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New Insights on the Fluorescent Emission Spectra of Prodan and Laurdan

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Abstract Prodan and Laurdan are fluorescent probes largely used in biological systems. They were synthetized to be sensitive to the environment polarity, and their fluorescent emission spectrum shifts around 120 nm, from cyclohexane to water. Although accepted that their emission spectrum is composed by two emission bands, the origin of these two bands is still a matter of discussion. Here we analyze the fluorescent spectra of Prodan and Laurdan in solvents of different polarities, both by decomposing the spectrum into two Gaussian bands and by computing the Decay Associated Spectra (DAS), the latter with time resolved fluorescence. Our data show that the intensity of the lower energy emission band of Prodan and Laurdan (attributed, in the literature, to the decay of a solvent relaxed state) is higher in cyclohexane than in water, showing a decrease as the polarity of the medium increases. Moreover, in all solvents studied here, the balance between the two emission bands is not dependent on the temperature, strongly suggesting two independent excited states. Both bands were found to display a red shift as the medium polarity increases. We propose here a new interpretation for the two emission bands of Prodan and Laurdan in homogeneous solvents: they would be related to the emission of two independent states, and not to a pair of non-relaxed and solvent relaxed states.

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Introduction

The fluorescent probes Prodan (2-dimethylamino-6-propionylnaphthalene) and Laurdan (2-dimethylamino-6-dodecanoylnaphthalene) have been widely used in biological relevant systems [1–7]. They were synthetized [1] to be sensitive to the environment polarity, so their emission spectra shifts about 120 nm from cyclohexane to water [1, 8, 9]. Moreover, when inserted into membranes, their emission spectra is extremely dependent on the lipid bilayer phase (gel or fluid), the maximum of the spectrum shifting around 50 nm from one phase to the other [4, 6, 10].

Although Prodan and Laurdan have been extensively used, their structure and electronic distribution, in different solvents, are still a matter of discussion [11–13]. For example, one very controversial point is the electronic structure of the excited state. With time resolved fluorescence, at least two lifetimes were found to be present in Prodan or Laurdan emission spectra in solvents like butanol [14], n-butanol [15], water, cyclohexane, and some other solvents [16], indicating the presence of two emission states. This dual emission has been assigned to the first excited state, S_1. Therefore, these two states are supposed to be a solvent-non-relaxed S_1 state, with a lower emission wavelength (called locally excited state, LE) and a solvent-relaxed S_1 state, with a higher emission wavelength (called internal charge transfer, ICT, possibly with an internal twist of the fluorophore, TICT) [17–31]. In the TICT state, the dimethylamine and the propanoyl groups would be rotated out of the naphthalene ring, due to the high charge separation. However, several authors do not agree with the above explanation [8, 11, 12, 13, 16, 32, 33], and insist that more investigation is necessary.

Considering that Prodan and Laurdan are used as fluorescent probes, to yield information about the micro-environment where they are located, it is fundamental to have a very good knowledge of their structure and electronic properties, so

changes on these properties can be correctly interpreted. Having that in mind, in the present work, we revisited the fluorescent spectra of Prodan and Laurdan in solvents of different polarities. The spectra were decomposed into two Gaussian bands, and the behavior of these two bands (their relative area and position) was analyzed as a function of the solvent polarity. Moreover, spectral decomposition was confirmed by the analyses of the Decay Associated Spectra (DAS) for both probes, using time resolved fluorescence.

Based on the experimental data presented here, a new interpretation for the two excited states of Prodan and Laurdan in homogeneous solvents is proposed. The data strongly support the hypothesis that the two emission states are related to two different excited states, S_1 and S_2. The emission from two excited states, S_1 and S_2, is an anomalous behavior that is an exception to Kasha's rule [9]. However, it was observed before for some aromatic molecules [34–40] and was assigned to a high fluorescent transition probability from S_2 or a suitable energy gap between S_2-S_1 [41]. Therefore, we conclude that Prodan and Laurdan belong to this class of molecules with anomalous dual emission from two different excited states.

Materials and Methods

Experimental Procedures

Materials

Fluorescent probes Prodan and Laurdan (Fig. 1) were purchased from Molecular Probes Inc. (Eugene, OR, USA), and the solvents cyclohexane, chloroform, dichloromethane, acetonitrile and methanol from Sigma-Aldrich (St Louis, MO, USA). Water was Milli-Q Plus (Millipore), pH~6.0.

Sample Preparation

Stock solutions of Prodan and Laurdan in chloroform (1.5 mM) were used in all experiments. Appropriated amounts
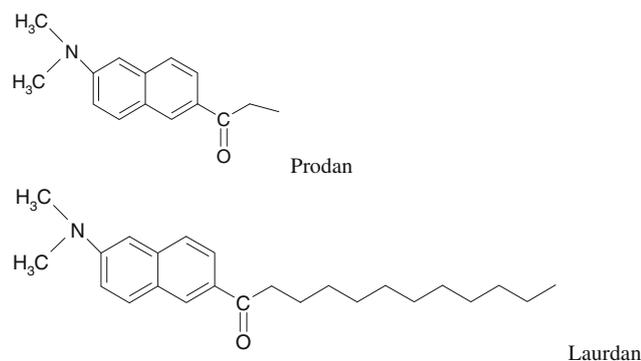
Fig. 1 Prodan and Laurdan molecular structures

of these solutions were transferred to glass flasks using calibrated glass microsyringes. Chloroform was evaporated under a stream of dry N_2. The dry residue was dissolved in the desired solvent to obtain the fluorophore concentration of 4.0 μM. pH was measured for all samples in water, and no alteration was observed (pH~6.0).

Steady-State Fluorescence Measurements

The fluorescent emission spectrum was measured in a VarianCary Eclipse Fluorescence spectrophotometer (Varian Australia PTY LTD, Mulgrave, VIC, Australia), with automated polarizers and a Peltier temperature controller. Prodan and Laurdan were excited at 340 nm in cyclohexane, 355 nm in chloroform, dichloromethane and acetonitrile, and 360 nm in methanol and water. For all measurements, a quartz cuvette with optical path of 1 cm was used. Bandpass was 5 nm.

All data shown are means of at least three experiments, and uncertainties are calculated standard deviations. If not shown, the uncertainty was found to be smaller than the graphic symbol.

Spectral analysis

Prodan and Laurdan fluorescent spectra were decomposed into two Gaussian bands, according to [10]. The obtained fluorescence spectrum was transformed from wavelength, $I(\lambda)$, to wavenumber, $I(\lambda^{-1})$, taking into account that the spectrum is recorded with constant wavelength resolution, not energy, so $I(\lambda^{-1})=\lambda^2 I(\lambda)$ [9, 42]. (It is important to have in mind that some spectrophotometers can display the spectrum in wavenumber, but do not perform the right conversion). Upon spectrum decomposition, the population of molecules relaxing from each excited state is proportional to the area under the corresponding band. All spectra fittings were performed with *Origin 8.5*.

Time-Resolved Fluorescence Measurements

Time-resolved fluorescence measurements were performed using the time-correlated single photon counting method (TCSPC). The excitation source used was a titanium-sapphire laser Tsunami 3950 from SpectraPhysics (Newport Corporation, Irvine, CA, USA), pumped by a solid state laser Millenia Pro model J80 also from SpectraPhysics. The repetition rate was set to 8000 kHz using a pulse picker (SpectraPhysics, model 3980-25). The Tsunami was set to give an output of 990 nm and a third harmonic generator BBO crystal (GWN-23PL SpectraPhysics) was used to generate the excitation beam at 330 nm. This beam was direct to a spectrophotometer FL900CDT (Edinburgh Instruments Ltd, Livingston, UK). The emitted light was detected at 90° from the excitation beam. The emission wavelength was selected by
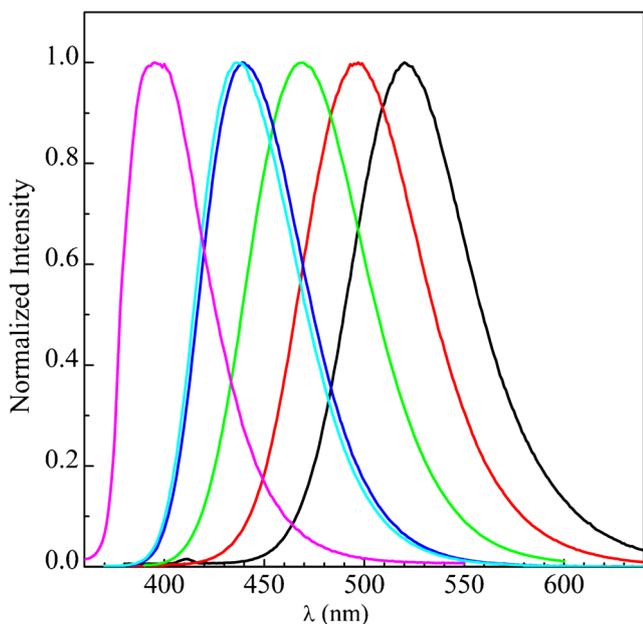
Fig. 2 Emission spectra of Prodan in several solvents. From *left* to *right*: cyclohexane (*magenta*), chloroform (*cyan*), dichlorometane (*blue*), acetonitrine (*green*), methanol (*red*) and water (*black*). Spectra are normalized, and measured at 25 °C. See values of λ_{exc} in Material and methods

a refrigerated microchannel plate photomultiplier (Hamamatsu R3809U). The FWHM of the instrument response function was 90 – 110 ps. Time resolution was 12 ps per channel. The temperature control used was a Julabo F25-HP water bath (JULABO USA Inc., Allentown, PA, USA).

Software from Edinburgh Instruments Ltd was used to analyze the decay curves. The intensity decays were fitted by:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i} \tag{1}$$

where τ_i is the lifetime of the ith component of decay and α_i is the respective pre-exponential factor.

Fluorescence intensity decays were analyzed by global analysis. Namely, for a given sample, a set of lifetime decay

profiles, measured at different wavelengths, was fitted with the same lifetimes, but different pre-exponential factors. The adequacy of the multiexponential decay fitting was judged by inspection of the residuals distribution and by statistical parameters, such as reduced chi-square (χ^2).

The fraction of each excited state decay was calculated by the equation:

$$f_i = \frac{\alpha_i \tau_i}{\sum \alpha_i \tau_i} \tag{2}$$

The decay associated spectrum (DAS) for each component was calculated considering the intensity of the steady-state fluorescent spectrum at the same wavelength that the lifetime decay was measured, I(λ), and the fraction of each excited state decay, given by Eq. 2 [9]:

$$I_i(\lambda) = f_i \, I(\lambda) \tag{3}$$

Results and Discussions

The well-known strong dependence of Prodan fluorescent emission spectra on the solvent polarity [1, 8, 12, 43, 44, 45] is evinced in Fig. 2. Here the solvents used are cyclohexane, chloroform, dichloromethane, acetonitrile, methanol and water. Apart from water, where Laurdan is not soluble and Prodan aggregates [46], Laurdan and Prodan display very similar fluorescence spectrum in all solvents studied [47]. The simplest way to define a solvent polarity is the use of the dielectric constant, ε. Another highly used parameter is the more comprehensive Reichardt normalized polarity scale, E_T^N [48], which takes into account not only solvent polarizability but all interactions that cause solvatochromic shift (Table 1). It is well known that Prodan and Laurdan emission maximum shows a significant red-shift as the polarity of the solvent increases, going from ~395 nm in cyclohexane to 495 nm in methanol and 520 nm in water. Considering, this

Table 1 Maximum of the emission spectra (in 10^3 cm^{-1} and, in parentheses, in nm) in several solvents, for Prodan and Laurdan

| Solvents	Polarity		Prodan		Laurdan	
	ε	E_T^N	λ_{max}[a]	λ_{max}[b]	λ_{max}[c]	λ_{max}[a]
Water	78.9	1.000	19.2 (520)	18.8 (531)	19.0 (528)	–
Methanol	33.0	0.762	20.1 (497)	19.8 (505)	19.9 (503)	20.0 (500)
Acetonitrile	36.6	0.460	21.3 (469)	21.6 (462)	21.9 (456)	21.2 (472)
Dichloromethane	8.9	0.309	22.5 (444)	–	22.7 (440)	22.6 (442)
Chloroform	4.8	0.259	22.7 (440)	22.7 (440)	23.0 (434)	22.7 (441)
Cyclohexane	2.0	0.046	25.0 (400)	24.9 (401)	25.5 (392)	25.2 (397)

[a] This work, [b] [1], [c] [8]

Table 2 Maximum intensity position (in 10^3 cm^{-1} and, in parentheses, in nm) of the two components obtained from spectrum decomposition and DAS analysis, for Prodan and Laurdan emission spectra in several solvents, and their respective intensities. Errors in the position are ~3 nm. For the intensities, the errors in the Gaussian decomposition are smaller than 1 %, and for DAS they are ~15 %

Solvents	Gaussian decomposition				DAS			
	λ_1	Int.	λ_2	Int.	λ_1	Int.	λ_2	Int.
Prodan								
Water	19.1 (522)	0.96	17.3 (579)	0.15	–	–	–	–
Methanol	20.1 (496)	0.82	18.9 (528)	0.24	20.0 (500)	0.73	19.6 (510)	0.27
Acetonitrile	21.4 (467)	0.75	20.3 (493)	0.34	21.3 (470)	0.67	20.8 (480)	0.36
Dichloromethane	22.9 (437)	0.75	21.5 (466)	0.46	22.7 (440)	0.82	21.7 (460)	0.19
Chloroform	23.1 (433)	0.66	21.8 (459)	0.54	23.3 (430)	0.40	22.2 (450)	0.67
Cyclohexane	25.5 (393)	0.65	24.1 (415)	0.62	–	–	–	–
Laurdan								
Methanol	20.1 (496)	0.82	19.0 (527)	0.25	20.0 (500)	0.69	19.6 (510)	0.32
Acetonitrile	21.8 (458)	0.84	20.5 (488)	0.24	21.7 (470)	0.75	21.3 (470)	0.25
Dichloromethane	22.9 (436)	0.75	21.5 (465)	0.45	22.7 (440)	0.66	22.2 (450)	0.32
Chloroform	23.1 (434)	0.70	21.7 (461)	0.51	23.2 (430)	0.16	22.2 (450)	0.86
Cyclohexane	25.7 (389)	0.54	24.5 (409)	0.76	–	–	–	–

large shift, in a recent review [47], we discussed the calculation of Prodan and Laurdan electric dipole moments based on Lippert-Mataga equations. Values for the dipole moment of the ground state were found to be 5.5 and 5.9 D for Prodan and Laurdan, respectively. For the excited state the values were 20 and 20.9 D, respectively. This result shows a large increase in the dipole moment after the excitation. These values were compared to those obtained by quantum mechanics
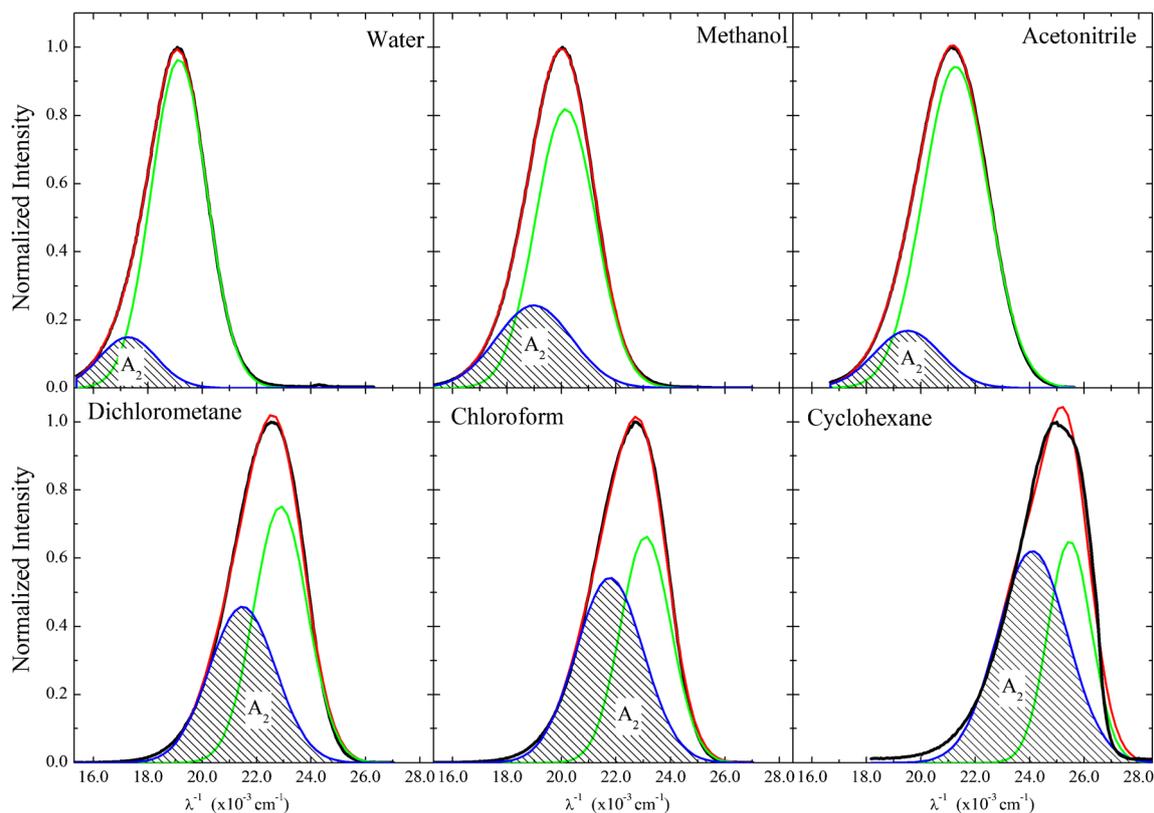
Fig. 3 Gaussian fitting for the fluorescent emission spectra of 4 µM of Prodan in several solvents, at 25 °C. Experimental data in *black*, total fitting in *red*, Gaussian curve for band 1 in *green* and for band 2 in *blue*
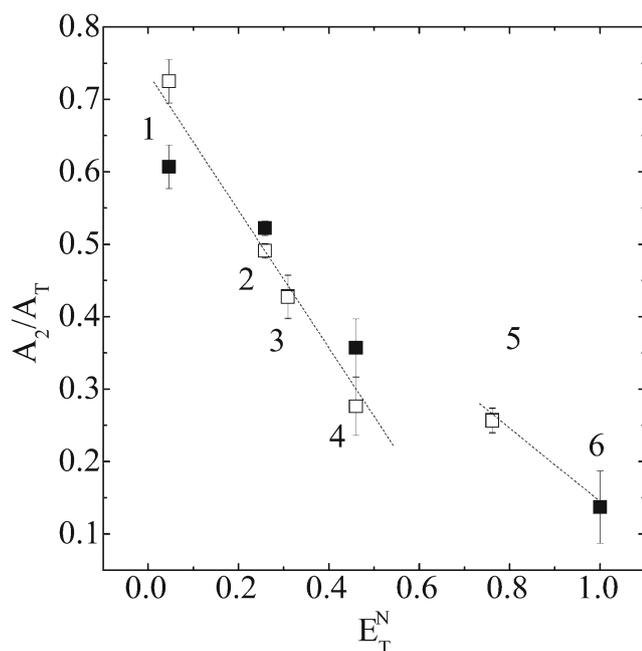
calculations, considering Prodan in vacuum, in a polarizable continuum solvent, and using a hybrid quantum mechanics-molecular mechanics methodology [47].

In all solvents studied here, emission spectra of Prodan and Laurdan were found to be asymmetric (plotted Intensity versus λ^{-1}), which indicates the presence of two or more emission bands. That was extensively discussed for Prodan in polar solvents, such as water and acetonitrile, through the analysis of at least two excited state lifetimes [11, 14, 15, 16, 24, 44, 49]. Two emission bands were also identified for Prodan in cyclohexane and toluene, solvents of low polarity, as well as in water and ethanol [16].
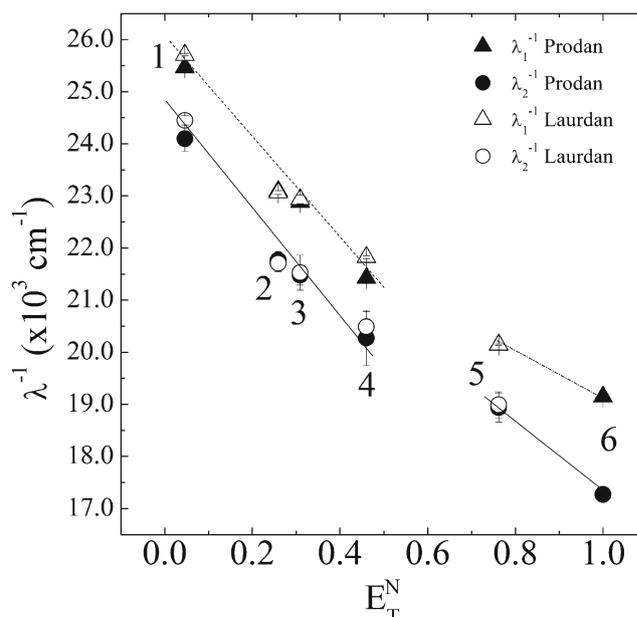
Spectrum decomposition

To characterize the emission bands present in Prodan and Laurdan spectra, spectrum decomposition was performed, as discussed in Materials and Methods. Spectra yielded by the fluorescent probes in all solvents studied here were well fitted with two Gaussians ($\chi^2 \sim 1.00$). The maxima and the intensities at the maxima of these two decomposed bands are presented in Table 2.

Figure 3 shows decompositions of Prodan emission spectra (decompositions of Laurdan spectra are presented in supplementary material), where (2) and (1) indicate the emission bands of the components of lower and higher energies,

Fig. 5 Maximum intensity position of each component obtained from the experimental spectrum decomposition as a function of the Reichardt normalized polarity scale, E_T^N: cyclohexane (*1*), chloroform (*2*), dichloromethane (*3*), acetonitrile (*4*), methanol (*5*) and water (*6*). λ_1^{-1} and λ_2^{-1} correspond to the higher and lower energy bands, respectively (values are presented in Table 2). Two linear behaviors could be identified: one for the solvents that form hydrogen bonds and another one for the remaining solvents

respectively (or higher and lower wavelengths). The areas under the bands, A_2 and A_1, are proportional to the number of molecules emitting from these excited states. Hence, the fraction of molecules decaying from band 2 can be calculated as A_2/A_T, where A_T is the total area under the fluorescent spectrum, $A_T = A_2 + A_1$.

The fraction of molecules emitting from the lower energy excited state (2), for Prodan and Laurdan, as a function of the polarity of the solvent (E_T^N), is shown in Fig. 4. A decrease on A_2/A_T as E_T^N increases is observed, clearly indicating that the probability of Laurdan, or Prodan, emission from the lower excited state energy decreases as the solvent polarity increases. In other words, the area of the emission component

Fig. 4 The area fraction of the lower energy emission component, of Prodan (*black square*) and Laurdan (*white square*), as a function of the Reichardt normalized polarity scale, E_T^N: cyclohexane (*1*), chloroform (*2*), dichloromethane (*3*), acetonitrile (*4*), methanol (*5*) and water (*6*). Two linear behaviors could be identified: one for the solvents that form hydrogen bonds and another one for the remaining solvents

Table 3 Lifetime values obtained for Prodan and Laurdan in several solvents

| Solvent	Prodan		Laurdan	
	τ_1 (ns)	τ_2 (ns)	τ_1 (ns)	τ_2 (ns)
Water	0.6(1)	2.1(2)	–	–
Methanol	2.4(3)	3.7(2)	2.5(3)	3.7(3)
Acetonitrile	3.2(3)	4.0(2)	3.2(2)	4.0(3)
Dichloromethane	3.1(3)	4.5(4)	3.0(3)	3.8(4)
Chloroform	2.3(3)	3.2(4)	1.8(4)	3.0(3)
Cyclohexane	0.2(1)	0.8(1)	0.2(1)	0.8(1)

The values in parentheses are the errors in the first decimal position

at lower energy, A_2, decreases going from cyclohexane to water. This is a very interesting and important result, as the lower emission energy has been attributed to solvent-relaxed S_1 state [1]. However, experimental data presented here show that this band is mostly present in less polar solvents, where a solvent-relaxed excited state of the fluorophore should not be expected.

The emission spectra in Fig. 2 were also measured at two different temperatures (25 and 40 °C), and found to be very similar, apart from their intensities (see Supplementary Material). (As expected, the spectrum intensity decreases as

temperature increases, due to non-radiative electron relaxation [9, 42]). It is well known that the increase in temperature favors solvent relaxation [9]. Therefore, if the dual fluorescence of these probes were associated with a pair of non-relaxed and solvent-relaxed S_1 state, a change in the shape of the fluorescent band with temperature would be expected. But we observed that the shape of the fluorescent spectra is nearly temperature independent. Again, this strongly suggests that fluorescent emission is from two independent excited states, and not from a pair of solvent relaxed and non-relaxed S_1 state.

Fig. 6 Spectra decomposition for Prodan in (**a**) water, (**b**) methanol, (**c**) acetonitrile, (**d**) dichloromethane, (**e**) chloroform and (**f**) cyclohexane. *Black line* —— is the experimental emission spectrum, *green* —— and *blue lines* —— are the Gaussians bands obtained by spectrum decomposition (same shown in Fig. 3). Obtained DAS lines are indicated with square symbols, for the shorter ——■—— and longer ——◼—— lifetime decays
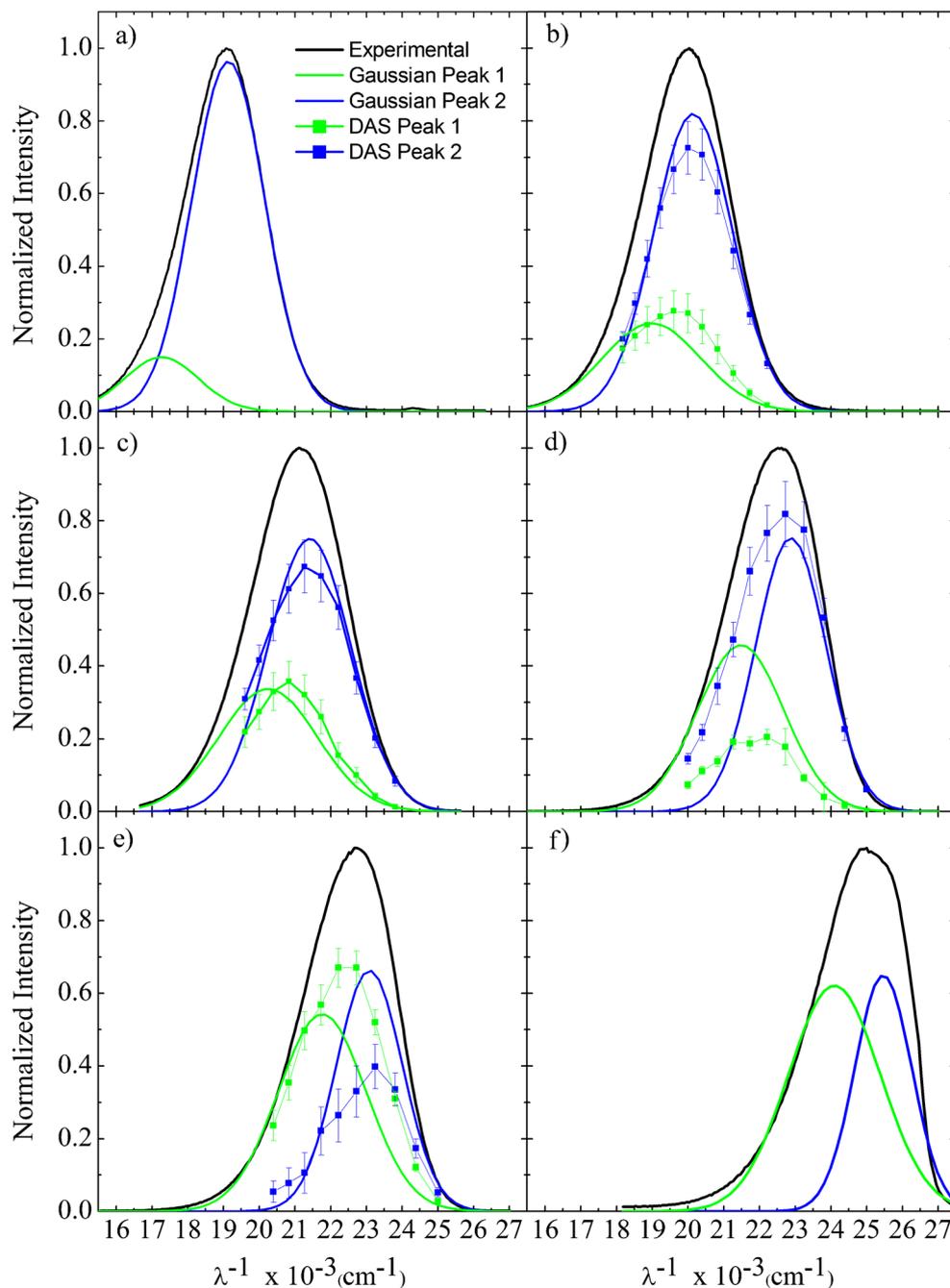
Another parameter to be analyzed is the maximum intensity position of each band as a function of the solvent polarity (Fig. 5). Interestingly, the two bands exhibit a red shift with the increase of solvent polarity, which results in the observed red shift of the whole spectrum, as presented in Fig. 2. This shows that the two excited states are equally affected by changes in solvent polarity. The red shift of a emission band as E_T^N increases is typical of $\pi - \pi*$ transitions [42, 50].

Absorption and excitation spectra of Prodan and Laurdan are very similar (SM), indicating that the emission spectra are independent of excitation wavelength, so the same states are present in the absorption and emission process.

Lifetime analyses

To further attest the presence of two emission bands, lifetime decays for Prodan and Laurdan were measured in all solvents studied. Using multiexponential decay fittings (Eq. 2), it was possible to fit the decays with two exponentials decays, confirming that the fluorescent emission of the fluorophores come from two excited states. Lifetime values are shown in Table 3. Two lifetimes for Prodan in water were measured before [11, 16, 44, 45], and are similar to those presented in Table 3. In cyclohexane, our values (τ=0.2 and 0.8 ns) agree with those published by Rowe et al. (τ=0.2 and 0.8 ns) [16], which are different from the values published by Balter et al. [11], who found one lifetime only (0.18 ns). Laurdan lifetimes were found to be very similar to those of Prodan (Table 3).

In the highly non-polar cyclohexane, both probes present short lifetimes: for Prodan they are even shorter than those found in water. As solvent relaxation is not expected to happen in cyclohexane, probably Prodan and Laurdan intramolecular non-radiative decay processes are favored in this solvent [9, 51]. Though very interesting, this subject is out of the scope of the present work.

From lifetime decays at several wavelengths, it is possible to obtain the decay associated spectra (DAS), as described in Materials and Method, which is the calculated emission spectrum of each excited state. Accordingly, DAS related to each excited state is compared with the Gaussian bands obtained by spectrum decomposition, as discussed in the previous section. In Fig. 6, it is shown that, for all solvents used, the DAS of Prodan are in very good agreement with the presence of the two Gaussian components, strongly supporting the decomposition analysis. The DAS of Laurdan is presented in supplementary material. For water and cyclohexane, the short lifetimes (Table 3) are in the lower limit of the equipment sensitivity, making the DAS analyses unreliable (the lamp-decay deconvolution process can introduce errors in the probe decay analysis [9]), so they are not presented here. In Table 2, the maximum of each band and its intensity calculated with the DAS analysis and the Gaussian decomposition are presented, and shown to be in excellent agreement. It is

interesting to stress that the positions of the two bands are different from the maximum of the spectra showed in Table 1 (see Figs. 3 or 6). So, to analyze Laurdan or Prodan emission spectra it is important to observe both bands behavior, and not only the changes in the emission spectrum. Also, theoretical calculations of the electronic transition energies for the excited states of these probes should be compared with both bands and not only with the general behavior presented by the emission spectrum.

Conclusions

Spectrum decomposition into Gaussians and the use of DAS demonstrate the presence of two emission bands for Prodan and Laurdan, in all solvents studied. Spectrum decomposition is a very good tool for spectrum characterization, much easier to perform than DAS, which needs time resolved fluorescence.

Experimental data presented here show that, in Prodan and Laurdan fluorescence spectra, the fraction of the lower energy emission band is higher in cyclohexane than in water, decreasing as the polarity of the medium increases. Therefore, it seems highly unlikely that the corresponding excited state would be a solvent-relaxed one, as has been considered (see, for instance, [17–19, 24, 30]). Also, in all solvents studied, the shape of Prodan and Laurdan emission spectrum does not change with temperature, strongly suggesting that the two emission bands are not related to a pair of solvent non-relaxed and solvent-relaxed S_1 state. A comparison between the absorption and the excitation spectra shows that they are very similar (SM) showing that the quantum yields and emission spectra are independent of excitation wavelength, so the same states are present in the absorption and emission process.

Therefore, we propose here a new interpretation for the two emission bands of Prodan and Laurdan in homogeneous solvents: they would be related to the emission of two independent excited states, S_1 and S_2. Similar anomalous behavior was found for a few aromatic molecules, and different explanations have been suggested [34–41]. In a recent publication [46] (see Table S1), theoretical calculations for the electronic absorption transition energies of Prodan in water showed that there are three transitions in the region of 340–360 nm, composing a broad absorption band. Therefore, this preliminary theoretical result supports the possibility of having two different excited states S_1 and S_2 populated in the excitation process of these probes in solution. The theoretical reason for Prodan and Laurdan atypical behavior is presently under investigation, through molecular modeling together with quantum mechanics calculations.

Conflict of Interest Authors C. C. Vequi-Suplicy, K. Coutinho and M. T. Lamy declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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