hoover, and M. J. Karnovsky (1980) Lipid domains in membranes, *J. Biol. Chem.* **255**, 1286–1295.
16. T. Parasassi, F. Conti, M. Glaser, and E. Gratton (1984) Detection of phospholipid phase separation. A multifrequency phase fluorimetry study of 1,6-diphenyl-1,3,5-hexatriene fluorescence, *J. Biol. Chem.* **259**, 14011–14017.
17. R. Fiorini, M. Valentino, S. Wang, M. Glaser, and E. Gratton (1987) Fluorescence lifetime distributions of 1,6-diphenyl-1,3,5-hexatriene in phospholipid vesicles, *Biochemistry* **26**, 3864–3870.
18. T. Parasassi, F. Conti, E. Gratton, and O. Sapora (1987) Membranes modification of differentiating proerythroblasts. Variation of 1,6-diphenyl-1,3,5-hexatriene lifetime distributions by multifrequency phase and modulation fluorimetry, *Biochim. Biophys. Acta* **898**, 196–201.
19. T. Parasassi, O. Sapora, A. M. Giusti, G. De Stasio, and G. Ravagnan (1991) Alterations in erythrocyte membrane lipids induced by low doses of ionizing radiation as revealed by 1,6-diphenyl-1,3,5-hexatriene fluorescence lifetime, *Int. J. Radiat. Biol.* **59**, 59–69.
20. T. Parasassi, G. Ravagnan, O. Sapora, and E. Gratton (1992) Membrane oxidative damage induced by ionizing radiation detected by diphenylhexatriene fluorescence lifetime distributions, *Int. J. Radiat. Biol.* **61**, 791–796.
21. L. A. Sklar, G. P. Miljanich, and E. A. Dratz (1979) Phospholipid lateral phase separation and the partition of *cis*-parinaric acid and *trans*-parinaric acid among aqueous, solid lipid, and fluid lipid phases, *Biochemistry* **18**, 1707–1716.
22. T. Parasassi, F. Conti, and E. Gratton (1984) Study of heterogeneous emission of parinaric acid isomers using multifrequency phase fluorometry, *Biochemistry* **23**, 5660–5664.
23. C. R. Mateo, J.-C. Brochon, M. P. Lillo, and A. U. Acuña (1993) Lipid clustering in bilayers detected by the fluorescence kinetics and anisotropy of *trans*-parinaric acid, *Biophys. J.* **65**, 2237–2247.
24. C. R. Mateo, P. Tauc, and J.-C. Brochon (1993) Pressure effect on the physical properties of lipid bilayers detected by *trans*-parinaric acid fluorescence decay, *Biophys. J.* **65**, 2248–2260.
25. G. Weber and F. J. Farris (1979) Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-Propionyl-2-(dimethylamino)naphthalene, *Biochemistry* **18**, 3075–3078.
26. R. B. MacGregor 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.
27. T. Parasassi, 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.
28. T. Parasassi, F. Conti, and E. Gratton (1986) Fluorophores in a polar medium: Time dependence of emission spectra detected by multifrequency phase and modulation fluorometry, *Cell. Mol. Biol.* **32**, 99–102.
29. T. Parasassi, F. Conti, and E. Gratton (1986) Time-resolved fluorescence emission spectra of Laurdan in phospholipid vesicles by multifrequency phase and modulation fluorometry, *Cell. Mol. Biol.* **32**, 103–108.
30. T. Parasassi, G. De Stasio, A. d'Ubaldo, and E. Gratton (1990) Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence, *Biophys. J.* **57**, 1179–1186.
31. T. Parasassi, and E. Gratton (1992) Packing of phospholipid vesicles studied by oxygen quenching of Laurdan fluorescence, *J. Fluorescence* **2**, 167–174.
32. T. Parasassi, G. Ravagnan, R. M. Rusch, and E. Gratton (1993) Modulation and dynamics of phase properties in phospholipid mixtures detected by Laurdan fluorescence, *Photochem. Photobiol.* **57**, 403–410.
33. S. Mitaku, T. Jippo, and R. Kataoka (1983) Thermodynamic properties of the lipid bilayer transition, *Biophys. J.* **42**, 137–144.
34. L. Davenport, J. R. Knutson, and L. Brand (1988) Time-resolved fluorescence anisotropy of membrane probes: Rotations gated by packing fluctuations, in J. R. Lakowicz (Ed.), *Time-Resolved Laser Spectroscopy in Biochemistry*, Proceedings SPIE **909**, pp. 263–270.

35. J. H. K. Ipsen, K. Jörgensen, and O. G. Mouritsen (1990) Density fluctuations in saturated phospholipid bilayers increase as the acyl-chain length decreases, *Biophys. J.* **58**, 1099–1107.
36. T. Parasassi, M. Di Stefano, G. Ravagnan, O. Sapora, and E. Gratton (1992) Membrane aging during cells growth ascertained by Laurdan generalized polarization, *Exp. Cell Res.* **202**, 432–439.
37. T. Parasassi, M. Loiero, M. Raimondi, G. Ravagnan, and E. Gratton (1993) Absence of lipid gel-phase domains in seven mammalian cell lines and in four primary cell types, *Biochim. Biophys. Acta* **1153**, 143–154.
38. M. Levi, D. Jameson, P. Wilson, and J. Cooper (1993) Effect of lipid–protein and lipid–lipid interactions on lipid dynamics and lipid phases in renal apical membranes, *Biophys. J.* **64**, 165a.
39. T. Parasassi, M. Di Stefano, M. Loiero, G. Ravagnan, and E. Gratton (1994) Influence of cholesterol on phospholipid bilayers phase domains as detected by Laurdan fluorescence, *Biophys. J.* **66**, 120–132.
40. M. R. Vist and J. H. Davis (1990) Phase equilibria of cholesterol/dipalmitoyl-phosphatidyl-choline mixtures: ^{2}H nuclear magnetic resonance and differential scanning calorimetry, *Biochemistry* **29**, 451–464.
41. M. B. Sankaram and T. E. Thompson (1990) Interaction of cholesterol with various glycerophospholipids and sphingomyelin, *Biochemistry* **29**, 10670–10675.
42. M. B. Sankaram and T. E. Thompson (1990) Modulation of phospholipid acyl chain order by cholesterol. A solid-state ^{2}H nuclear magnetic resonance study, *Biochemistry* **29**, 10676–10684.
43. W. K. Subczynski, W. E. Antholine, J. S. Hyde, and A. Kusumi (1990) Microimmiscibility and three-dimensional dynamic structure of phosphatidylcholine–cholesterol membranes: Translational diffusion of a copper complex in the membrane. *Biochemistry* **29**, 7936–7945.
44. R. Tampé, A. von Lukas, and H.-J. Galla (1991) Glycophorin-induced cholesterol–phospholipid domains in dimyristoyl-phosphatidylcholine bilayer vesicles, *Biochemistry* **30**, 4909–4916.
45. K. M. Keough, B. Giffin, and P. L. Matthews (1989) Phosphatidylcholine–cholesterol interactions: Bilayers of heteroacid lipids containing linoleate lose calorimetric transitions at low cholesterol concentrations, *Biochim. Biophys. Acta* **983**, 51–55.
46. D. L. Melchior, F. J. Scavitto, and J. M. Steim (1980) Dilatometry of dipalmitoyl-lecithin–cholesterol bilayers *Biochemistry* **19**, 4828–4834.
47. N. K. Mortensen, W. Pfeiffer, E. Sackmann, and W. Knoll (1988) Structural properties of a phosphatidylcholine–cholesterol system as studied by small-angle neutron scattering: Ripple structure and phase diagram, *Biochim. Biophys. Acta* **945**, 221–245.
48. F. Schroeder, J. R. Jefferson, A. B. Kier, J. Knittel, T. J. Scallen, W. Gibson Wood, and I. Hapala (1991). Membrane cholesterol dynamics: Cholesterol domains and kinetic pools, *Proc. Soc. Exp. Biol. Med.* **196**, 235–252.
49. V. Ben-Yasar and Y. Barenholz (1989). The interaction of cholesterol and cholest-4-en-3-one with dipalmitoylphosphatidyl-choline. Comparison based on the use of three fluorophores, *Biochim. Biophys. Acta* **985**, 271–278.
50. G. Nemecz and F. Shroeder (1988). Time-resolved fluorescence investigation on membrane cholesterol heterogeneity and exchange, *Biochemistry* **27**, 7740–7749.
51. J. H. Ipsen, G. Karlstrom, O. G. Mouritsen, H. Wennerstrom, and M. H. Zuckermann (1987) Phase equilibria in the phosphatidylcholine–cholesterol system, *Biochim. Biophys. Acta* **905**, 162–172.
52. O. G. Mouritsen (1991) Theoretical models of phospholipid phase transitions, *Chem. Phys. Lipids* **57**, 179–194.
53. P. L. Yeagle (1985) Cholesterol and the cell membrane, *Biochim. Biophys. Acta* **822**, 267–287.
54. S. A. Simon, T. J. McIntosh, and R. Latorre (1982) Influence of cholesterol on water penetration into bilayers, *Science* **216**, 65–67.
55. T. Parasassi, M. Di Stefano, M. Loiero, G. Ravagnan, and E. Gratton (1994) Cholesterol modifies water concentration and dynamics in phospholipid bilayers. A fluorescence study using Laurdan probe, *Biophys. J.*, in press.
56. P. J. Somerharju, J. A. Virtanen, K. K. Eklund, P. Vainio, and P. K. J. Kinnunen (1985). 1-Palmitoyl-2-pyrenedecanoyl glycerophospholipids as membrane probes: Evidence for regular distribution in liquid-crystalline phosphatidylcholine bilayers, *Biochemistry* **24**, 2773–2781.
57. D. Tang and P. L. Chong (1992). E/M dips. Evidence for lipids regularly distributed into hexagonal super-lattices in pyrene-PC/DMPC binary mixtures at specific concentrations, *Biophys. J.* **63**, 903–910.
58. J. M. Beechem and E. Gratton (1988) Fluorescence spectroscopy data analysis environment: A second generation global analysis program, in *Time-Resolved Laser Spectroscopy in Biochemistry*, Proceedings SPIE **909**, pp. 70–81.