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LOG-NORMAL DECONVOLUTION OF LAURDAN FLUORESCENCE SPECTRA – A TOOL TO ASSESS LIPID MEMBRANE FLUIDITY

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Abstract. Deconvolution of complex steady-state fluorescence spectra is a key subject in analytical fluorescence spectroscopy. The shape of the spectra is generated by the presence in the analyzed solution of a mixture of several fluorophores or by a single fluorophore found in different excited states. The spectra shape of most of the fluorophores is asymmetric, even in a homogeneous solution, where only one excited state is presumed to be present. Due to this, fluorescence spectra can be analyzed much better by a log-normal (LN) distribution than by a Gaussian one. Laurdan is a membrane fluorescent probe who has the advantage of detecting changes in bilayer phase properties. Laurdan typical red-shift (~ 50 nm) is observed during the phospholipid phase transition, and is originating from the probe sensitivity to its environment polarity. In this study, we propose a comparison between Gaussian and LN deconvolution of fluorescence spectra of Laurdan, inserted in large unilamellar vesicles, prepared from lipids with different hydrocarbon tails. We used a new parameter, namely, the difference of relative areas of the elementary peaks (ΔSr) to assess lipid membrane fluidity. We found that the results give a better characterization of the hydration level of the environment surrounding Laurdan.

Key words: Laurdan fluorescence, generalized polarization, Gauss *vs*. log-normal deconvolution, lipid membrane fluidity, phospholipid phase transition.

1. INTRODUCTION

The emission spectra of most fluorophores have a complex structure and an asymmetric shape which can be much better analyzed by a log-normal (LN) function. But, only a few studies have proposed this alternative, and very often, the fluorescence spectra are analyzed using symmetric functions like Gaussian or Lorentzian functions. The use of the LN function to describe the absorption spectra was first proposed by Siano and Metzler [1] and later on adjusted by Burstein to analyze the emission spectra of fluorophores [2]. The procedure was first

implemented for the analysis of Tryptophan emission spectrum in proteins [3] and later modified to allow the analysis of other fluorophores, like Prodan and Acrylodan [4]. In our previous study, we addressed this procedure for the first time to Laurdan (6-dodecanoyl-2-dimethylamino naphthalene) [5].

The solvatochromic dye, Laurdan, has been intensively used in the exploration of both model and natural membrane properties [4, 6–8]. Laurdan 12 carbon aliphatic tail facilitates its insertion into lipid membranes. The naphthalene moiety of Laurdan is generally considered to be located at the level of the glycerol backbone [7, 9] and its dipole moment oriented along the normal to the membrane [6, 7, 10]. Laurdan fluorescence properties depend strongly on the relaxation of the polar solvent molecules around the increased dipole moment of the probe in the excited state [11]. When inserted into the lipid membranes, the relaxation is due to the water molecules that can penetrate the lipid membrane which depends on the state of the lipid phase [10, 12]. Thus, the lipid phase of the membrane can be assessed by Laurdan [13].

Considering this, for a membrane with a higher order of lipid packing (in the gel phase), only a few water molecules can reach the naphthalene moiety of Laurdan, resulting in only a slight effect of dipolar relaxation. Conversely, the fluid phase is characterized by a poor lipid packing which allows more water molecules to penetrate the membrane at the level of the glycerol backbone [10, 12]. These can be observed as a red shift in Laurdan emission maximum, which goes from around 440 nm, in the gel phase, to around 490 nm, in the fluid phase [7, 10, 14].

Using the fluorescence intensities of Laurdan at 440 nm (I_{blue}) and 490 nm (I_{green}), the so-called generalized polarization (GP) was defined as [13]:

$$\mathrm{GP} = (I_{blue} - I_{green})/(I_{blue} + I_{green}). \quad (1)$$

Theoretically, GP values can range from +1 (no solvent effects) to –1 (strong solvent effects) in homogenous solutions (using solvents covering a large range of polarity from highly apolar cyclohexane to largely polar alcohols) (for details see [8]). In the lipid bilayer, it was proved experimentally that *GP* goes from 0.6 to –0.4, irrespective of spectroscopy method [8, 12, 13, 15] or microscopy measurements [14, 16–19]. GP values cannot reach the extreme values in the lipid membranes because of the spectral overlapping of the emission bands from the relaxed and non-relaxed states. Nevertheless, if instead of using the fluorescence intensities at 440 and 490 nm, we will use the number of emitting molecules from each state, we could generate a more reliable parameter. Such information can be provided by decomposition of Laurdan spectra in a superposition of two elementary peaks. Thereafter, we can use the relative area of the elementary peaks (as an indirect measure of the number of molecules emitting from each excited states) to characterize changes in Laurdan spectra. This is usually done using a sum of two Gaussian functions to solve the fluorescence emission spectra [20–22]. However, in our previous study, we proposed instead, the use of LN functions to deconvolute the spectra of Laurdan inserted into model lipid membranes (DMPC LUVs) [5]. This method proved to describe better the complex spectra of Laurdan

in DMPC bilayers and to provide a more feasible picture concerning the hydration of these structures at the level of lipid backbone.

In this article, we extended our previously proposed model [5] to analyze the spectra of Laurdan incorporated into three other types of liposomes and we compared the results obtained using the LN decomposition with those generated using the Gaussian decomposition.

2. MATERIALS AND METHODES

2.1. MATERIALS

Laurdan was purchased from Invitrogen/Molecular Probes (Eugene, OR, USA). The lipids used, 1,2-ditridecanoyl-sn-glycero-3-phosphocholine (C13) 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). $Na_2HPO_4 \cdot 2H_2O$, KH_2PO_4 anhydrous, and NaCl were purchased from Sigma-Aldrich and used to prepare the phosphate-buffered saline (PBS, 10 mM, pH 7.4).

2.2. LUVS PREPARATION

LUVs with a final lipid concentration of 50 μM were prepared using the extrusion method according to the Avanti published protocol. Briefly, an appropriate amount of lipids was dried under nitrogen flow to remove the solvent and to obtain a lipid film. The lipid film was hydrated with PBS, heated above the transition temperature (T_m) of the lipids and vigorously vortexed to form a suspension of multilamellar vesicles (MLVs). The MLV suspension was repeatedly freeze–thawed (5 cycles) and extruded (25 times) through a 200-μm filter using a standard extruder (Avanti Polar Lipids). The extrusion was performed also at a temperature above T_m of the lipids, resulting in a suspension of LUVs. Laurdan was added into the LUV suspension to a final lipid/probe ratio of 500:1.

2.3. FLUORESCENCE SPECTROSCOPY MEASUREMENTS

Steady-state fluorescence measurements were performed using a FluoroMax 3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ, USA) equipped with a Peltier thermostated cell holder. The emission spectra of the Laurdan were recorded in the range, 1–70 °C, depending on the lipids used. The spectra were recorded in the range, 400–600 nm, with the excitation set at 378 nm. The recorded spectra were corrected for the spectral sensitivity of the emission channel of the spectrofluorimeter and for Raman and scattering artifacts. All of the emission recordings have been done at a suspension absorption smaller than 0.05.

Consequently, no correction for the inner effect was needed [23]. Before the fitting, all the spectra were converted in the wavenumber scale using the relation $I = I_\lambda \times \lambda^2$, where I is the intensity in wavenumber scale, I_λ is the intensity in wavelength scale, and λ is the wavelength [24].

2.4. SPECTRA DECOMPOSITION PROCEDURE

Spectra recorded for Laurdan inserted into the lipid bilayer of LUVs were fitted with a superposition of two LN functions as reported earlier [5] in a script written using MatLab 9b software (MathWorks, Natick, MA, USA). For comparison, the complex spectra in LUVs have been decomposed by two Gaussian functions using the Origin 8.0 software package. GP values were calculated using Origin 8.0.

3. RESULTS AND DISCUSSIONS

We recorded the emission spectra of Laurdan incorporated into pure C13, DPPC or DSPC LUVs. The recordings were performed at different temperatures in such a way that, for each type of lipids used, their transition from the gel phase to the fluid phase was well covered. Thus, for C13 the spectra were recorded between 1 and 60 ^{o}C, for DPPC between 20 and 60 ^{o}C, while for DSPC were between 35 and 70 ^{o}C. In addition, the spectra recorded for each type of lipids were normalized with the values recorded at the first temperature (Fig. 1).

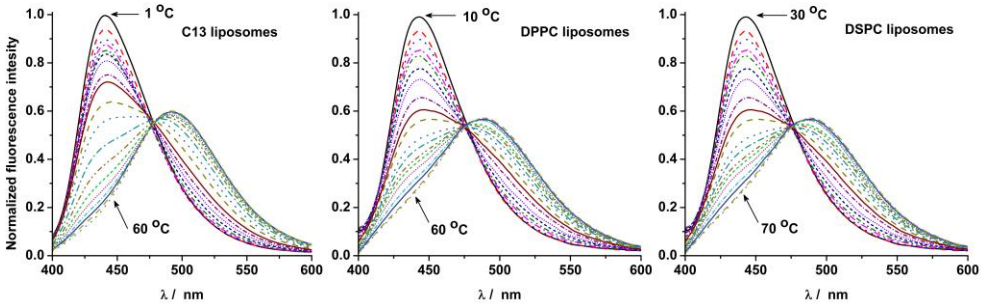

Fig. 1 – Normalized fluorescence emission spectra of Laurdan inserted into pure C13, DPPC, and DSPC LUVs, at different temperatures.

Analyzing the family curves presented in Fig. 1 we can observe that for all three lipids we have the usual behaviour of the fluorescence emission of Laurdan incorporated into lipid membranes. Namely, the emission maximum shifts from 440 nm, when the lipids are in the gel phase to 490 nm, when the membrane is in the fluid phase.

To describe the contribution of the non-relaxed and relaxed excited states to the Laurdan emission we deconvoluted the spectra in a sum of two LN functions using the procedure described previously [5]. Hereinafter, we present our results obtained by LN decomposition for all experimental conditions and we will also make a comparison with the results obtained by much more utilized Gaussian decomposition.

In Fig. 2, the comparison between the decomposition of Laurdan spectrum done by LN and Gaussian functions for three representative spectra recorded on DPPC LUVs, one at 20 °C (A), the second at 41 °C (B), and the last one at 60 °C (C) is presented.

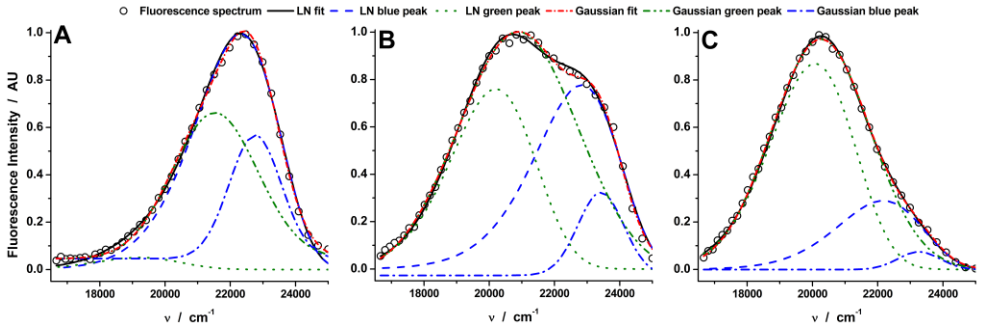

Fig. 2 – Comparison between fitting the spectra of Laurdan inserted into DPPC LUV, at different temperatures (A : 20 ^{o}C, B : 41 ^{o}C, and C: 60 ^{o}C), using the LN or Gaussian functions. The data are presented in wavenumber scale.

The position of the two peaks originated after the Gaussian decomposition starts from around 435 nm (the blue peak) and 465 nm (the green peak) and varies greatly with the increase of temperature, reaching 425 nm and 490 nm respectively (data not shown) in agreement with previous results [18, 21, 22]. Even though these studies give an explanation for the increase of the position of the second peak, due to the presence of more water molecules around Laurdan, none of them explains the decrease observed for the first band. This decrease is not supported by physical evidence, meaning that with the increasing temperature the region around some of Laurdan molecules becomes more rigid with less water molecules surrounding it. We think that our approach, using the LN decomposition, can give a more accurate insight into the hydration level in the membrane as sensed by Laurdan molecules.

In contrast, the outcome from the LN decomposition are two peaks located around 440 nm for the blue peak and 490 nm for the green peak, which remain almost independent of the temperature or lipid composition (data not shown). At 20 °C (Fig. 2A) the lipids are found in the gel phase (for DPPC, T_m is 41 °C) and it is expected that Laurdan molecules emit mostly from a nonrelaxed state. From the LN analysis, the area of the blue peak, located at approximately 440 nm, is as expected, higher compared with the one of the green peak. On the other hand, the

peaks obtained after Gaussian decomposition does not show the same trend. But even more surprisingly, is that the area of the green peak is much larger than the one of the blue peak. These results mean that even when the bilayer is in the gel phase, most of the Laurdan molecules emit from a relaxed state. For the spectra recorded at 41 °C (Fig. 2B), where it is expected that the gel and fluid phase of the lipids co-exist [25], we obtain, from the Gaussian decomposition, preponderantly a green peak, whereas from the LN decomposition the area of the two peaks are almost equal. Similar tendencies (the green peak obtained using Gaussian decomposition is larger than the one obtained using LN decomposition) are also found for all the other analyzed spectra. For the last condition, when the spectra were recorded at 60 °C (at this temperature, the lipids are in the fluid phase) we notice that for the Gaussian deconvolution there is almost no contribution coming from the nonrelaxed state, whereas for the LN decompositions, the contribution diminishes but it still has an important level.

Analyzing the evolution of the elementary peaks areas for the LN decomposition, we observed for all tested conditions, that at lower temperatures the blue peak prevails against the green one. Also, with increasing temperatures the area of the blue peak is reduced while that of the green peak becomes larger, until they become equal around the transition temperature while, at higher temperatures, the green peak is the most predominant. These results prove that even at low temperature, when the lipid membrane is in the gel phase, there are Laurdan molecules that emit from a relaxed state. This description is very similar with that already presented for DMPC LUVs [5], proving that the LN decomposition can be easily extended to analysis of Laurdan spectrum in various lipid environments.

In more details, the temperature dependence of elementary peak areas of the data recorded for the three types of LUVs is presented in Fig. 3, for both LN and Gaussian deconvolution. The relative area of the peak in the blue channel (emission from non-relaxed state) (Sr_B) is ~ 0.92 for C13 LUVs, ~ 0.96 for DPPC LUVs, and ~ 0.97 for DSPC LUVs at low temperatures, which decreases with increasing temperature and reaches ~ 0.25 for all types of vesicles in the fluid phase, whereas, the Sr_B values after Gaussian deconvolution starts always from around 0.35, at low temperatures, and decreases to nearly 0, when the membrane is in the fluid phase, independent of the type of lipids. The relative area of the peak in the green channel (Sr_G) has an opposite variation to the Sr_B one. In Fig. 3, we observe that the peak areas obtained by LN decomposition have a point where they meet. Around the temperature, characteristic to this point, the gel and fluid phases are coexisting in the lipid bilayer. In the case of Gaussian decomposition, the green peak area is higher than that of the blue peak for all the temperatures suggesting that the fluid phase is more present in the bilayer even at temperatures below main transition temperature. This description is difficult to fit into the accepted models of the hydration during main transition of the lipid bilayer as we already proved for DMPC LUVs [5].

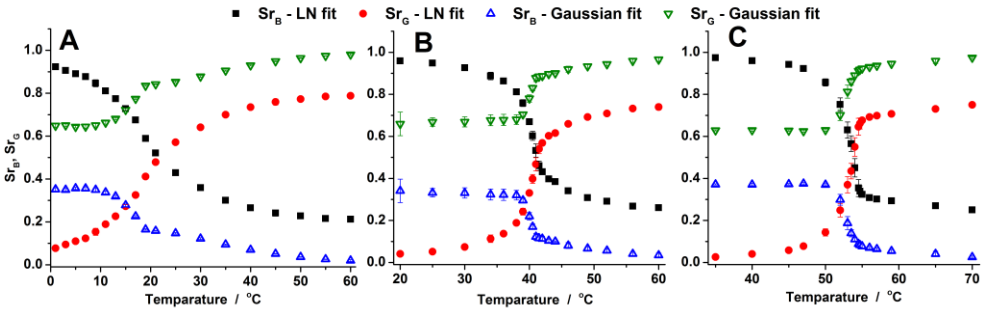

Fig. 3 – Comparison of peak relative area resulted from fitting procedures with LN and Gaussian functions (A: C13, B: DPPC, and C: DSPC). The error bars are the standard deviations resulting from at least three repeated measurements for each condition.

Analyzing in more details the LN peaks area curves presented in Fig. 3, some differences (additionally to the main transition temperature) can be noticed among the curves characterizing the lipids used in this study: the relative area peak at low and high temperature and the temperature range of the transition. The values of all these parameters are presented in the Table 1. Additionally to the lipids used in this report, in Table 1 we added also the DMPC, based on the data reported in our previous work [5].

Firstly, there are slightly differences among the values of Sr_B obtained for the LUVs of different lipids at lowest temperature. This parameter monotonically increases with the length of the fatty acid chains. This effect is not observed at the highest temperature. Secondly, the transition temperature range (obtained fitting the curve with a sigmoid) is monotonically decreasing with the length of the fatty acid chains.

Table 1

Comparison between Sr_B at extreme values of temperature and ΔT range obtained from sigmoidal fit of Sr_B curves (data from Fig. 3 and [5] for DMPC)

Lipid	Sr_B at T_{min}	Sr_B at T_{max}	ΔT
C13	0.92	0.21	7.45
DMPC	0.95	0.26	5.31
DPPC	0.96	0.26	2.02
DSPC	0.97	0.25	1.57

These effects may have a simple explanation when one compares the length of Laurdan hydrophobic tail (12 carbons) with those of the lipids where it is inserted, The DSPC lipid chains have 18 carbons compared with 16 carbons of DPPC, 14 of DMPC and 13 of C13. As a result, the longer lipids can accommodate better the Laurdan molecules, that is, less water molecules reaches them in the case of membranes composed of lipids with longer aliphatic tails in the well packaged

gel phase. On the contrary, in the fluid phase, the results indicate that the length of the lipid chains is not important, so that when the membrane becomes more fluid, Laurdan "senses" similar environments. These differences generated by the length of the lipid tails can also be observed around the transition temperature. Thus, for the C13 we notice a wider transition region, while with the increase of the lipid tails, the transition becomes steeper (Fig. 3). Such an effect cannot be related only to the lipids themselves, because one of the most used methods for analyzing the main transition (differential scanning calorimetry) has not evidenced similar results [26].

The curves of peak area against the temperature can be fitted using a sigmoidal function and used to obtain information about T_m. The point where the relative areas become equal represents the experimental condition where the gel and fluid phase of the lipids co-exist and the same number of Laurdan molecules (in the hypothesis of the same quantum yield for emission from the relaxed and non-relaxed state) are found in both of them. This should happen at T_m of the lipids and using the plots from Fig. 2, we can determine T_m for each lipid (Table 2).

Table 2

T_m of lipids obtained using the GP or the parameters resulted by the fitting with LN function, compared with the ones reported in the literature

	T_m / °C		
	C13	DPPC	DSPC
GP[a]	22.01	40.93	53.35
ΔSr[a]	19.27	40.32	53.04
S_{rB}, Sr_G[b]	30.83	42.81	54.06
S_{rG}/Sr_B[a]	23.00	41.50	53.70
Ref [27]	13.50	41.30	54.50

[a] – sigmoidal fit
[b] – intersection of Sr_B and Sr_G curves

Using the relative areas of the elementary peaks we can define a parameter similar to the GP, namely, the difference of relative areas: $\Delta Sr = Sr_B - Sr_G$. This parameter describes Laurdan accessibility to water molecules when inserted into lipid membranes and depends on the fractions of emitting molecules in each state, theoretically ranging from +1 (emission from non-relaxed state only) to –1 (emission from relaxed state only). GP values were also calculated using equation (1). Thus, both ΔS_r and GP values obtained for the recordings of Laurdan inserted in the liposomes prepared from C13, DPPC and DSPC are plotted in Fig. 4. The ΔSr values start from high values, 0.85 for C13, 0.91 for DPPC, and ~ 0.95 for DSPC and drop to ~ –0.5. Compared with ΔSr values, GP values are 0.44, 0.45 and 0.48, respectively and decrease to ~ –0.4. As we can observe, for all tested conditions, the range covered by ΔSr is significantly larger than the one covered by GP. The larger range suggests that ΔSr can be a more sensitive parameter in detecting and characterizing the hydration level in the lipid membranes.

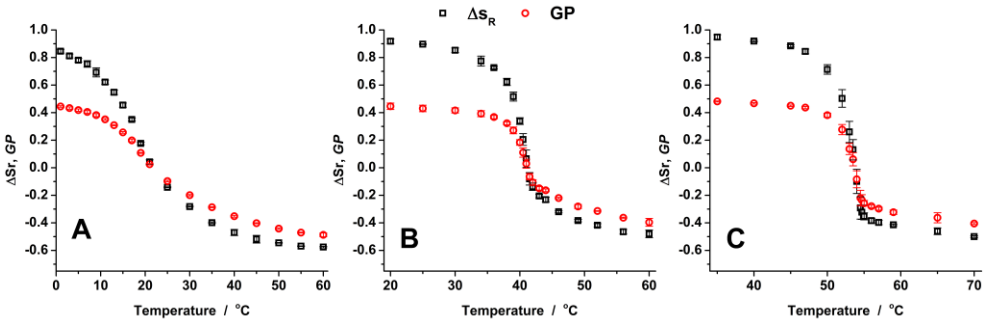

Fig. 4 – Comparison between Δ*S*r and GP (A: C13, B: DMPC, C: DPPC, and D: DSPC). The results are means ± standard deviations on three repetitions of the recordings.

The GP values calculated for the LUVs used in this study (Fig. 4) are in agreement with the literature [6]. Previous studies on liposomes showed that T_m of the lipids can be accurately determined by the temperature dependence of GP [6, 22]. Thus, both GP and ΔSr were fitted with a sigmoidal function and we determined T_m of the lipids used to prepare the LUVs (Table 2). The T_m values reported in Table 2 for ΔSr are consistent with those derived from GP curves and also with those reported in literature [27].

We also calculated the ratio of elementary peak areas (Sr_G/Sr_B), which is another parameter proposed in the literature to characterize the changes in Laurdan spectrum [22]. These parameters calculated for the LN and Gaussian fitting peaks, for all the LUVs used, are depicted in Fig. 5. This parameter has a sharp increase starting close to the phase transition temperature.

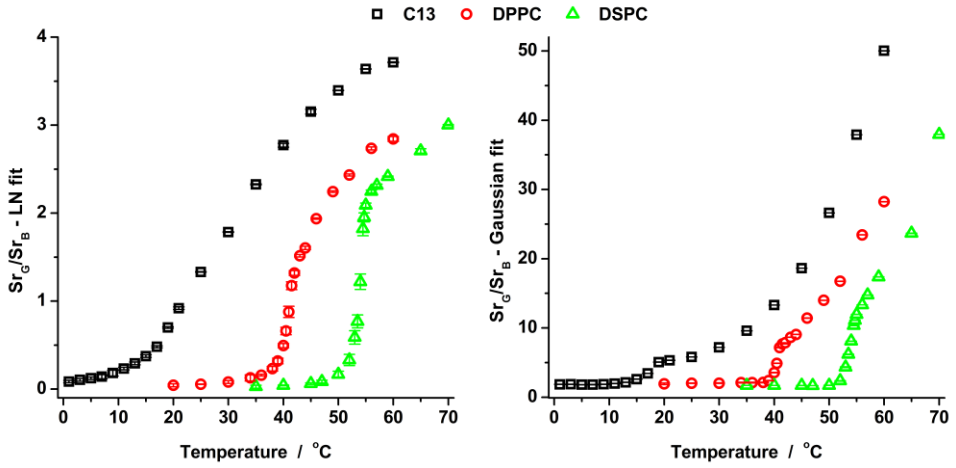

Fig. 5 – Relative area ratio dependence on temperature for pure C13, DPPC, and DSPC LUVs as obtained for LN or Gaussian decomposition. The results are means ± standard deviations on three repetitions of the recordings.

3. CONCLUSIONS

In this article, we used a method for analysis Laurdan spectrum presented in a previous work [5] based on a decomposition of spectra using two asymmetric LN functions to evaluate the spectra of Laurdan inserted into the membrane of pure LUVs prepared from C13, DPPC or DSPC.

The temperature dependence of LN elementary peak area is providing a more realistic image for the main transition phase comparing with the Gaussian deconvolution for all the lipids used in this work. All the results are consistent with our findings on DMPC LUVs [5] proving that LN decomposition method is consistent in describing the Laurdan emission when inserted into lipid bilayers,

The new parameter proposed to characterize the state of the liposome membrane, ΔSr, is a more sensitive with respect to the hydration level sensed by Laurdan. The peak area values in gel phase and the range of temperature characteristic for the main transition are depending on the length of lipid hydrophobic chains.

The method used in this paper will be directed to the analysis of more complex systems and the changes induced in membrane fluidity by different factors and also to the interactions between proteins and lipid bilayers.

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