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ORIGINAL PAPER

Laurdan Spectrum Decomposition as a Tool for the Analysis of Surface Bilayer Structure and Polarity: a Study with DMPG, Peptides and Cholesterol

Aline D. Lúcio · Cíntia C. Vequi-Suplicy · Roberto M. Fernandez · M. Teresa Lamy

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Abstract The highly hydrophobic fluorophore Laurdan (6-dodecanoyl-2-(dimethylaminonaphthalene)) has been widely used as a fluorescent probe to monitor lipid membranes. Actually, it monitors the structure and polarity of the bilayer surface, where its fluorescent moiety is supposed to reside. The present paper discusses the high sensitivity of Laurdan fluorescence through the decomposition of its emission spectrum into two Gaussian bands, which correspond to emissions from two different excited states, one more solvent relaxed than the other. It will be shown that the analysis of the area fraction of each band is more sensitive to bilayer structural changes than the largely used parameter called Generalized Polarization, possibly because the latter does not completely separate the fluorescence emission from the two different excited states of Laurdan. Moreover, it will be shown that this decomposition should be done with the spectrum as a function of energy, and not wavelength. Due to the presence of the two emission bands in Laurdan spectrum, fluorescence anisotropy should be measured around 480 nm, to be able to monitor the fluorescence emission from one excited state only, the solvent relaxed state. Laurdan will be used to monitor the complex structure of the anionic phospholipid DMPG (dimyristoyl phosphatidylglycerol) at different ionic strengths, and the alterations caused on gel and fluid membranes due to the interaction of cationic peptides and cholesterol. Analyzing both the emission spectrum decomposition and anisotropy it was possible to distinguish between effects on the packing and on the hydration of the lipid membrane surface. It could be clearly detected that a more potent analog of the melanotropic hormone α-MSH (Ac-Ser^{1}-Tyr^{2}-Ser^{3}-Met^{4}-Glu^{5}-His^{6}-Phe^{7}-Arg^{8}-Trp^{9}-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2) was more effective in rigidifying the bilayer surface of fluid membranes than the hormone, though the hormone significantly decreases the bilayer surface hydration.

Keywords Laurdan · Fluorescence · Spectrum decomposition · Fluorescence anisotropy · DMPG · Melanocortin peptides · α-MSH · [Nle^{4}, D-Phe^{7}]α-MSH · Cholesterol

Introduction

Since designed by Weber and Farris (1979) [1], Prodan (6-propionyl-2-(dimethylaminonaphthalene)) and its more hydrophobic derivative, Laurdan (6-dodecanoyl-2-(dimethylaminonaphthalene)), have been extensively used due to their high sensitive to solvent polarity. When incorporated into lipid bilayers, their fluorescence emission spectrum is clearly composed of two bands, which have been attributed to emission from two different excited states: a solvent-non-relaxed, and a solvent-relaxed state [2, 3].

Considering the gel-fluid thermal transition of lipid bilayers, the short-wavelength band (solvent-non-relaxed) is predominantly present when Laurdan is incorporated into

A. D. Lúcio · C. C. Vequi-Suplicy (✉) · R. M. Fernandez · M. T. Lamy
Instituto de Física, Universidade de São Paulo,
CP 66318, CEP 05314-970 São Paulo, SP, Brazil
e-mail: cintia@if.usp.br

A. D. Lúcio
Departamento de Exatas, Universidade Federal de Lavras,
CP 3037, CEP 37200-000 Lavras, MG, Brazil

R. M. Fernandez
Depto. de Física e Biofísica, Instituto de Biociências da Universidade Estadual Paulista,
CP 510, CEP 18618-000 Botucatu, SP, Brazil

a lipid gel phase, as compared to the predominance of the longer wavelength band (solvent-relaxed) when the probe is in fluid bilayers [4]. Taking that into consideration, together with the high sensitivity of Prodan or Laurdan to solvent polarity, these two bands have been mostly attributed to the fluorophore incorporated in the less hydrated lipid gel phase and in the more hydrated fluid phase [3]. However, it has been shown that Laurdan fluorescence in lipid bilayers is extremely sensitive to the bilayer packing, even in a pure lipid fluid phase, and that even in a lipid gel phase the two bands are almost equally present [5]. Hence, the emission spectrum of these fluorophores seems to be a reporter of both the presence of water molecules and the mobility of these molecules at the bilayer surface, where the naphthalene moiety is supposed to reside [4–6].

In the present work Laurdan was used to monitor the structure of the anionic phospholipid DMPG (dimyristoyl phosphatidylglycerol). At high ionic strength (1 mM DMPG in 500 mM NaCl), DMPG presents a sharp gel-fluid thermal transition around 23 °C. However, at low ionic strength, DMPG presents a rather peculiar thermal behavior, displaying a large gel-fluid transition region, with several calorimetric peaks, between the beginning (T_m^{on}~17 °C) and the end (T_m^{off}~35 °C) of the melting process (see, for instance [7, 8]). Although the lipid structure of this transition region is not well understood, some of its characteristics are known, like low turbidity, high viscosity and high electric conductivity [9–11]. Moreover, electron spin resonance (ESR) of spin labels intercalated into the lipid phase evidenced the presence of two structurally different domains: rigid and highly hydrophobic gel domains coexisting with rather fluid and hydrated domains, possibly high curvature regions [7]. The ESR data mentioned above, together with SAXS data [12] and the study of giant vesicles by optical microscopy [13] suggest that DMPG bilayers are perforated along the transition region, explaining the coexistence of gel bilayers patches with micelle like structures, the latter would be along the rim of the pores. This proposal is in accord with the formation of pores in most lipid bilayers, but over a very short interval of temperature, at the gel-fluid transition [14, 15].

Considering that Laurdan fluorescence has been used for detecting phases coexistence in lipid membranes, through the analysis of a parameter called Generalized Polarization [2, 3], this probe seemed appropriate for detecting the possible presence of the two phases over the gel-fluid transition region of DMPG at low ionic strength.

Moreover, the present paper discusses the sensitivity of Laurdan fluorescence to the interaction of DMPG with two cationic peptides: the melanocortin hormone α-MSH (Ac-Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2) and the biologically more potent analog [Nle^4, D-Phe^7]α-MSH (NDP-MSH) [16–18]. Apart from acting as skin darkening regulator in most vertebrates, α-MSH is involved in many physiological and neurological processes [19–21]. The hormone and its derivatives are known to interact with receptors at the membrane surface [22], but their biological activity can be modulated by the interaction with membrane lipid domains. Both α-MSH and NDP-MSH were found to interact with DMPG bilayers at low ionic strength, at the highly charged bilayer surface. It was found that the peptide-lipid interaction is primarily electrostatic, but leads to a partial peptide penetration into the lipid bilayers [23–26]. The peptide penetration into the bilayer core was monitored by spin labels intercalated into the bilayer, and by the peptide Trp^9 fluorescence. It was found that Trp^9 in the more potent analog, NDP-MSH, is in a somewhat deeper position inside the DMPG bilayer [27]. In the present paper Laurdan fluorescence was used to compare the structural changes produced by these two peptides, α-MSH and NDP-MSH, at the bilayer surface of DMPG membranes in the gel and fluid phases. The data is compared with those yielded by spin labels intercalated into the hydrophobic core of the membrane [27]. As done before [27], results obtained with cholesterol, which is totally inserted into the bilayer, are shown for comparison.

It will be shown here that the analysis of the decomposition into two bands of the Laurdan emission spectrum is more sensitive to bilayer structural changes than the Generalized Polarization parameter. Moreover, it will be shown that the two bands decomposition needs to be done with the spectrum as a function of energy, and not wavelength.

Materials and methods

Materials

DMPG (1,2–Dimiristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol)]) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). Laurdan (2-dimetilamino-6-lauroilnaftaleno) was from Molecular Probes Inc. (Eugene, OR, USA). α-MSH (Ac-Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2), [Nle^4, D-Phe^7]-α-MSH, cholesterol, Hepes (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) and EDTA (ethylenediaminetetraacetic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Milli-Q Plus water (Millipore), pH~6, was used for buffer preparation.

Sample preparation

A lipid film was formed from a solution of DMPG and Laurdan (0.1 mol% relative to lipid concentration) in chloroform. When used, cholesterol was added to the chloroform solution (30 mol% relative to lipid concentra-

tion). Chloroform was evaporated under a stream of N_2, and the sample left under reduced pressure for a minimum of 2 h, to remove all traces of organic solvent. Vesicles were prepared by the addition of the 10 mM Hepes solution with 1 mM EDTA, pH 7.4, to a final lipid concentration of 1 mM (low ionic strength samples). After hydration, the sample was vortexed for ~2 min, and extruded 10 times through polycarbonate filters (100 nm pore) at a temperature above the lipid phase transition, to yield large unilamellar vesicles. For the high salt concentration samples (high ionic strength samples), 500 mM NaCl was added to the buffer solution, before lipid hydration. Peptides, when used, were also added to the buffer before lipid film hydration, at 10 or 20 mol% relative to lipid concentration.

Fluorescence measurements

Samples were placed in quartz cuvettes with 2 mm optical pathway. The excitation and emission spectra were measured with a Fluorolog 3 Jobin YvonSPEX, model FL3-11, equipped with a xenon lamp, with 1.5 nm wide bandpass. The excitation spectra were corrected with a quantum counter (Rhodamine B in 3 mg/mL ethylene glycol). The temperature was controlled with a thermal bath model Julabo HP 25, and was measured by a digital thermometer in the sample. The emission spectra were obtained with the excitation wavelength at 340 nm. The steady-state anisotropy, r, is given by

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where I_{VV} and I_{VH} are the intensities with the excitation polarizer on vertical position, and the emission polarizer at vertical and horizontal position, respectively, and G represents the ratio of the sensitivity of the system for vertically and horizontally polarized light [28]. The anisotropy measurements were done using the same equipment with automated polarizers in front of the excitation and emission light. The excitation wavelength was 340 nm and the emission was measured at 430 nm and 480 nm.

The presence of tryptophan (Trp), a fluorescent residue of α-MSH and NDP-α-MSH, did not alter the Laurdan emission spectra profile (Trp has maximal excitation at 296 nm [27]). Emission spectra were decomposed into two Gaussian bands, using the software *Origin 6.1*. When necessary, the fluorescence spectrum was transformed from wavelength to energy, and the intensity multiplied by λ^2 taking into account that the spectrum is recorded with constant wavelength resolution, not energy [28].

The Generalized Polarization (GP_{exc}) was calculated by [2, 3]:

$$GP_{exc} = \frac{(I_{440} - I_{490})}{(I_{440} + I_{490})}$$

where I_{440} and I_{490} are the fluorescence emission intensities at 440 and 490 nm, respectively. When indicated (Fig. 3), GP_{exc} was calculated as a function of the excitation wavelength, by directly subtracting the two excitation spectra obtained with the emission wavelength at 440 nm and at 490 nm.

Results and discussion

Laurdan in DMPG bilayers at low and high ionic strength

Figure 1 shows typical fluorescence emission spectra of Laurdan in 1 mM DMPG dispersion at low ionic strength (see Material and methods), at different temperatures. As observed for Laurdan incorporated in other lipids, its emission spectra is extremely sensitive to temperature, changing from a spectrum centered around 430 nm, in the lipid gel phase, to a wide band centered around 480 nm when the lipid is in the fluid phase [2, 3, 5]. (The present paper will not focus on the lipid pre-transition, centered around 11 °C for DMPG [7]. Hence, the lipid gel and ripple phases, present at temperatures below the lipid gel-fluid main transition, will be generally called "gel phase").

The temperature dependence of the fluorescent spectra of Laurdan incorporated in DMPG at low and high ionic strength (see "Materials and methods") was analyzed as it

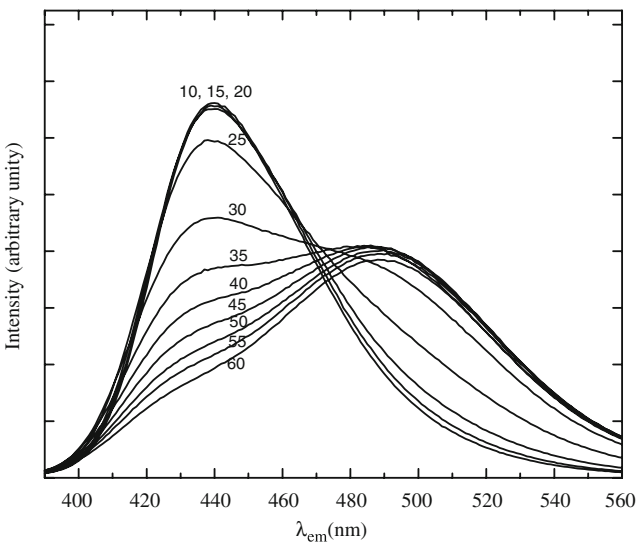

Fig. 1 Fluorescence emission spectra of Laurdan in DMPG bilayers at low ionic strength, at different temperatures (°C). λ_{exc}=340 nm

usually is, using the GP_{exc} [2, 3], (see "Materials and methods"). Figure 2 shows that this parameter is unable of detecting the known large difference between the temperature transition profile of the two DMPG dispersions, at low and high salt (see, for instance, [7, 8]). Moreover, considering that GP_{exc} (λ_{exc}) has been largely used for the discussion of phases coexistence in lipid dispersions [2, 3], and the proposed coexistence of domains with different packing structures over a large interval of temperature (17–35 °C) for DMPG at low ionic strength [7, 8], GP_{exc} values were calculated for all temperatures, as a function of the excitation wavelength (see "Materials and methods"). Figure 3 shows the dependence of GP_{exc} with λ_{exc}, for DMPG at low salt. Three different samples were analyzed, and the results were rather reproducible. It can be clearly seen that GP_{exc} does not vary with λ_{exc} up to around 25 °C. At and above 30 °C, GP_{exc} decreases with λ_{exc}. Taking into account the usual analysis of GP_{exc} [2, 3], the bilayer would be in the gel phase up to around 25 °C (no GP_{exc} variation with λ_{exc}), and in the fluid state above that temperature (a decrease of GP_{exc} with λ_{exc}). However, this analysis is not in accord with DSC, ESR, and other techniques [9, 13, 29, 30], as mentioned above.

Hence, as the GP_{exc} analysis did not seem to be sensitive to the complex structure of DMPG bilayers, we decided to analyze the Laurdan fluorescent spectrum through the decomposition of the spectrum into two bands, as discussed before [5]. This has the advantage over the GP_{exc} calculation of clearly separate the fluorescence emission of the two different excited states of Laurdan. Different

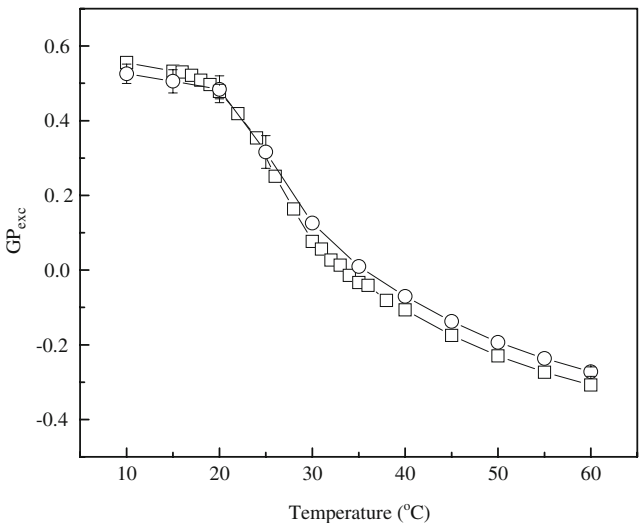

Fig. 2 Excitation GP as a function of the temperature for Laurdan in DMPG bilayers at low (□) and high (○) ionic strength. The data are mean values of two different samples, and the uncertainties are the standard deviations, not shown if they are smaller than the symbols. λ_{exc}=340 nm

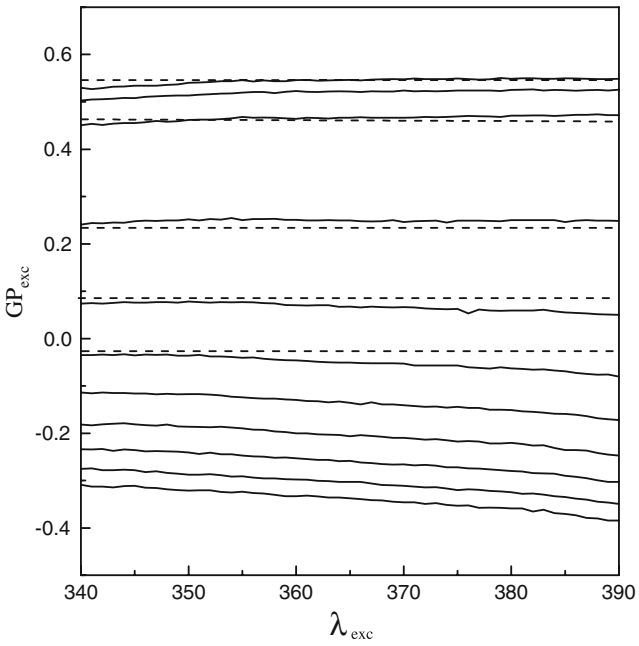

Fig. 3 Excitation GP as a function of the excitation wavelength, for Laurdan in DMPG at low ionic strength. The full lines from *top* to *bottom* correspond to temperatures 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 °C, respectively. The dashed lines are lines of constant GP_{exc}, drawn just for helping the visualization of GP_{exc} variation with λ_{exc}.

from the previous work [5], we would like to discuss here the importance of making the analysis with the spectrum as a function of energy, and not wavelength. Figures 4a and b show the spectrum decomposition into Gaussian bands as a function of energy and wavelength, respectively. It can be clearly seen that the relative percentage of the bands are different in the two cases: for the lower energy band we have 80 % for the decomposition in energy and 90 % for the decomposition in wavelength. Hence, in the present paper all the decompositions were performed with the emission spectra in energy, as, theoretically, this is the correct way of decomposing a spectrum into different emission bands. From now on, the bands will be named shorter and longer wavelength bands, as they are known in the literature, corresponding to the bands with higher and lower energy, respectively, in the spectra decomposition.

The spectra for all temperatures could be well decomposed into two Gaussians, (χ^2~1.00). Figure 5a shows the variation of the longer wavelength area fraction with temperature, for DMPG at low and high ionic strength. The centers of the two Gaussian lines were found to vary from 438 to 430 nm (2.84 to 2.89 eV) and 463 to 493 nm (2.68 to 2.52 eV) for the shorter and longer wavelength bands, respectively, as the temperature increases from 10 to 60 °C, for all samples studied here. As observed before [5], the fraction of the longer wavelength band is higher in the lipid fluid phase as compared with the gel phase (Fig. 5a).

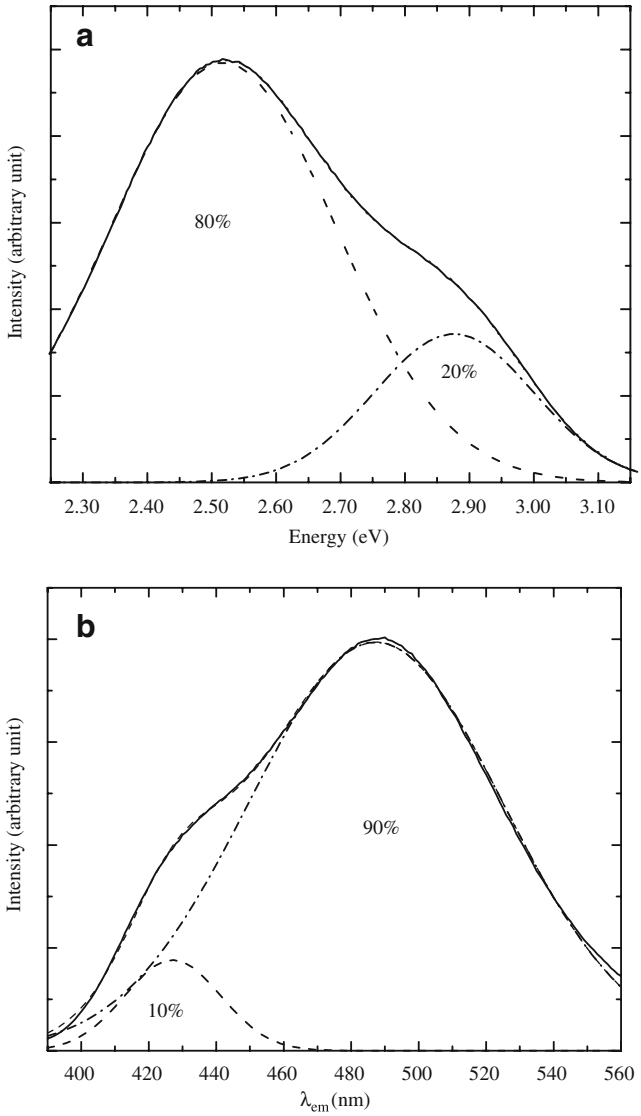

Fig. 4 A typical decomposition of Laurdan emission spectrum into two Gaussian lines, in energy and in wavelength, for 1 mM DMPG vesicles at 45 °C. λ_{exc}=340 nm

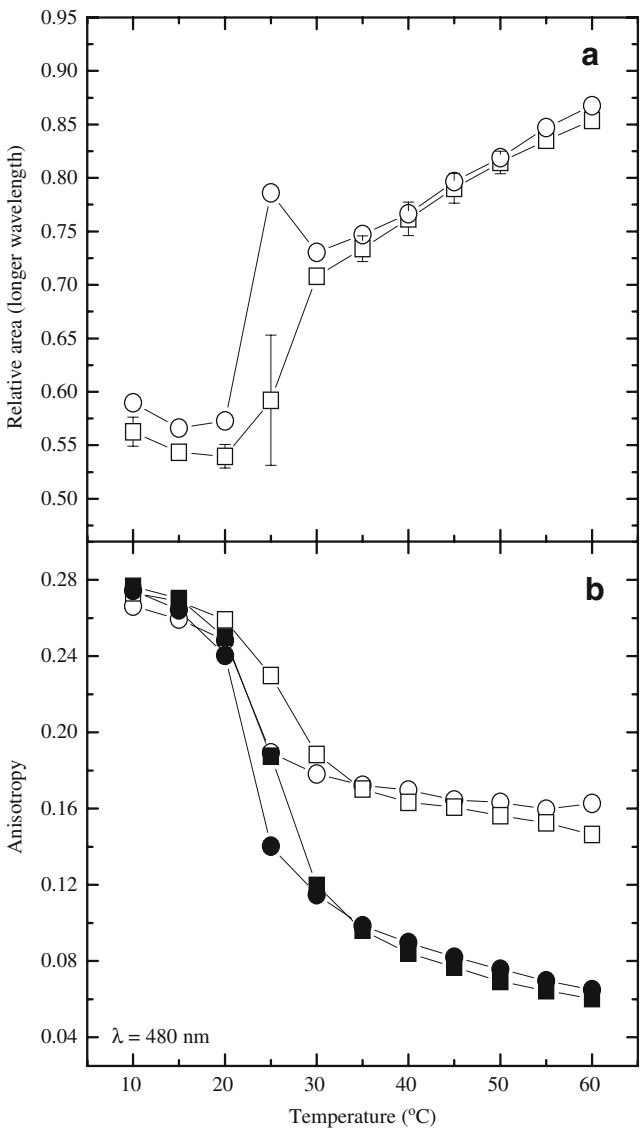

Fig. 5 **a** Temperature variation of the area fraction of the Laurdan emission component at lower energy (longer wavelength, ~480 nm), for DMPG at low (□) and high (○) ionic strength (see "Material and methods"). **b** Laurdan fluorescence anisotropy at 430 nm (*open symbols*) and 480 nm (*full symbols*), for DMPG at low (■ and □) and high (● and ○) ionic strength. The data are mean values of at least three different samples, and the uncertainties are the standard deviations, not shown if they are smaller than the symbols. λ_{exc}= 340 nm

This longer wavelength band has been attributed to the fluorescence emission from a more relaxed Laurdan excited state, which would be favored by a looser and/or more hydrated micro-environment [4, 5]. However, it is important to have in mind that even in the rigid lipid gel phase, the contribution of this band for Laurdan spectrum in DMPG is around 0.5–0.6 (Fig. 5a), showing that half of the emission energy comes from the so called relaxed excited state. Similar result was obtained with DPPG [5]. (The presence of more than one Laurdan excited state in lipid gel phase was found before [31] and confirmed by us (Vequi-Suplicy et al., unpublished results))

In contrast to the GP_{exc} analysis, here there is a clear difference in the relative areas of the two Laurdan fluorescent bands over the lipid transition region for the two systems studied, namely DMPG at low and high ionic strength. Hence, even at a bilayer shallow position where Laurdan is considered to reside [5], the fluorophore detects the anomalous gel-fluid transition region of DMPG at low salt.

For the high ionic strength DMPG sample, there is a sharp increase on the fraction of the longer wavelength band between 20–25 °C (Fig. 5a), following the lipid gel-fluid transition temperature (T_m~23 °C). However, though difficult to explain, there is a small but significant decrease

on the fraction of the longer wavelength band from 25 to 30 °C. The result obtained with DMPG at low salt is rather interesting, as the fraction of the Laurdan longer wavelength band slightly decreases up to 20 °C, increasing from 25 °C upwards. This is curious, because DSC indicates that the gel-fluid transition of low ionic strength DMPG starts around 17 °C going up to 35 °C [7–9, 29]. Moreover, ESR of spin labels indicates the coexistence of phases with different packing along this transition region (17–35 °C) [7], one of them being rather loose and hydrated. Hence, the difference observed with Laurdan in low and high ionic strength DMPG dispersions (Fig. 5a) indicates that Laurdan fluorescence is sensitive to the anomalous gel-fluid transition region present at low ionic strength DMPG, but the interpretation of the data is rather complex, and requires a better understanding of the origin of the two emitting states of Laurdan.

In the lipid fluid phase (35 to 60 °C), the Laurdan fluorescence is very similar for both systems, with DMPG at low and high salt presenting similar spectra decomposition (Fig. 5a). The similarity between these two systems, concerning the lipid bilayer structure and polarity in the fluid phase, was also observed with spin labels intercalated into the bilayers [27]. It is interesting to note that, for DMPG fluid phase, the contribution of the longer wavelength band increases with temperature, at least up to 60 °C, as measured here. At 60 °C, the fraction of the shorter wavelength band is still 0.15 of the total Laurdan fluorescent spectrum (Fig. 5a), although this band has been frequently attributed to the gel phase only [2, 3].

Considering that the Gaussian lines were somehow centered around 430 nm (2.84 eV) and 480 nm (2.54 eV) for all temperatures, the fluorescence anisotropy of Laurdan was measured at those two wavelengths (Fig. 5b). Like Laurdan in DPPG [5], the measured fluorescence anisotropy at the two wavelengths are rather similar for DMPG gel phase, but for the fluid phase the Laurdan anisotropy is much higher at 430 than at 480 nm. It is important to have in mind that the measured anisotropy at 480 nm is almost completely due to the longer wavelength emission band, as the contribution of this band at 480 nm was found to be around (95±5) %, for all temperatures studied. At 430 nm, the anisotropy is mostly due to the shorter wavelength band, (70±5) %, but not entirely. As discussed before [5], the much higher anisotropy observed for the shorter wavelength band (at 430 nm) for Laurdan in the lipid fluid phase could possibly be related to the shorter lifetime of the excited state associated to this emission, but needs further investigation.

As expected, the Laurdan fluorescence anisotropy decreases as a function of temperature, monitoring the lipid gel-fluid transition, consistent with a looser fluid bilayer surface. Laurdan anisotropy also indicates a sharper gel-fluid transition for DMPG at high ionic strength as compared to DMPG at low salt, as found with other techniques [7–9, 29].

Lets focus on the Laurdan anisotropy at 480 nm (Fig. 5b), which is mostly due to the longer wavelength band, and compare its temperature dependence with that of the fraction area of this band (Fig. 5a). Similar to the results obtained with the Laurdan emission band decomposition, for the fluid phase of DMPG (35 to 60 °C), Laurdan fluorescence anisotropy is rather similar when incorporated in high and low ionic DMPG bilayers, confirming the similar bilayer packing and hydration for the two DMPG systems. However, considering the saturating effect on Laurdan anisotropy as temperature increases (Fig. 5b), and the steep increase on the relative area of the longer wavelength band (Fig. 5a), it seems that the packing of DMPG bilayer surface does not change much for temperatures above 45 °C, whereas the bilayer hydration keeps increasing (Fig. 5a).

At low temperatures, from 10 to 20 °C, Laurdan fluorescence anisotropy presents a small decrease with temperature (Fig. 5b), for both high and low ionic DMPG samples, indicating an small increase in average bilayer fluidity with temperature (the data are rather reliable, as the values are mean values of three different samples, and the standard deviations are not shown because they are smaller than the symbols). These results should be compared with those obtained with Laurdan spectrum decomposition (Fig. 5a), which indicated a decrease on the fraction of the longer wavelength band from 10 to 20 °C (low ionic strength DMPG) or 10 to 15 °C (high ionic strength DMPG). Hence, it is clear that the two parameters (Figs 5a and b) give complementary information about the lipid bilayer surface: as the decrease in fluorescence anisotropy indicates that Laurdan is monitoring, in average, a less packed bilayer surface as temperature increases from 10 to 20 °C, the decrease on the fraction of Laurdan longer wavelength band seems to be solely related to a decrease on bilayer surface hydration.

Though a rationalization about possible structures of DMPG aggregates at low ionic strength is out of the scope of the present work (see, for instance, [11, 13]), the data presented here will have to be taken into account for further discussions about this peculiar lipid system. Due to the complexity of the system, results obtained with Laurdan fluorescence emission need to be compared with data from other techniques (Lamy et al., in preparation)

Laurdan in DMPG bilayers in the presence of cationic peptides and cholesterol

In this section we will apply the methodology presented above on the discussion of the modifications on Laurdan

fluorescence spectrum due to the interaction of two cationic melanotropic peptides, α-MSH and the more potent analogue, NDP-α-MSH, with low ionic strength DMPG bilayers. DMPG has to be used at low salt concentration otherwise the cationic peptide–anionic lipid interaction is not observed [23, 25]. It was found that the peptides reside at the bilayer surface, but partially penetrate the membrane [23–27]. Results obtained with cholesterol, which is totally incorporated into the bilayer, are shown for comparison. The information yielded by Laurdan fluorescence will be compared with the results obtained with spin labels inside the DMPG bilayer (at the bilayer core and at the 5th C-atom position [27]), regarding α-MSH and cholesterol interaction with DMPG bilayers.

Figure 6 shows that the Laurdan emission spectra is sensitive to the interaction of DMPG with α-MSH (20 mol %), NDP-α-MSH (20 mol %), and cholesterol (30 mol %), at different temperatures. As analyzed for pure DMPG samples, the spectra as a function of energy could be well decomposed into two Gaussian lines, at all temperatures (χ^2~1.00). The temperature dependence of the fraction of the longer wavelength emission band for Laurdan incorporated in pure DMPG bilayer, and in DMPG bilayers in the presence of α-MSH, NDP-α-MSH, and cholesterol are shown in Fig. 7a. As expected from the changes on the shape of the Laurdan emission spectra (Fig. 6), the spectrum decomposition is dependent on the presence of the cationic peptides and cholesterol. The results related to the Laurdan fluorescence band decomposition will be discussed together with Laurdan emission anisotropy at 480 nm (Fig. 7b), which corresponds to the anisotropy of the longer wavelength emission band, as discussed before.

Considering the complexity of the gel-fluid transition region of DMPG (17 to 35 °C), and the present controversy about the structure of the bilayer along this temperature range [11, 13], alterations on the Laurdan fluorescence emission caused by the interacting molecules on DMPG bilayers over this temperature interval will not be discussed here, but will be the subject of future studies, together with other spectroscopic techniques. In the present paper, we focus on the alterations caused by the cationic peptides, and cholesterol, on the Laurdan spectra when the probe is incorporated in the gel (10–15 °C) and fluid (40–60 °C) phases of DMPG bilayers.

Focusing on the fluid phase of DMPG bilayers (at and above 40 °C), the increase on the fluorescence anisotropy of Laurdan in DMPG bilayers with NDP-α-MSH and cholesterol indicates an increase on the bilayer packing/order at the bilayer surface, monitored by Laurdan. Clearly, cholesterol is much more effective in increasing the bilayer surface packing than the peptides (higher anisotropy). Different from NDP-α-MSH, the hormone α-MSH does not change the DMPG membrane packing at the bilayer

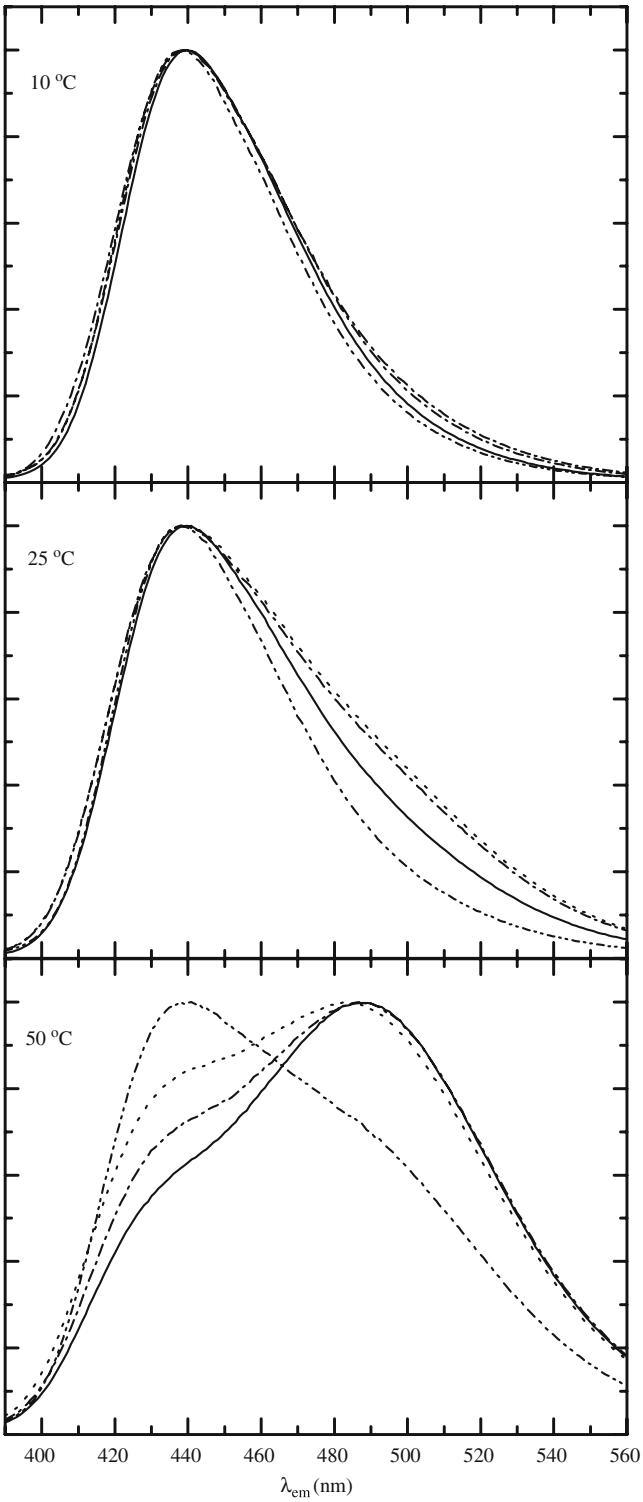

Fig. 6 Typical fluorescence emission spectra of Laurdan in low ionic strength DMPG (—), and in the presence of 20 mol% of α-MSH (-·-), 20 mol% of NDP-α-MSH (···) and 30 mol% of cholesterol (-···-), at 10, 25 and 50 °C. λ_{exc}=340 nm

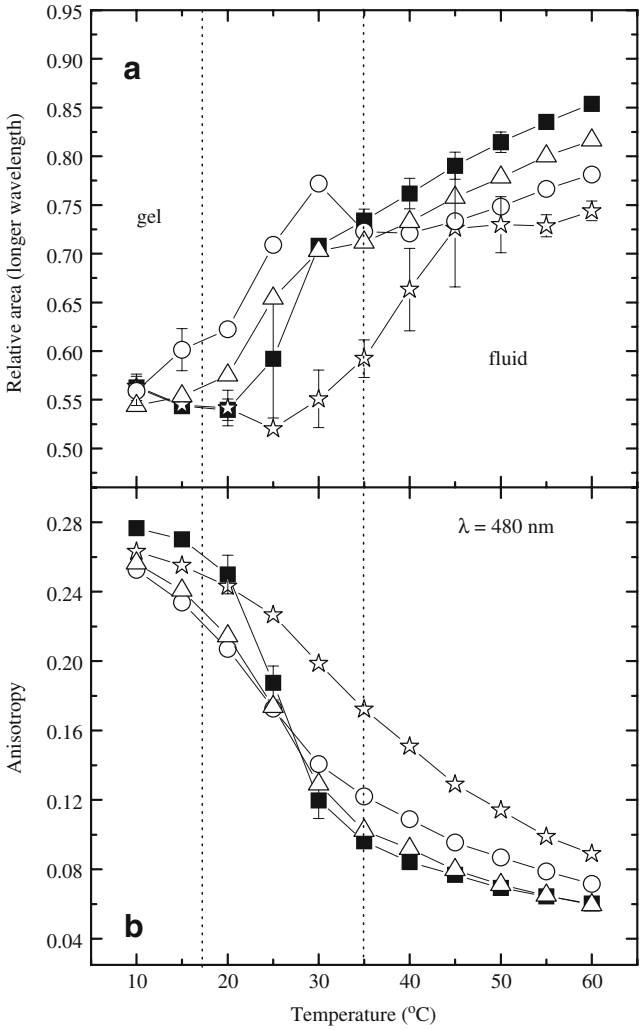

Fig. 7 **a** Temperature variation of the area fraction of the Laurdan emission component at lower energy (longer wavelength, ~480 nm), for DMPG at low ionic strength (■), and in the presence of 20 mol% of α-MSH (Δ), 20 mol% of NDP-α-MSH (○) and 30 mol% of cholesterol (ψ). **b** Laurdan fluorescence anisotropy measured at λ_{em}= 480 nm (same symbols as in (**a**)). The data are mean values of at least three different samples, and the uncertainties are the standard deviations, not shown if they are smaller than the symbols. λ_{exc}= 340 nm

surface. But spin labels localized at the 5th C-atom and at the bilayer core indicated that both cholesterol and α-MSH increase the packing of fluid DMPG hydrocarbon chains [27].

The Laurdan anisotropy results discussed above (at and above 40 °C) for DMPG membranes with and without peptides and cholesterol can be compared with the calculated area of the longer wavelength Laurdan emission band in the same conditions (Fig. 7a). Clearly, the two peptides and cholesterol decrease the fraction of the longer wavelength band, with cholesterol yielding a stronger effect. Considering that this longer wavelength emission band has been associated with Laurdan in a looser and/or more hydrated environment, it can be said that cholesterol clearly increases the packing of the DMPG bilayer surface and/or decreases the bilayer hydration (increases Laurdan fluorescence anisotropy and decreases the fraction of the longer wavelength band). Though not inside the membrane, but at the bilayer surface, NDP-α-MSH also increases the packing of the bilayer (increases the Laurdan fluorescence anisotropy), and decreases the fraction of the Laurdan longer wavelength band emission (Fig. 7a). Considering that α-MSH does not change the Laurdan emission anisotropy (Fig. 7b), but significantly decreases the Laurdan longer wavelength band emission (Fig. 7a), it can be concluded that the presence of the hormone at the DMPG bilayer surface decreases the hydration of Laurdan without much changing the bilayer surface packing. Interestingly, this is an effect quite different from that observed with spin labels at the 5th carbon atom position, or at the bilayer core: the hormone was found to increase the bilayer packing and increase the bilayer hydration at the micro-environment monitored by the labels [27]. Hence, it seems that the presence of α-MSH at the bilayer surface of DMPG pushes the water molecules from the bilayer surface to the bilayer core.

On DMPG gel phase, at 10 and 15 °C, it is interesting to see that the known fluidizing effect of cholesterol (for instance, see [32]) can be well detected through the decrease of the Laurdan emission anisotropy (Fig. 7b). However, the percentage of the longer wavelength Laurdan emission band is not changed with the presence of cholesterol, indicating a similar water environment for Laurdan (Fig. 7a). Considering the decrease on Laurdan fluorescence anisotropy, the two peptides, the hormone α-MSH and its more potent analogue NDP-α-MSH, similarly disturb the structure of the membrane gel phase, increasing the bilayer surface fluidity (Fig. 7b). Interestingly, the peptides are more effective in decreasing the gel bilayer surface packing than cholesterol. Similar to cholesterol, the presence of α-MSH does not alter the Laurdan spectrum decomposition (Fig. 7a). Hence, though the hormone decreases the DMPG gel phase packing it does not seem to alter the bilayer surface water distribution. Only with NDP-α-MSH at 15 °C, the effect of increasing the fraction of the longer wavelength emission band (related to the more relaxed Laurdan excited state) is observed.

Conclusions

In the present paper we discuss the decomposition of the Laurdan fluorescent spectrum into two Gaussian bands, and we show that this decomposition should be done with the spectrum as a function of energy, and not wavelength. This methodology has the advantage over the calculation of the

Excitation Generalized Polarization, GP_{exc}, by clearly separating the fluorescence emission of the two different excited states of Laurdan. We present here an example with DMPG at low and high ionic strength, where this methodology was found to be better for detecting alterations on bilayer structure and/or polarity than the calculation of GP_{exc}: even at a bilayer shallow position, Laurdan can detect the anomalous gel-fluid transition region of DMPG at low salt. Though the interpretation of the data is rather complex, it could be clearly seen that Laurdan anisotropy and Laurdan spectra decomposition give complementary information.

The presence of the two emission bands in Laurdan spectrum should also be considered in the calculation of the fluorescence anisotropy. At 480 nm the fluorescence anisotropy measures the anisotropy of the fluorescence emission from the more relaxed excited state only, but at 430 nm the fluorescence anisotropy measures the anisotropy of the emission from the two excited estates. It is important to have in mind that the origin of the two emission bands of Laurdan and Prodan, when incorporated into lipid bilayers, is still not well understood. They can be related to two different excited states of the molecule in a certain microenvironment, but they could also be related to different populations of the fluorophore, positioned at two different regions of the membrane [33–35], which would differently favor the more relaxed or the less relaxed excited state.

In a fluid bilayer (DMPG above 40 °C), Laurdan fluorescence, through the analysis of both the spectrum decomposition and anisotropy, could detect that the hormone α-MSH does not alter the bilayer surface packing, but significantly decreases the surface hydration. Comparatively, the more potent analog NDP-α-MSH was found to increase the surface packing and, possibly, also decrease the surface hydration. The surface rigidifying effect of NDP-α-MSH could be related to its somewhat more rigid structure as compared to that of the hormone [36, 37]. The presence of the two peptides at the bilayer surface clearly decreases the packing of the highly ordered gel bilayer surface (DMPG at 10–15 °C, Fig. 7b). However, the expected increase on the fraction of the longer wavelength band, which is associated to an emission from a more relaxed excited state, is not observed for all temperatures. The above information may be important for future melanotropin analogs design. However, a better understanding of the origin of the two populations present in Laurdan emission spectra is still necessary, and would strongly contribute to disentangle the effect of the two peptides on the lipid bilayer surface.

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