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Surface properties of cholesterol-containing membranes detected by Prodan fluorescence

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Abstract

The fluorescent membrane probe 6-propionyl-2-dimethylaminonaphthalene (Prodan) displays a high sensitivity to the polarity and packing properties of lipid membrane. Contrary to 6-lauroyl-2-dimethylaminonaphthalene (Laurdan), Prodan can also monitor the properties of the membrane surface, i.e., the polar-head pretransition. In bilayers composed of coexisting gel and liquid–crystalline phases, Prodan shows a preferential partitioning in the latter, so that the detected membrane properties mainly belong to fluid domains. In the presence of cholesterol, the packing properties of the gel phase phospholipids are modified in such a way that Prodan can penetrate and label the membrane. Although Prodan labeling of the gel phase is a function of cholesterol concentration, 3 mol percent cholesterol is sufficient for a 60% Prodan labeling with respect to the maximum labeling reached at 15 mol percent cholesterol. We present steady-state and dynamical fluorescence measurements of Prodan in bilayers in the presence of cholesterol. Our results fit the liquid-ordered/liquid-disordered phase model for cholesterol-containing membranes and show that the presence of cholesterol, in addition to modification to the phase state of the hydrophobic portion of the bilayer, strongly affects the packing and the polarity of the membrane hydrophobic–hydrophilic interface. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Dipolar relaxation; Generalized polarization; Laurdan; Partition coefficient; Pretransition; Polarity

1. Introduction

The naphthalene fluorescent moiety of 6-propionyl-2-dimethylaminonaphthalene (Prodan), as well as that of 6-lauroyl-2-dimethylaminonaphthalene (Laurdan), renders these probes particularly sensitive to the polarity of their environment [1–5]. When used to study phospholipid bilayers, both Laurdan and Prodan monitor relevant differences in the polarity of the different phase states [6–9]. The two probes differ for the length of their acyl residues, 3 and 12 carbon atoms in Prodan and Laurdan, respectively. Based on this difference, the two probes detect membrane properties at different depths so that the phospholipid polar-head pretransition is well evidenced only using Prodan. In addition, also the two probes' partitioning between the bilayer and water differs. Laurdan completely partitions into the membrane while Prodan shows appreciable fluorescence and partitioning also in water, which in turn, depends

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on the phospholipid phase state and on membrane 'fluidity' [4,10]. Indeed, Prodan partition coefficient between the membrane and water is higher in the liquid–crystalline phase with respect to the gel for a factor of about 30. The low partition coefficient of Prodan in gel phase phospholipids has been attributed to the tight packing of the bilayer [4]. These findings have been confirmed by a direct visualization of fluorescent and dark areas in mixed-phase giant unilamellar vesicles (GUVs) labeled with Prodan and imaged with two-photon excitation microscopy [11].

For both Laurdan and Prodan, the dependence on polarity of the emission red-shift lead to the development of the generalized polarization (GP) function that allows a quantitative measurement of membrane polarity, related to the membrane phase state [12,13]. In the case of Prodan, due to its appreciable partitioning in water, since the GP function must report on membrane properties, the wavelength corresponding to the gel and to the liquid–crystalline phase had to be corrected for the contribution of Prodan in water and a three-wavelength GP function (3wGP) was developed [4]. The 3wGP was shown to effectively subtract the fluorescence arising from Prodan molecules in water. An additional attractive advantage of the 3wGP function is the possibility of directly determining the ratio between Prodan fluorescence arising from the bilayer and from water, as well as the probe partition coefficient between the membrane and water [4], both parameters being a function of the membrane properties and composition.

With the purpose of extending the use of Prodan to investigations on the surface properties of cholesterol-containing membranes, we present here a study on the effect of different cholesterol concentrations on the surface packing of the phospholipid bilayer. Indeed, the liquid-ordered and liquid-disordered phases, previously defined on the basis of evidence from several spectroscopic techniques [14,15], can be rapidly and directly characterized by simple steady-state measurements of Prodan emission. We confirmed the spectroscopic results obtained in cholesterol-containing membranes by their direct visualization in GUVs labeled with Prodan, using two-photon excitation microscopy.

2. Materials and methods

Multilamellar phospholipid vesicles were prepared by mixing the appropriate amounts of solutions in chloroform (spectroscopic grade) of phospholipids (Avanti Polar Lipids, Inc., Alabaster, AL, USA), cholesterol (Sigma Chem. Co, St. Louis, MO, USA) and Prodan (Molecular Probes Inc., Eugene, OR, USA), then evaporating the solvent by nitrogen flow. The dried samples were resuspended in Dulbecco's phosphate-buffered saline solution, pH 7.4 (PBS, Sigma Chem. Co.), heated at 60°C for 10 min and vortexed. All samples were prepared in red light and immediately used. Unless differently specified, the final lipids and probe concentrations were 0.3 mM and 0.3 μM, respectively. To subtract the background contribution from the emission, samples of the same phospholipids and at the same concentration were prepared, omitting the addition of Prodan.

For the preparation of GUVs stock chloroform solutions of equimolar dilauroyl- and dipalmitoylphosphatidylcholine (DLPC and DPPC, respectively), with or without 30 mol percent cholesterol, at the final lipid concentration of 0.2 mg/ml were used. The electroformation method developed by Angelova and Dimitrov [16–18] was followed, and a previously described [11,19] home-made temperature-controlled chamber was used. About 3 μl of the lipid stock solution were spread on each Pt wire under a stream of N_2. Residues of organic solvent were removed by lyophilization for about 2 h. To add the aqueous solvent (Millipore water, 17.5 MΩ/cm) inside the chamber, the bottom part of the chamber was sealed with a coverslip. The water was previously heated to 50°C. Sufficient water was added into the chamber to cover the Pt wires (∼4 ml). The Pt wires were then connected to a function generator (Hewlett-Packard, Santa Clara, CA, USA) and a low frequency AC field (sinusoidal wave function with a frequency of 10 Hz and amplitude of 2 V) was applied for 90 min. After the vesicle formation, the AC field was turned off and the temperature scan (from high to low temperature) initiated. The experiments were carried out in the same chamber after the vesicle formation using an inverted microscope (Axiovert 35, Zeiss, Thornwood, NY, USA) as previously reported [11,19]. A CCD color video camera

(CCD-Iris, Sony) in the microscope was used to follow the vesicle formation and to select the target vesicle. The temperature was measured inside the sample chamber using a digital thermocouple (model 400B, Omega Inc., Stamford, CT, USA) with a precision of 0.1°C. To label the vesicles, a small amount (less than 1 μl) of Prodan in DMSO was added after the vesicle formation (final Prodan:lipid ratio = 1:700 mol/mol). The GUVs yield was approximately 95% and their mean diameter was about 30 μm. To check for the lamellarity of the GUVs we used a previously reported method [11,19,20]. Shortly, up to 20 vesicles in different regions of the Pt wires were imaged, labeled either with Prodan or with lissamine rhodamine B-1,2-dihexadecanoyl-glycero-3-phosphoethanolamine (*N*-Rh-DPPE), using the two-photon excitation microscope described below. We found that the fluorescence intensity histogram of the lipid layer constituting the perimeter of the imaged vesicles sections were similar in the liquid–crystalline phase (similar center value in the histogram). Due to the presence of various Prodan-labeled lipid bilayers, the existence of multilamellar vesicles would have give rise to different intensity histograms. In agreement with previous observations [11,19,20], we concluded that the vesicles were unilamellar.

Fluorescence steady-state spectra were obtained using a GREG 200 fluorometer (ISS Inc., Champaign, IL, USA) equipped with photon counting electronics (P01X, ISS Inc.) and a xenon arc lamp. The cell compartment was thermostated by a circulating water bath to ±0.1°C. Monochromators bandpasses were 8 nm. The GP value was calculated from the excitation spectra, using fixed emission wavelengths of 440 nm = I_g, and of 480 = I_{lc} [12], following:

$$\mathrm{GP} = \frac{I_g - I_{lc}}{I_g + I_{lc}} \tag{1}$$

Due to the appreciable partitioning and fluorescence of Prodan in water [4], to obtain GP values only related to Prodan emission from membranes, the subtraction of the emission arising from the probe molecules in water was performed by the 3wGP method. In this method we measure the emission intensity at three wavelengths, chosen as to maximize the separation of the fluorescence emission from the different Prodan environments, i.e., $I_1 = 420$ nm, $I_2 = 480$ nm, $I_3 = 530$ nm. For clarity, we reproduce below the derivation of the 3wGP:

$$3\mathrm{wGP} = \frac{R_{12} - 1}{R_{12} + 1} \tag{2}$$

where:

$$R_{12} = \frac{I_1 k_{32}}{I_2 k_{32} - I_3 + I_3 R_{31}} \tag{3}$$

with:

$$k_{32} = I_{3\mathrm{W}}/I_{2\mathrm{W}} \tag{4}$$

$$R_{31} = I_{3\mathrm{M}}/I_{1\mathrm{M}} \tag{5}$$

and where $I_{2\mathrm{W}}$ and $I_{3\mathrm{W}}$ are the emission intensities at 480 and 530 nm, respectively, of Prodan in water, separately measured and which gave $k_{32} = 2.8$; $I_{1\mathrm{M}}$ and $I_{3\mathrm{M}}$ are the components of the intensities at 420 and 530 nm, due only to the probe emission in the membrane after the subtraction of the intensity arising from the probe in water. The 3wGP can be simplified by assuming that at 530 nm the contribution to the total emission intensity of Prodan molecules in the membrane is negligible, i.e., $I_{3\mathrm{M}} = 0$. We obtain:

$$R_{12} = I_1 k_{32}/I_2 k_{32} - I_3 \tag{6}$$

As discussed previously [4], by using the intensities I_2 and I_3 and the $k_{32} = 2.8$ value we can also measure the ratio between Prodan fluorescence arising from the membrane and from the aqueous phase:

$$R_F = \frac{F_\mathrm{M}}{F_\mathrm{W}} = \frac{I_2 k_{32} - I_3}{I_3 - I_2 R_{32}} \tag{7}$$

where F_M and F_W are the fractional intensities of Prodan fluorescence in the membrane and in water, respectively, and:

$$R_{32} = I_{3\mathrm{M}}/I_{2\mathrm{M}} \tag{8}$$

with $I_{2\mathrm{M}}$ and $I_{3\mathrm{M}}$ representing the component of the intensities at 480 and 530 nm due only to the probe emission in the membrane, i.e., after subtraction of the intensity arising from the probes' molecules in water [4].

If we assume that at 480 and at 530 nm the contribution of probe emission in water compared to its

emission in the membrane is small, i.e., $R_{32} = 0$, then Eq. 7 can be written as:

$$R_F = \frac{I_2 k_{32} - I_3}{I_3} \cong \frac{2.8\, I_2}{I_3} - 1 \quad (9)$$

By assuming that the lifetime is proportional to the quantum yield, and by using the measured values of the probe lifetime in water, τ_W, and in the membrane, τ_M, a quantitative determination of the ratio between Prodan molecules, R_M, in the two environments can be achieved:

$$R_M = R_F \frac{\tau_W}{\tau_M} \quad (10)$$

as well as the partition coefficient of the probe, C_p, between the bilayer and water can be directly determined if the phospholipid concentration is known:

$$C_p = R_M \frac{[W]}{[\text{lipids}]} \quad (11)$$

Fluorescence lifetime measurements were performed using a K2 phase fluorometer (ISS Inc.), with a xenon arc lamp as the light source. Excitation was at 360 nm and emission was observed through a Janos 418 cutoff filter. A solution of 2,2′-*p*-phenylene-*bis*-(5-phenyl)oxazole (POPOP) in ethanol was used as the reference (lifetime = 1.35 ns). During measurement, samples were continuously stirred. Sample compartment was kept at 25 ± 0.1°C by a water-circulating bath. Phase and modulation data were collected for 10 modulation frequencies, logarithmically spaced in the range from 0.6 to 150 MHz. For time-resolved spectra, the emission was observed through a series of seven interference filters, from 420 to 540 nm, with 10 nm bandwidth and at 20 nm intervals. Phase and modulation data were acquired at each emission wavelength using a set of 10 frequencies, logarithmically spaced from 25 to 250 MHz. Data were analyzed using the Globals Unlimited software™ (Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, IL, USA).

Two-photon excitation fluorescence microscopy images of GUVs were obtained using a scanning microscope developed in the Laboratory for Fluorescence Dynamics [21,22] employing a LD-Acroplan 20× long working distance air objective (Zeiss, Homldale, NJ, USA) with a N.A. of 0.4. A titanium-sapphire laser (Mira 900, Coherent, Palo Alto, CA, USA) pumped by a frequency-doubled Nd:Vanadate laser (Verdi, Coherent) produced excitation light at 780 nm which was attenuated to 50 mW and polarized before entering the microscope objective. The sample received about 1/10 of the incident power. To change the polarization of the laser light from linear to circular, a quarter-wave plate (CVI Laser Corporation, Albuquerque, NM, USA) was placed before the light entered the microscope. The fluorescence emission was observed through a broad band-pass filter, from 350 to 600 nm (BG39, Chroma Technology Inc., Battleboro, VT, USA). A miniature photomultiplier (R5600-P, Hamamatsu, Bridgewater, NJ, USA) was used for light detection in the photon counting mode. The diameter of the vesicles was measured by size-calibrated fluorescent spheres (latex FluoSpheres™, polystyrene, blue fluorescent 360/415, diameter 15.5 μm, Molecular Probes Inc.). In our experiments, the pixel size corresponded to 0.31 μm.

3. Results

Prodan emission spectra in phospholipid vesicles composed of one single phospholipid, DLPC or DPPC, or of an equimolar mixture of the two, in the absence and in the presence of 30 mol percent cholesterol and at 25°C are reported in Fig. 1. For

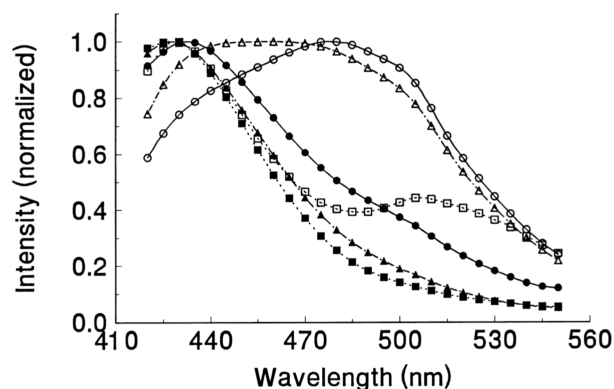

Fig. 1. Prodan emission spectra obtained in multilamellar phospholipid vesicles composed of DPPC (□,■), of DLPC (○,●) and of an equimolar mixture of the two phospholipids (△,▲) at 25°C, in the absence (open symbols) and in the presence (filled symbols) of 30 mol percent cholesterol. Excitation at 360 nm.

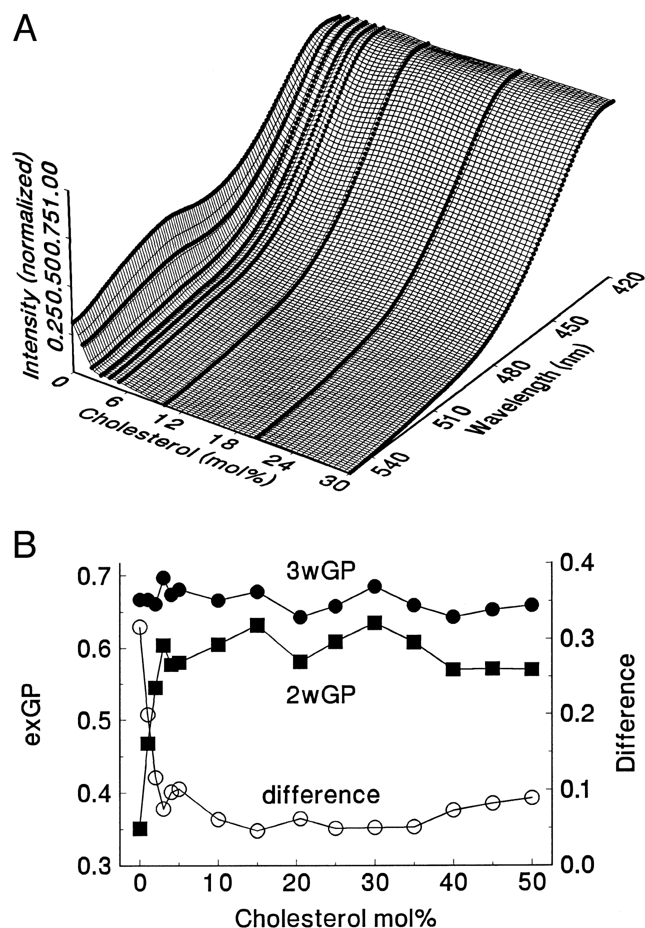

Fig. 2. (A) Normalized Prodan emission spectra in DPPC vesicles at 25°C as a function of cholesterol concentration. Measurements using cholesterol concentrations of 0, 1, 2, 3, 4, 5, 10, 20, and 30 mol percent. Numbers 0, 1, and 2 indicate the corresponding cholesterol mol percent. (B) Values of the 2wGP and 3wGP, respectively as a function of cholesterol concentration in DPPC vesicles. The difference between the values of the 3wGP and the 2wGP is also reported. Excitation at 360 nm.

the presence of cholesterol, Prodan emission spectra in the samples composed of DLPC and of the mixture of the two phospholipids showed an appreciable blue-shift (Fig. 1). Of relevance, in the sample composed of DPPC, the red emission band at about 510 nm, arising from Prodan molecules in water [4,10], disappeared, suggesting that the presence of cholesterol favors Prodan partitioning in DPPC. To verify this point, emission spectra of the probe in DPPC vesicles, at 25°C, have been acquired as a function of cholesterol concentration. Already in the presence of 1 mol percent cholesterol, the red emission band decreased (Fig. 2A) and was barely detectable at 3 mol percent cholesterol. Judging by these spectra, in bilayers with a cholesterol concentration >3 mol percent we could not detect Prodan molecules in water.

The 3wGP function was previously developed to correct the two-wavelengths GP value for the contribution of Prodan fluorescence arising from water [4]. We calculated 2wGP and 3wGP values (Eqs. 1 and 2, respectively, and using R_{12} values calculated following Eq. 6) from the emission spectra of Prodan in DPPC as a function of cholesterol concentration, from 1 to 50 mol percent (Fig. 2B). A large difference between the 2wGP and the 3wGP was observed at cholesterol concentration between 0 and 3 mol percent. At low cholesterol concentrations, the 2wGP value increased with cholesterol concentration, while the 3wGP value was nearly constant. Above 3 mol percent cholesterol, constant values were obtained using both GP functions, but the 2wGP values were constantly lower than the 3wGP values, suggesting that also above 3 mol percent cholesterol some Prodan molecules are present in the aqueous buffer. To verify this point, dilution experiments have been performed with a procedure similar to that previously used [4] for phospholipids without cholesterol and the results (not reported) confirmed that a low partitioning of Prodan in the buffer still occurs at 30 mol percent cholesterol and that a GP value corrected for Prodan fluorescence in water must be used.

The values of the 3wGP as a function of temper-

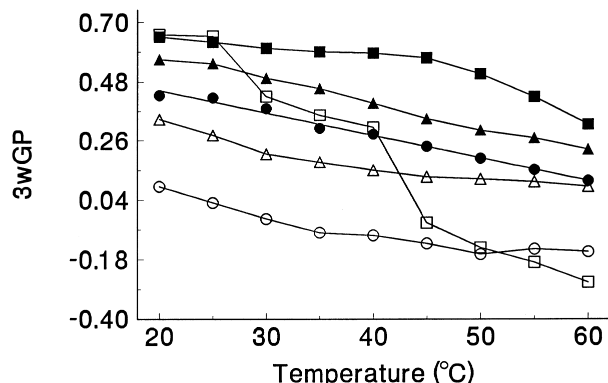

Fig. 3. Prodan 3wGP values in vesicles composed of DPPC (□,■), DLPC (○,●) and of the equimolar DLPC–DPPC mixture (△,▲) as a function of temperature, in the absence (open symbols) and in the presence of 30 mol percent cholesterol (filled symbols). Excitation at 360 nm.

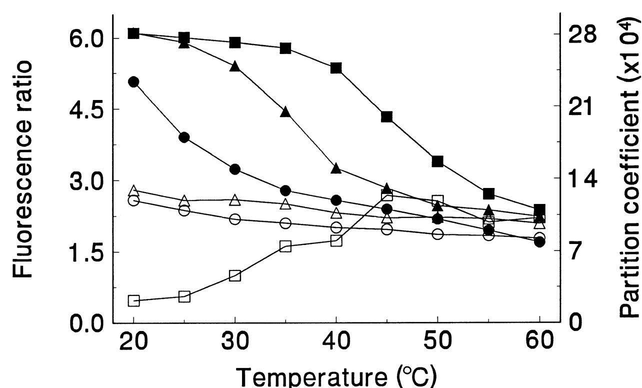

Fig. 4. Ratio of Prodan fluorescence arising from membrane and from water, R_F, (Eq. 9), and Prodan partition coefficient (Eq. 11) in vesicles composed of DPPC (□,■), DLPC (○,●) and of an equimolar mixture of DLPC and DPPC (△,▲) as a function of temperature, in the absence (open symbols) and in the presence (filled symbols) of 30 mol percent cholesterol.

ature in the three phospholipid samples in the presence of 30 mol percent cholesterol are reported in Fig. 3, together with the values obtained in the absence of cholesterol. As reported [14,15] and consistent with the results obtained using Laurdan [23], the presence of 30 mol percent cholesterol causes the disappearance of the DPPC phase transition. Also the DPPC pretransition, clearly monitored by Prodan in the absence of cholesterol, is no longer visible. As for the DLPC sample and for the equimolar mixture of the two phospholipids, the 3wGP values are higher in the presence of cholesterol.

As discussed in Section 2, from the emission intensities at the wavelengths used for the 3wGP calculation we can also determine the ratio between Prodan fluorescence in the bilayer and in water, R_F (Eq. 9), as well as the probe partition coefficient between the phospholipids and water, C_p (Eq. 11). The results for the three samples and as a function of temperature are reported in Fig. 4, together with the values obtained using the same phospholipids without cholesterol. The R_F values have been calculated using $k_{32} = 2.8$, as measured for Prodan in water at 25°C, and the C_p values have been calculated using the measured Prodan lifetime values in the three samples at 25°C (Table 1). For all samples and at low temperature, the R_F value increased in the presence of cholesterol. In the DLPC and DLPC/DPPC samples, both in the presence and in the absence of cholesterol, the R_F value decreased with the increase of temperature (Fig. 4), and this trend was particularly clear in the presence of cholesterol. By comparing Prodan R_F values in DPPC with 30 mol percent cholesterol and without, an opposite trend was observed as a function of temperature (Fig. 4). In the absence of cholesterol, low R_F values were observed at low temperatures, increasing at the polar-head pretransition and at the main DPPC transition. Instead, in the presence of 30 mol percent cholesterol, higher R_F values were obtained at low temperatures. In both samples, at temperatures higher than 40°C, the R_F value decreased with temperature.

Prodan lifetime values have been measured in the DPPC sample, at 25°C and using excitation at 360 nm, as a function of cholesterol concentration. The results are summarized in Table 2. Prodan decay is described by two lifetime components, the short-lived one arising from the probe fluorescence in water [4]. In general, by increasing cholesterol concentration, the value of the main lifetime component increased. A noticeable effect due to the presence of cholesterol is the decrease of the fractional intensity of the short-lived component, from about 30% without cholesterol to 18% with 1 mol percent cholesterol, reaching a minimum of about 2% at 50 mol percent cholesterol (Table 2). These lifetime values have been used to

Table 1
Prodan fluorescence decay in phospholipid with cholesterol

	τ_1 (ns)	w_1 (ns)	f_1	τ_2 (ns)	w_2 (ns)	f_2	χ^2
DLPC	5.52	–	0.966	1.44	–	0.034	1.98
DPPC	6.41	–	0.961	1.58	–	0.039	1.01
DLPC:DPPC = 1:1	6.05	0.21	0.963	1.50	–	0.027	1.18
Water	1.41	1.94	1.00	–	–	–	1.07

Prodan lifetime values (τ) were measured in different phospholipid samples with 30 mol percent cholesterol and in water, at 25°C using 360 nm excitation. Full width at half maximum of the lifetime continuous distribution, w, fractional intensity, f, and values of the reduced χ^2.

Table 2
Prodan fluorescence decay in DPPC as a function of cholesterol concentration

Cholesterol (mol percent)	τ_1 (ns)	f_1	τ_2 (ns)	f_2	χ^2
0	5.87	0.703	1.58	0.297	0.70
1	6.30	0.818	1.60	0.182	0.70
2	6.37	0.823	1.58	0.177	0.40
3	6.30	0.850	1.61	0.150	0.94
4	6.43	0.859	1.61	0.141	1.19
5	6.42	0.876	1.60	0.124	1.44
10	6.30	0.943	1.55	0.057	1.57
15	6.61	0.953	1.58	0.047	0.52
20	6.42	0.942	1.56	0.058	1.47
25	6.42	0.957	1.50	0.043	0.89
30	6.41	0.959	1.63	0.041	0.58
35	6.23	0.970	1.53	0.030	0.81
40	6.16	0.965	1.54	0.035	0.65
45	6.14	0.968	1.54	0.032	0.72
50	5.97	0.983	1.53	0.017	0.66

Prodan lifetime values (τ), measured at 25°C using 360 nm excitation. Fractional intensity, f, and values of the reduced χ^2.

calculate the probe partition coefficient as a function of cholesterol concentration at 25°C. The results are reported in Fig. 5 with a relevant increase of Prodan partitioning in DPPC from 0 to 15 mol percent cholesterol.

Prodan emission spectral shape as a function of the time after excitation has been measured in the three phospholipid samples in the presence of 30 mol percent cholesterol, at 25°C. The corresponding values of the center of mass are reported in Fig. 6, together with the values obtained in the same phospholipid samples without cholesterol. As previously reported [2,4,24], due to the dipolar relaxation phenomenon of solvent dipoles, in the absence of cholesterol a red-shift of Prodan emission is observed as a function of time after excitation in samples in the liquid–crystalline phase (DLPC) and in the presence of coexisting phases. The general effect of cholesterol in the three samples is reducing the time-dependent red-shift of the emission. In particular, in the DPPC sample, the disappearance of the short-lived probe molecules emitting from water occurs after 3 ns from the excitation, then an increase of the center of mass is observed, characteristic of the dipolar relaxation (Fig. 6). Instead, in the same sample but without cholesterol, the short component decreases more slowly with time, and no increase of the center of mass can be observed, also after 30 ns from excitation (Fig. 6). Similarly to what was observed using Laurdan in the same phospholipid mixture [12,24] and as previously reported for Prodan [4], in the sample composed of the equimolar DLPC/DPPC mixture without cholesterol a red-shift of the emission can be observed as a function of time, up to about 15 ns, then the emission shifts back to the blue. This behavior has been interpreted as indicative of a fluctuation between the two phospholipid phases [12]. In the presence of 30 mol percent cholesterol, in addition to an average decrease of the center of mass, only a continuous red-shift of Prodan emission can be observed as a function of time, up to

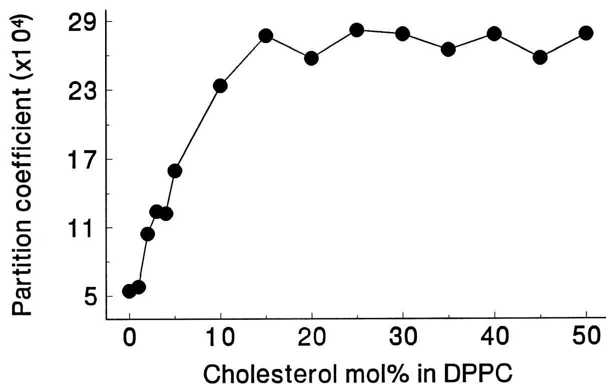

Fig. 5. Prodan partition coefficient, C_p (Eq. 11), at 25°C, in vesicles composed of DPPC as a function of cholesterol concentration.

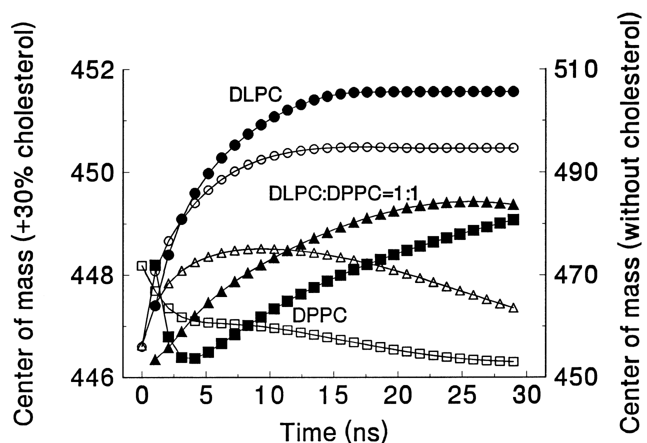

Fig. 6. Values of the center of mass (nm) of Prodan time-resolved spectra in vesicles composed of DPPC (□,■), DLPC (○,●) and of an equimolar mixture of DLPC and DPPC (△,▲), at 25°C, in the absence (open symbols) and in the presence (filled symbols) of 30 mol percent cholesterol.

30 ns after excitation. Also in the presence of 30 mol percent cholesterol, this result is in agreement with what obtained using Laurdan [23] and to the modification of the phospholipid phase state in the presence of cholesterol [14,15].

A direct visualization of the effect of cholesterol on Prodan partitioning in the membrane can be obtained by microscopy images of GUVs using two-photon excitation [11]. In Fig. 7 we report images obtained using vesicles composed of the equimolar DLPC–DPPC mixture labeled with Prodan. In the left column, the images obtained in the absence of cholesterol are reported. At low temperature (26.4°C, top), where domains of gel and of liquid–crystalline phase coexist, dark areas were observed. At high temperature (40.0°C, bottom), where only the liquid–crystalline phase occurs, the vesicle appeared uniformly bright. In the right column, the images obtained in the presence of 30 mol percent cholesterol are reported. An uniform distribution of the fluorescence intensity can be observed both at low (top) and at high (bottom) temperatures. Noticeably, the relatively low intensity observed at the center of the vesicles at low temperature (top) indicates a photoselection effect due to the direction of the propagation of the excitation beam [9] in the liquid-ordered lipid phase [14,15]. Indeed, in the equimolar DLPC–DPPC sample and in the presence of 30 mol percent cholesterol the restricted lipid molecular motion allows a preferential orientation of Prodan molecules along the membrane normal (Fig. 8B), as will be discussed below.

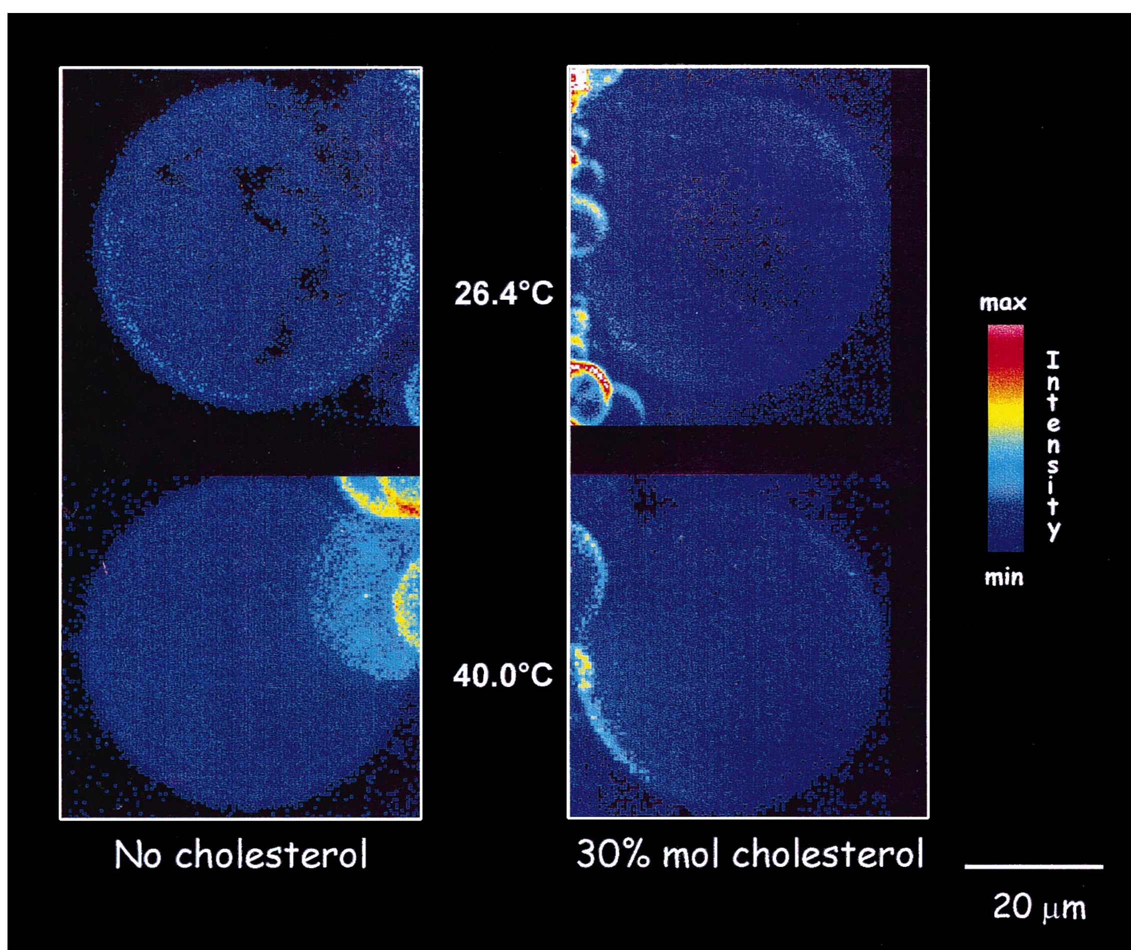

Fig. 7. Two-photon excitation fluorescence microscopy images of GUVs composed of an equimolar DLPC–DPPC mixture in the absence (left column) and in the presence (right column) of 30 mol percent cholesterol labeled with Prodan. Pseudo color intensity scale on the right.

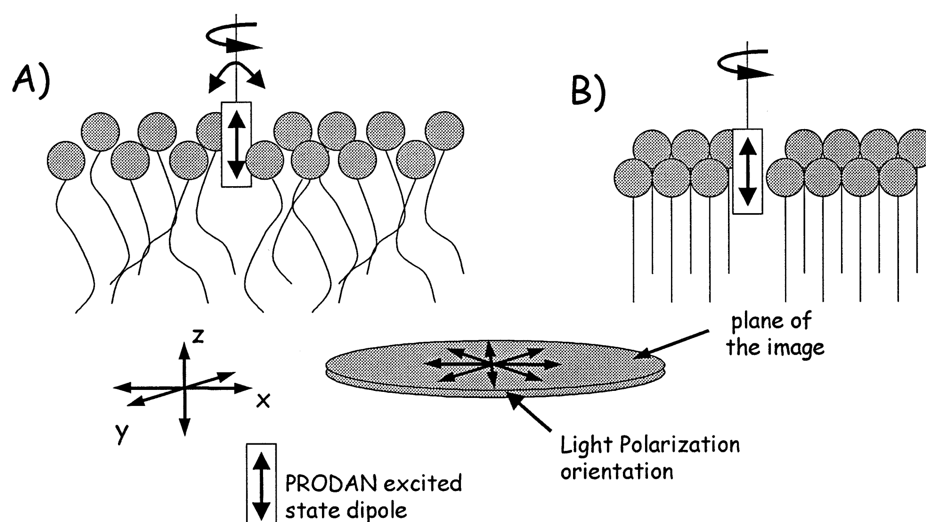

Fig. 8. Schematic representation of the excitation photoselection operated by the light polarization on Prodan at the top of lipid vesicles. In the liquid–crystalline phase (A), the relatively free molecular motion of Prodan allows a higher excitation than in the gel or in the liquid-ordered phase (B), where the probe motion is restricted.

4. Discussion

Similarly to Laurdan, Prodan is a membrane probe sensitive to the polarity of the bilayer [2,3–5,13]. While Laurdan is tightly anchored into the bilayer by its lauric acid tail of 12 carbon atoms, Prodan is more loosely anchored by its shorter propionic acyl chain. In addition and differently from Laurdan, Prodan chemical structure favors its partitioning also in the aqueous environment. Prodan partitioning is strongly influenced by the phase state of the bilayer, i.e., by its packing [4]. Thus, Prodan can detect polarity or packing variations of the bilayer surface not only by means of its GP value, but also by its partition coefficient. The small but detectable Prodan fluorescence arising from water can be subtracted by the 3wGP function [4,10], derived from the intensity at three selected emission wavelengths. These intensities can also be used for the calculation of the ratio between the Prodan fluorescence arising from the membrane and from water, thus allowing the calculation of its partition coefficient.

In a previous work [4] we showed that due to the tight packing of the phospholipid Prodan partitioning is extremely low in the gel phase. This result was directly observed in GUVs composed of different phospholipid binary mixtures, at the temperature corresponding to the phase coexistence, using two-photon excitation microscopy [11]. In the present work we show that in the presence of cholesterol, also at very low concentration, i.e., $\sim$3 mol percent, Prodan partitioning in the membrane increases dramatically, reaching an almost complete partitioning in favor of the membrane at cholesterol concentration of about 15 mol percent. This result adds information both on the partitioning behavior of Prodan and, more relevant, on the properties of the bilayer surface in the presence of cholesterol.

In the presence of cholesterol, a noticeable increase of Prodan partitioning in the gel phase is observed (Figs. 4 and 5) and this result fits the disordering effect of cholesterol in the hydrophobic environment of phospholipid gel phase [14,15,23] and adds new information on a similar disordering of the bilayer surface. The decreased packing of the gel phase bilayer due to cholesterol favors Prodan partitioning into the membrane, as clearly visible by the virtual disappearance of the red emission band of Prodan in water, in gel phase DPPC at cholesterol concentrations >3 mol percent (Fig. 2A). As a consequence, also the difference of its partitioning between the gel and the liquid–crystalline phase decreases in the presence of cholesterol. Nevertheless, we determined that Prodan partitioning into the bilayer is not complete, even in the presence of cholesterol, by: (i) dilution experiments; (ii) lifetime measurements (Table 2); (iii) measurements of the emission center of mass as a function of time after excitation (Fig. 6), so

that for the study of the bilayer surface the 3wGP is necessary.

The effect of cholesterol on the molecular dynamics and on the polarity of the phospholipid bilayer that we determined in the present work using Prodan is in agreement with previous reports where a variety of spectroscopic techniques were used [14,15,23]. The effect of cholesterol varies with the phase state of the bilayer, by increasing the molecular motion and the polarity of gel phase phospholipids that, instead, decrease in the liquid–crystalline phase. When using Prodan, together with the variation of the 3wGP value, that well corresponds to the variation of Laurdan GP value [23], more information on cholesterol effect is obtained by the variation of the C_p value. Both phospholipids phases with 30 mol percent cholesterol give high 3wGP values which, coupled with a high partition coefficient, indicates a decreased surface packing, i.e., a liquid-ordered phase. This result represents new evidence for the effect of cholesterol on the surface of the bilayer, at its hydrophobic–hydrophilic interface.

To further confirm our results, cholesterol effect on the bilayer properties has been verified by the time evolution of Prodan emission center of mass (Fig. 6). Similarly to Laurdan [12,24], in polar lipid environments the dipolar relaxation phenomenon occurs with Prodan [2,4], with an emission red-shift as a function of time after excitation. In the absence of cholesterol, this behavior can be observed in the liquid–crystalline phase and in the coexistence of both phases, and, when in the presence of 30 mol percent cholesterol, in all phospholipid phases. No emission red-shift is observed in the gel phase without cholesterol, i.e., in a non polar, tightly packed bilayer. In membranes composed of coexisting gel and liquid–crystalline phases and in the absence of cholesterol, the emission center of mass increases after excitation, up to about 15 ns, then decreases to shorter wavelengths. This behavior has been interpreted as due to phase fluctuation [4,12,24]. In the same sample and in the presence of cholesterol, the spectra center of mass shows a continuous small increase with time (Fig. 6) indicating a homogeneous, relatively polar phase without coexisting domains. These findings correspond to what was observed using Laurdan [23] and, again, add further information that cholesterol affects the molecular dynamics, the packing and the polarity also at the surface of the bilayer. None of Prodan fluorescence features in phospholipid bilayers in the presence of cholesterol, including spectra, lifetimes, 3wGP and time-resolved spectra suggest a preferential interaction between Prodan and cholesterol [25].

Images of GUVs labeled with Prodan directly show the effect of cholesterol on the surface packing of the bilayer [11]. In GUVs composed of the equimolar mixture of the two phases and in the absence of cholesterol, the fluorescence intensity is not uniform but shows dark areas corresponding to unlabeled gel phase domains (Fig. 7). After increasing the temperature to reach a homogeneous liquid–crystalline phase, Prodan fluorescence is uniformly distributed all over the vesicle. In the presence of cholesterol the GUVs are uniformly labeled, both at low and high temperature. For the photoselection effect [9,11], these images also clearly show that at low temperature and in the presence of 30 mol percent cholesterol, although the gel phase domains are no longer present, the overall lipid dynamics is reduced, as expected in a homogeneous liquid-ordered phase [14,15]. Indeed, at the top of the vesicle, the lipids are oriented perpendicularly to the plane of the membrane and Prodan fluorescent moiety is aligned with the lipids [9]. We used circularly polarized excitation, in the x–y plane, and the direction of the propagation of the excitation light is parallel to the plane of the membrane, thus perpendicular to Prodan dipole, aligned along the z-axis. In these conditions, a partial photoselection effect occurs so that at the top of the vesicle only those Prodan molecules that have an orientation not coincident with the membrane normal can be excited (Fig. 8) [9]. Excitation will only occur for Prodan molecules in the liquid-ordered phase and not in the packed gel phase. This explains the low fluorescence intensity observed at the top of the vesicle in the Fig. 7 (right column, top image) and is in agreement with the reported physical properties of mixed-phase phospholipids in the presence of 30 mol percent cholesterol [14,15,23].

In this work, to calculate the 3wGP and the R_F values we used the simplified Eqs. 6 and 9. We previously showed [4] that the simplified Eq. 6 does not introduce relevant changes in the calculation of the 3wGP. Instead, due to the increase of Prodan partitioning in water at high temperatures, with its emis-

sion overlapping the red-shifted emission of Prodan in the liquid–crystalline phase of phospholipids, for the determination of the R_F value the simplified equations can give different absolute values when in the presence of the phospholipid liquid–crystalline phase. As shown by our results, when in the presence of cholesterol, Prodan partitioning and emission in water is negligible with respect to the membrane, so that the use of the two simplified equations does not introduce relevant changes also in the liquid-disordered phase.

The attractiveness of using Prodan in membrane studies resides in the possibility of detecting the properties of the bilayer surface, eventually in parallel with the use of Laurdan [13], that is located more deeply in the bilayer, by steady-state measurements of emission intensity. Indeed, due to the sensitivity of Prodan 3wGP value and of its partition coefficient, Prodan seems particularly appropriate for the study of the polarity and packing of the bilayer surface.

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