Influence of DPPC Liposome Concentration on the Fluorescence Properties of PRODAN and LAURDAN

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Fluorescence spectral features of PRODAN and LAURDAN in phospholipid vesicles of different phase states were investigated. The results indicate that in the liquid crystalline phase the dominant emission results from the charge transfer (CT) excited state, whereas in the gel state of the membrane the emission from the locally excited (LE) state dominates. The fluorescence time studies point out that there are two radiation modes, one starting from only vibrationally relaxed excited states $S_1(LE)_v$ ($(S_1(CT)_v$) and the other from a totally thermally equilibrated state $S_1(LE)_{EQ}$ ($S_1(CT)_{EQ}$). In accordance with the obtained decay time dependencies, the fluorescence emission from total non-equilibrated excited states consists of a dominant or minor radiation process in the LE or CT band emission.

Key words: PRODAN, LAURDAN; Locally Excited and Charge Transfer States; Fluorescence Decay Time.

1. Introduction

PRODAN (6-propionyl-2-dimethylaminonaphtalene) and LAURDAN (6-dodecanoyl-2-dimethylaminonaphtalene) have been widely used to study the structure and dynamics of biological systems, e. g. membrane constituents, surfaces, large biological molecules, cells, etc. [1 – 5]. Particular attention has been paid to these organic molecules since they are capable of simultaneously creating locally excited (LE) and charge (CT) transfer excited states [5 – 9]. Therefore their luminescence properties are very sensitive to changes of the surrounding microenvironment, its heterogeneity, polarity, viscosity and ability to raise specific intermolecular interactions between the solute and solvent molecules [8 – 11].

It has been shown in a series of papers [8 – 12], that LAURDAN in glycerol solution is an inhomogeneous spectroscopic medium in which multi-channel luminescence phenomena take place. The spectroscopic inhomogenity is caused by a distribution of space conformational forms possessing different twisting angles φ between the mutual orientation planes of the dimethyl group and the naphthalene moiety (see Fig. 1), and by accompanying intra- and intermolecular charge transfer phenomena. At higher concentrations, LAURDAN, due to the aliphatic tail of -(CH_2) groups, forms micelles, a particular type of aggregates, in which the twisting rotations of the –N(CH_3)$_2$ group are damped [9]. The number of micelle aggregates and types of conformational forms of LAURDAN in glycerol solutions depends on temperature, and for a given temperature an equilibrium distribution is achieved [8, 9].

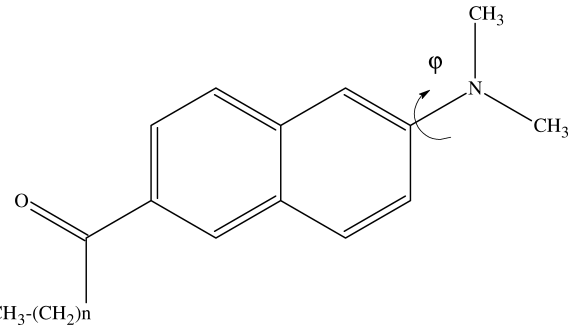

Fig. 1. Chemical structure of PRODAN ($n = 1$) and LAURDAN ($n = 10$).

In biological membranes, which are complex assemblies of lipids and proteins, the fluorescence probes are located in defined positions of the bilayer depth. This location depends on the phase state of the bilayer,

which can be changed by variation of the temperature or pressure [12, 13]. In practical studies, the biological membranes are often replaced by model systems, such as phospholipid vesicles forming single or multiple bilayers, which are dispersed in aqueous medium. In such solvent mixtures, the radiation of fluorescent probe molecules often differs from that in neat solvents [15, 16]. Thus the absorption and emission spectra result from ensembles in different environments. Luminescence studies allow to determine quantitatively the phase state of such phospholipid solutions [4, 12].

In the literature concerning the photoluminescence properties of PRODAN and LAURDAN in neat solvents and phospholipid vesicles, the distinguishable fluorescence states are defined differently, e.g., as nonrelaxed (blue fluorescence) and relaxed (red fluorescence) singlet states [14, 22]. Hereafter the above singlet excited states will be named locally excited S_1(LE) and charge transfer S_1(CT) states in accordance with [6–9, 19–21].

This paper is devoted to the characterization of the fluorescence emission properties of PRODAN and LAURDAN as functions of the phospholipid concentration and temperature of the solution. Different lengths of $(CH_2)_n$ residues in the molecules under study allow the determination of the influence of solvation processes and bilayer phase states on the fluorescence properties. The performed study includes steady state and time resolved spectroscopic measurements of both probe molecules in phospholipid solutions at 25 °C and 50 °C.

2. Materials and Methods

2.1. Chemicals

PRODAN and LAURDAN were purchased from Molecular Probes, Eugene, OR. High purity DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) was purchased from Lipoid KG (Ludwigshafen, Germany) and used without further purification. Ethanol and DMF (N,N-dimethylformamide) were of spectroscopic grade and were provided by Merck (Darmstadt, Germany).

2.2. Preparation of Liposomes

DPPC dissolved in ethanol was evaporated to deposit a thin lipid film on the wall of a glass tube. The final traces of residual solvent were removed after overnight storage under vacuum (Vacutherm, Heraeus Instr., Hannover, Germany) at 42 °C. The lipid film was resuspended in an appropriate amount of Tris buffer (0.1 M, pH 7.4), and vigorously vortexed at a temperature above the phase transition, giving a lipid concentration of approximately $2\,\mathrm{mg\cdot ml^{-1}}$. For the preparation of small unilamellar vesicles (SUV), the resulting multilamellar vesicle (MLV) dispersion was sonicated with a Bandelin sonoplus HD70 (Bandelin Electronics, Berlin, Germany) for 15 min at maximal power (cycle 30%) under nitrogen and transferred to a thermostatted membrane extruder system (Lipex Biomembranes Inc., Vancouver, Canada), which allowed the extrusion of unilamellar vesicles of 25 nm final diameter. The final lipid concentration of the SUV suspension was determined for each preparation [17] and stored under nitrogen in darkness at 4 °C to avoid lipid peroxidation. All liposomal preparations were used within 2 weeks.

2.3. Fluorescence Measurements

Fluorescence measurements of LAURDAN and PRODAN in SUV prepared from DPPC were carried out in a computer-controlled Perkin Elmer LS-50 spectrofluorimeter equipped with a thermostatted cuvette (Julabo Labortechnik, Seelbach, Germany). PRODAN and LAURDAN were added from a 1 mM stock solution in DMF. All samples were incubated for 1 h before the measurements. The excitation wavelength was 380 nm. A 5 nm slit width was set for both excitation and emission.

The fluorescence lifetime measurements were performed by means of the Time-Correlated Single Photon Counting Technique (TCSPC). We used a violet ($\lambda = 403$ nm) pulsed semiconductor laser LDH-C 400/PDL-800B (PicoQuant GmbH, Germany), emitting pulses of approximately 70 ps duration (FWHM). The average laser output power was about 1 mW. The laser repetition could be varied up to 40 MHz. To select the correct polarization plane for light illuminating the samples we decided to employ a Babinet-Soleil compensator (RKQ10, Bernhard Halle Nachfolger GmbH, Germany) rather than a "conventional" input polarizer. In this way we could easily rotate the polarization plane of the laser beam, keeping at the same time the highest available intensity of excitation. The fluorescence light emitted by the samples was directed to a monochromator slit by a carefully selected quartz lens. We applied a SpectraPro-300i monochromator (Acton Research Corporation, USA), equipped with three diffraction gratings (300, 600 and

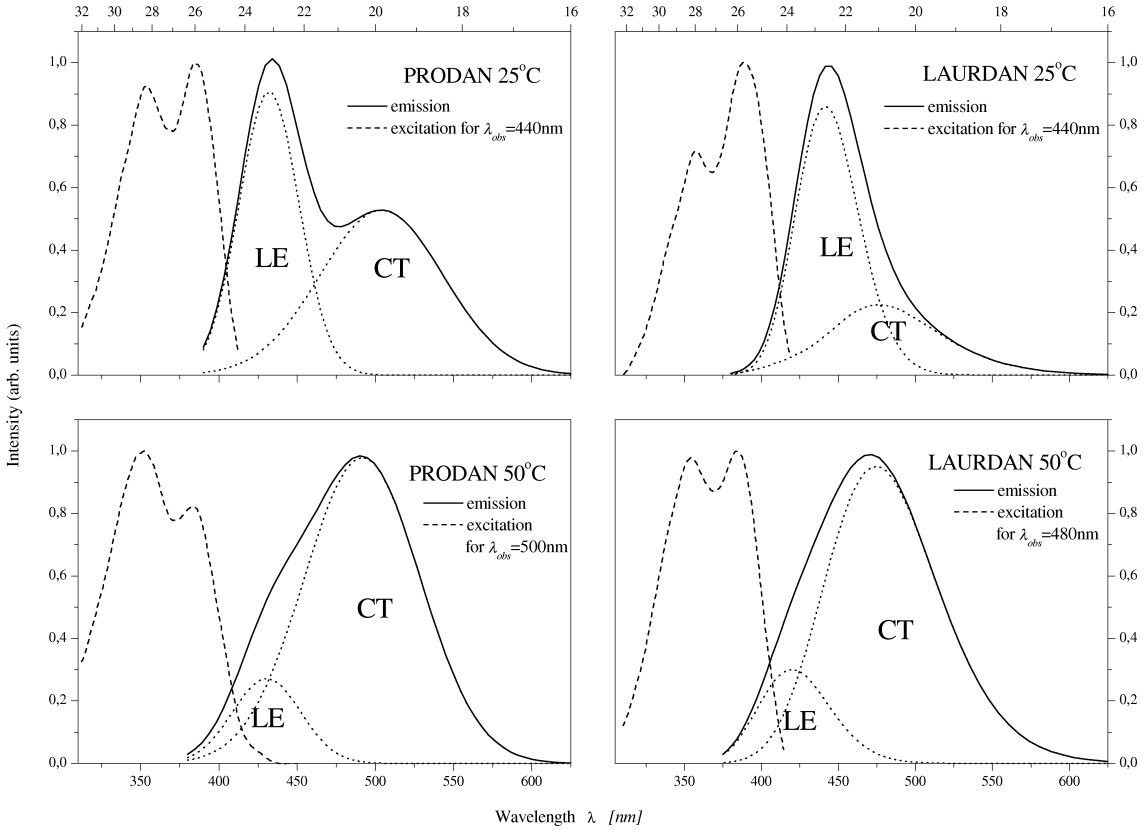

Fig. 2. PRODAN and LAURDAN excitation and emission spectra obtained in small unilamellar phospholipid vesicles composed of gel (25 °C) and liquid crystalline (50°) phases.

1200 grooves per mm; blaze 500 nm). The monochromator entrance and exit slits were adjustable from 10 μm to 3 mm (2.7 nm/mm spectral resolution for 1200 g/mm grating). The selected observation wavelengths were 440 nm and 500 nm. The measurements were performed at 25° and 50 °C. In order to eliminate the influence of anisotropy on the decay curves, an analyzer set at the magic angle was positioned between the monochromator and the sample. The fluorescence light was registered by a H5783P photomultiplier (Hamamatsu Photonics K.K., Japan). Next, the photomultiplier signal was captured by a TimeHarp 100 PC-board for TCSPC (4096 channels; 36 ps resolution) (PicoQuant GmbH, Germany). Finally, the fluorescence decay times were calculated (using the Marquardt algorithm) by means of "FluoFit" multiexponential fluorescence decay fitting software (PicoQuant GmbH, Germany). The quality of the fit was assessed over the entire decay and tested with a plot of weighted residuals and the reduced χ^2 value.

3. Results and Discussion

3.1. Excitation and Emission Spectra

Figure 2 shows excitation and emission spectra of PRODAN and LAURDAN in small unilamellar vesicles (SUV) composed of DPPC (at molar ratio 1/500) at 25 and 50 °C. Analyzing the spectra of both molecules in a phospholipid bilayer, it follows that:

• The fluorescence excitation spectra (in the spectral region of the long wavelength absorption band) have two peaks. At room temperature (gel phase state) the long wavelength maximum ($\lambda \cong 380$ nm) is higher than that at $\lambda \cong 350$ nm. At 50 °C (liquid crystalline state) the two intensity maxima show the reverse dependence. They are slightly shifted to shorter wavelengths (by about 10 nm). The fluorescence excitation spectra at 50 °C were measured by detecting radiation at $\lambda = 500$ nm emitted by the ensemble of molecules possessing twisted –N(CH_3)$_2$ functional groups.

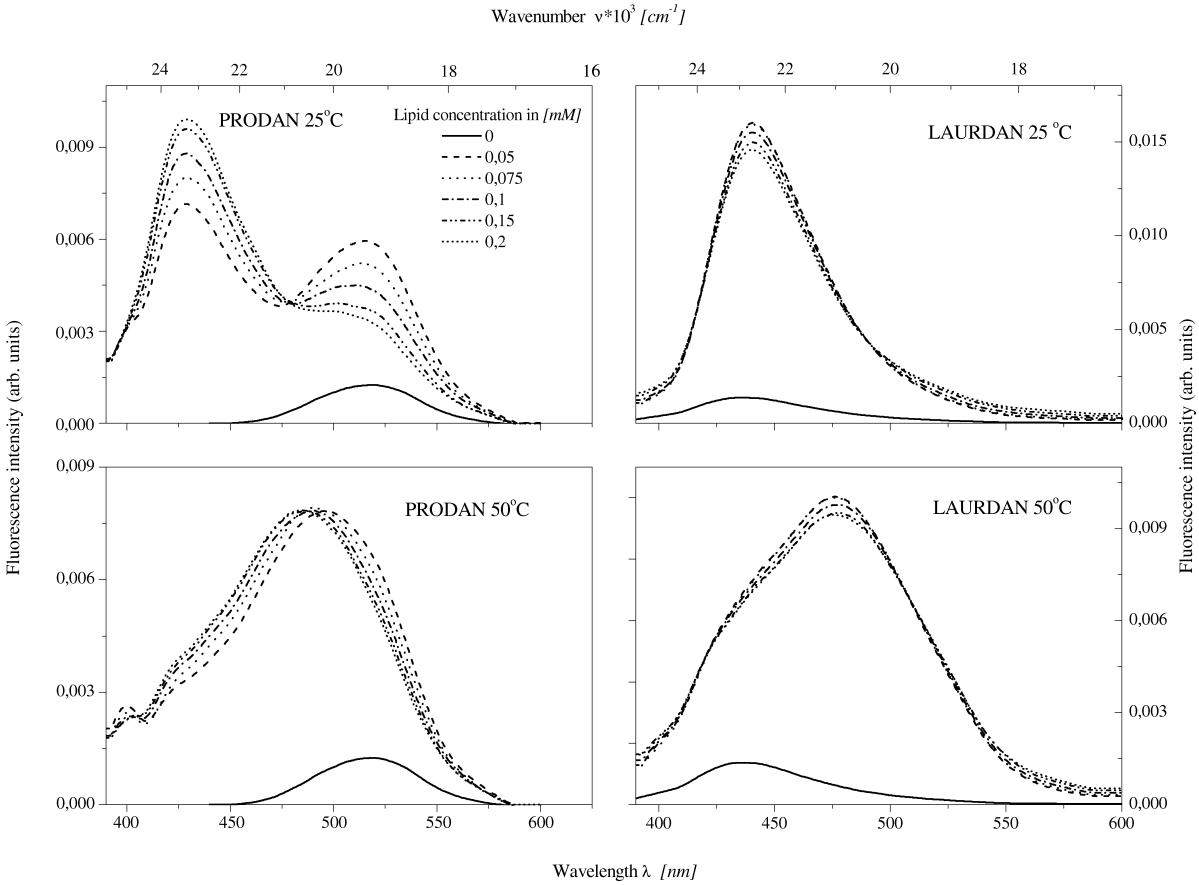

Fig. 3. PRODAN and LAURDAN emission spectra in DPPC vesicles at different phospholipid concentrations.

• The fluorescence spectra of PRODAN and LAURDAN in a DPPC bilayer in the gel and liquid crystalline phase consist of two bands (see Fig. 2). The separated bands, named, respectively, locally excited fluorescence band, $I_{(LE)}$, and charge transfer fluorescence band, $I_{(CT)}$, are emitted from ensembles of molecules with the plane of the dimethylamino group parallel and perpendicular to that of the naphthalene moiety [8, 9]. At room temperature the fluorescence intensity of the plane molecule is two to three times bigger than that possessing twisted $-N(CH_3)_2$ functional groups. This finding suggests that in the gel phase of the phospholipid bilayer, torsions of the $-N(CH_3)_2$ are more damped than in the liquid crystalline phase. The intensity ratio $I_{(LE)}/I_{(CT)}$, determined for the PRODAN and LAURDAN spectrum at 25 °C equals 1,8 and 3,1, respectively. This suggests that the LAURDAN molecules are tightly and deeper located in the bilayer than the PRODAN molecules.

The above fluorescence spectra of both molecules were obtained at constant dye concentration and different lipid concentrations in the solutions. In such solutions the number of dye molecules settled in the bilayer decreases with increasing lipid concentration. As a consequence, in such solutions the bulk dye concentration changes, whereas the nearest environment of the fluorescent probe does not change. Figure 3 shows families of fluorescence spectra of both molecules, obtained for five different concentrations of the lipid. The concentration of the lipid in the Tris buffer changes 4 times, e. g., from to 0,05 mM to 0,2 mM.

As can be seen on Fig. 3 the fluorescence spectra of PRODAN in aqueous Tris solution with dissolved DPPC liposomes at different concentrations show abrupt spectral changes at 25 °C, whereas in the liquid phase (at 50 °C) they show a small shift of the maximum intensity only, but their shape does not change. Analyzing the spectra more closely, we can

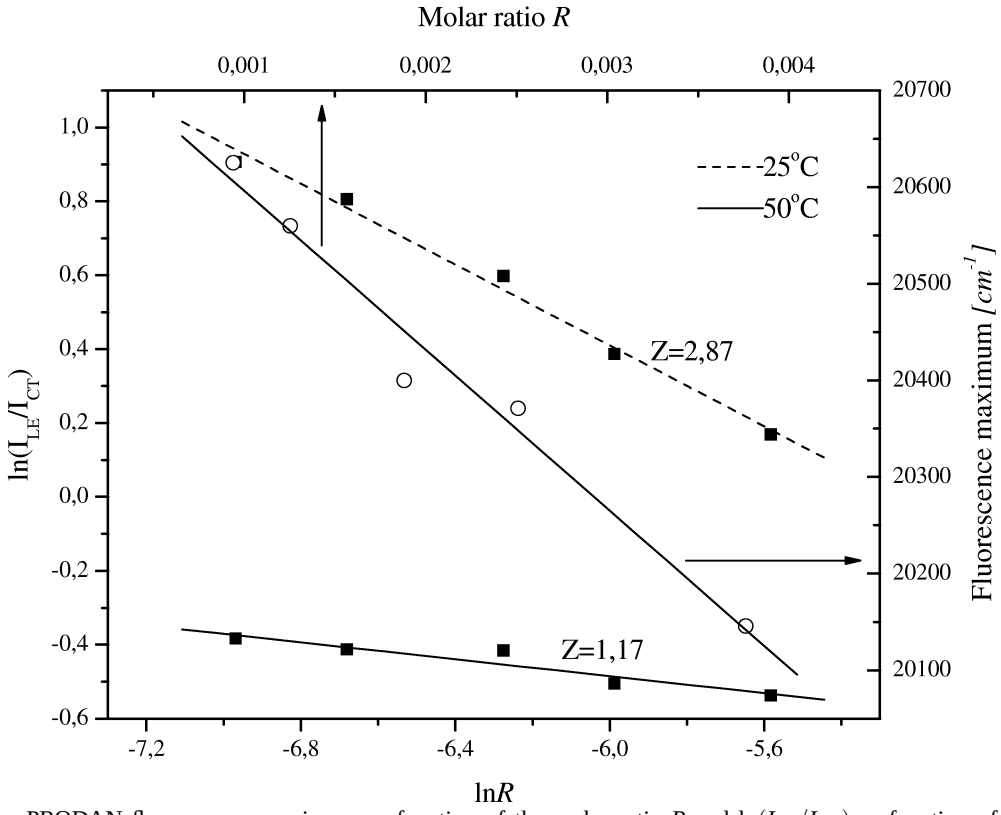

Fig. 4. The PRODAN fluorescence maximum as function of the molar ratio R and $\ln(I_{\mathrm{LE}}/I_{\mathrm{CT}})$ as function of $\ln R$ (R = fluorophor molecules/lipid molecules).

state that in the liquid phase the dominate emission results from the TICT state molecules, whereas in the gel state of the membrane the emission from the locally excited state S_1(LE) dominates. This is true for both molecules under study.

Figure 4 gives a graphical presentation of the noted changes, i. e., the logarithm of the fluorescence intensity ratio $\ln(I_{\mathrm{LE}}/I_{\mathrm{CT}})$, and the shift of the CT band maximum, obtained as a function of $\ln R$, R being the molar ratio of PRODAN molecules to DPPC molecules at 25 °C and 50 °C. It follows that the $\ln(I_{\mathrm{LE}}/I_{\mathrm{CT}})$ increases with increasing molar ratio of the gel and liquid crystalline phase states. The $\ln(I_{\mathrm{LE}}/I_{\mathrm{CT}})$ value changes less at higher temperature. This suggests that in an aqueous solution of dissolved DPPC liposomes in the liquid crystalline phase, the intensity of the TICT fluorescence band decreases only slightly with increasing molar ratio, contrary to the gel phase. In Tris solution, the fluorescence emission of PRODAN and LAURDAN appears from different excited states, i. e., PRODAN emits from S_1(CT) and LAURDAN from the S_1(LE). This different behavior of the probes is a consequence of their hydrophobic differences, caused by different lengths of the aliphatic tail of $-(CH_2)_n$ groups. Also, the fluorescence intensity (fluorescence quantum yield) in Tris buffer solution is smaller by one order of magnitude than in DPPC liposomes. This can be understood by taking into account that protic solvents, forming hydrogen bonded complexes, quench the fluorescence emission. Analyzing the fluorescence spectra of PRODAN and LAURDAN obtained for solutions with different lipid concentration at 50 °C (see Fig. 2), we state that the shift of the fluorescence maximum intensity of the CT band of PRODAN with increasing lipid concentration is well described by a linear function: $\tilde{\nu}_{\mathrm{F}} = \tilde{\nu}_0 + A[L]$, where $\tilde{\nu}_0$ is the wave number of the intensity maximum of the CT band in the Tris buffer, and $[L]$ is the lipid concentration. The noted dependence can be used to determine the lipid concentration and polarity of the probe microenvironment.

These findings, which are graphically presented in Fig. 4, suggest that PRODAN and LAURDAN in

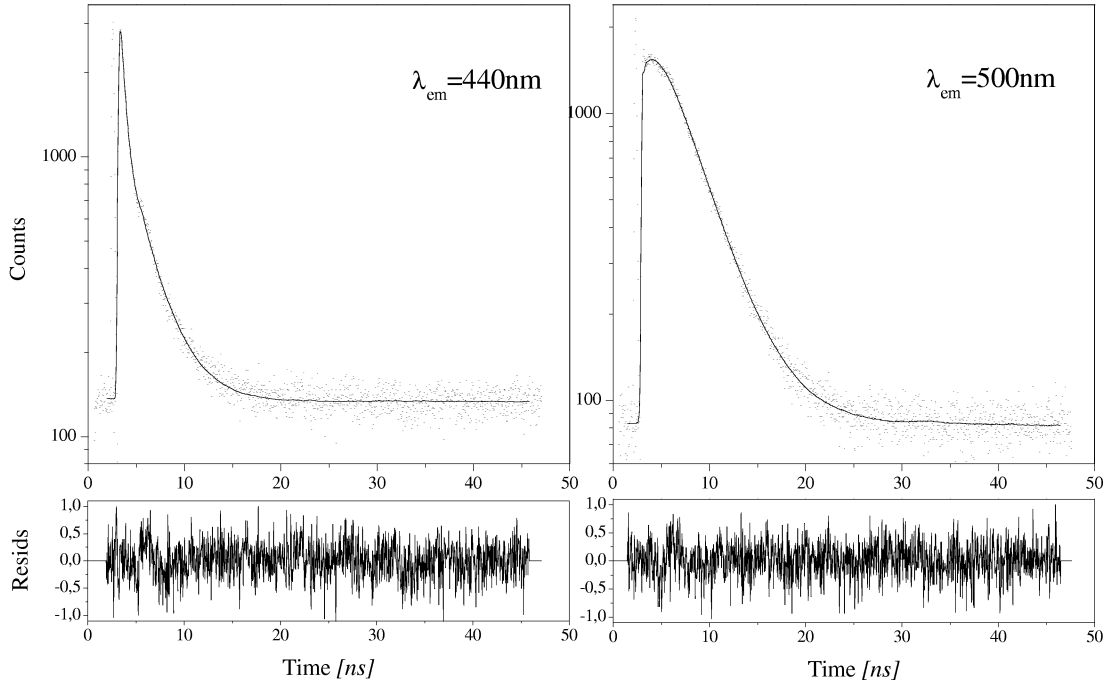

Fig. 5. Fluorescence decay curves of the LE ($\lambda_{em} = 440$ nm) and CT ($\lambda_{em} = 500$ nm) bands of the PRODAN luminescence spectrum at 50 °C.

DPPC liposome consists of a spectroscopic inhomogeneous medium, for which the CT fluorescence band shift and the intensity ratio I_{LE}/I_{CT} are linear functions of the lipid concentration $[L]$ in the Tris buffer. The fluorophore solution is a dielectric mixture of solvents. The composition in the near vicinity of the dipolar solute molecule, according to Suppan and co-workers [23], is described by

$$Y = Xe^{-Z}, \tag{1}$$

where Y and X in the single-shell approximations are the local and bulk mole fractions, and Z is the index of preferential solvation

$$Z = \frac{1}{4\pi\varepsilon_0}\frac{C\mu^2 M\Delta f(\varepsilon_{np})}{RT\delta \cdot r_{s-s}^6}. \tag{2}$$

In (2) μ is the solute dipole moment, M the mean molecular weight of the solvents used, $\Delta f(\varepsilon_{np})$ the difference of its Onsager polarity functions, δ the mean density of the two components, and r_{s-s} the mean separation between the solute and solvent molecules.

From fitting the experimental points $\ln(I_{LE}/I_{CT})$ versus $\ln R$ (see Fig. 4), we get Z equal to 2,87 and 1,17 for spectroscopic data determined at $T = 25$ °C and 50 °C, respectively. This result agrees well with the expected ratio Z_{25}/Z_{50} calculated using (2). It confirms our supposition that the noted changes in the fluorescence spectra of PRODAN and LAURDAN caused by solvent heterogeneity can be used to determine the lipid concentration and its polarity.

3.2. Fluorescence Decay Times

The depopulation kinetics of the excited states, e. g., S_1(LE) and S_1(CT), have been measured detecting the fluorescence decay curve at $\lambda_{em}^{CT} = 500$ nm and $\lambda_{em}^{LE} = 440$ nm. Respective decays are shown in Figure 5. Tables 1 – 3 contain the results of the fluorescence decay curve analysis. As has been mentioned earlier, the experimental fluorescence decay curves in all cases could not be fitted by a single exponential function. The fitting quality is satisfactorily confirmed by small values of weighted residuals and χ^2 around one. The curves detected at the maximum intensity of the LE or CT bands are deconvoluted by the sum of double and treble exponential functions. Deconvolution of the fluorescence decay data obtained separately for the two bands results in components in the sub- and nanosecond time range with different participation.

Table 1. Fluorescence decay times of Prodan in DPPC at 25 °C.

Lipid Concentration [mM]		$\lambda_{em} = 440$ nm τ_i [ns]	A_i [%]	χ^2	$\lambda_{em} = 500$ nm τ_i [ns]	A_i [%]	χ^2
	τ_1	0,85	2889 (33%)		0,46	3933 (28%)	
0,05	τ_2	6,40	773 (67%)	1,067	6,10	235 (22%)	1,094
	τ_3				1,80	1772 (50%)	
	τ_1	0,67	5681 (32%)		0,45	4200 (27%)	
0,075	τ_2	6,26	1280 (68%)	1,076	6,84	241 (24%)	1,171
	τ_3				1,98	1710 (49%)	
	τ_1	0,36	20524 (36%)		0,36	3985 (22%)	
0,1	τ_2	6,44	2031 (64%)	0,99	6,37	328 (34%)	1,149
	τ_3				1,77	1601 (44%)	
	τ_1	0,29	35686 (34%)		0,24	4159 (17%)	
0,15	τ_2	6,32	3129 (66%)	1,036	5,57	476 (47%)	1,093
	τ_3				1,45	1400 (36%)	
	τ_1	0,69	7097 (30%)		0,16	4315 (18%)	
0,2	τ_2	6,52	1752 (70%)	1,018	5,14	382 (53%)	1,117
	τ_3				1,30	838 (29%)	

$i = 1,2,3$ for the decay function $I(t) = \sum_{i=1}^{3} A_i \exp(-t/\tau_i)$.

Table 2. Fluorescence decay times of Prodan in DPPC at 50 °C.

Lipid Concentration [mM]		$\lambda_{em} = 440$ nm τ_i [ns]	A_i [%]	χ^2	$\lambda_{em} = 500$ nm τ_i [ns]	A_i [%]	χ^2
	τ_1				0,50	40548 (39%)	
0[a]	τ_2	–	–	–	1,83	15133 (54%)	1,18
	τ_3				0,08	−46089 (7%)	
	τ_1	0,84	2815 (70%)		0,37	6323 (36%)	
0,05	τ_2	4,86	201 (30%)	1,072	2,70	1257 (54%)	1,128
	τ_3				0,10	−5308 (6%)	
0,075	τ_1	0,59	10421 (71%)		0,39	5700 (32%)	
	τ_2	4,53	550 (29%)	0,99	2,85	1512 (62%)	1,218
	τ_3				0,07	−5308 (6%)	
0,1	τ_1	0,65	7954 (70%)		0,41	4155 (35%)	
	τ_2	4,53	483 (66%)	1,058	2,62	1143 (60%)	1,181
	τ_3				0,06	−3872 (5%)	
0,15	τ_1	0,38	29233 (67%)		0,40	4652 (32%)	
	τ_2	3,80	14182 (33%)	1,061	3,70	1021 (64,5%)	1,635
	τ_3				0,07	−3000 (33,5%)	

[a] Measured at 25 °C. $i = 1,2,3$ for the decay function $I(t) = \sum_{i=1}^{n} A_i \exp(-t/\tau_i)$.

Analysing the τ data (see Tables 1 and 2) obtained by measuring the fluorescence decay of PRODAN at $\lambda = 440$ nm (maximum intensity of the LE fluorescence band) for different lipid concentrations and temperatures, we can see that the experimental decay curves are well fitted by a bi-exponential function. The fast decaying fluorescence component of PRODAN, with $\tau < 1$ ns, participates by 1/3 in the gel and 2/3 in the liquid crystalline phase in the total radiation of the LE. The long lifetime decay component of the LE fluorescence band possesses different τ values in the gel (6,39 ns) and liquid crystalline (4,44 ns) phases. The decrease of the fluorescence decay time by about 20% in the liquid crystalline phase is coupled with a conspicuous increase in its participation (about 70%)

Table 3. Fluorescence decay times of LAURDAN in DPPC and Tris buffer.

	Temp.	λ_{em} [nm]	τ_1 [ns]	A_1	τ_2 [ns]	A_2	χ^2
DPPC	25 °C	450	0,75	51342 (10%)	6,95	47441 (90%)	1,307
DPPC	50 °C	450	0,51	20620 (53%)	3,03	3013 (47%)	1,067
		520	1,45	−1978 (19%)	3,54	3396 (80%)	1,025
Tris buffer	25 °C	460	0,76	20289 (74%)	4,60	1143 (26%)	1,167

in the total LE emission in the gel phase. As has been shown in [8], in inhomogeneous solution, PRODAN and LAURDAN in the ground state S_0 and first singlet excited state S_1 form space conformers possessing two distinct distributions of the electron density in the molecule. The two border configurations of these molecules are the LE and CT conformers. This creates strong inhomogenity in the solution because the probe molecules possess different environments, e.g. aqueous, gel and liquid crystalline phase states. In the different micro domains the luminescent molecules have different fluorescence spectra and decay times [18].

On the basis of earlier measurements [8] and data collected in Tables 1 and 2, we notice that the fast component ($\tau < 1$ ns) indicates the radiation of molecules possessing in the S_0 and S_1(LE) states parallel donor-acceptor planes, whereas the assemble of molecules with perpendicular planes decays with average time constants of about 6,40 ns and 4,0 ns if PRODAN molecules are in a phospholipid bilayer being in the gel or liquid crystalline state. The decay process of the CT band is described by three components. Determined τ values for the CT band indicate a conspicuous dependence on the lipid concentration and its phase state. They point out that in the liquid crystalline phase of a DPPC bilayer, PRODAN molecules form three assembles of molecules possessing different space configurations caused by their localization in the membrane and nearest environment. An accurate analysis of the decay data detected at a maximum of the CT band ($\lambda = 500$ nm) indicates that:

- PRODAN fluorescence decay curves for the CT band in a neat Tris buffer and in its DPPC lipid solution at 25 and 50 °C can be decomposed into three exponential functions, possessing distinctly different τ values.
- The decay component with τ values altering from 0,50 to 0,35 ns results from an ensemble of space conformers possessing the TICT structure in the ground and excited states. In the S_1(CT) state this ensemble of

molecules is only vibrationally equilibrated [8, 16]. In the gel state (25 °C) of the membrane about 1/4 of the dye molecules possess this structure. Their number decreases with increasing DPPC concentration (from 28 to 17%). In the liquid crystalline state (50 °C) the number of such conformers increases, varying from 28 to 35%. By increasing the DPPC concentration, the participation of its fluorescence emission decreases in the same manner as at 25 °C.

• The components with the longest decay time (about 6 ns for the gel and 4 ns for the liquid crystalline phase) are responsible for the emission of the molecules, completely thermally and vibrationally equilibrated in the $S_1(CT)_{EQ}$ state.

• The fluorescence decay component possessing τ_3 values ranging from 1,83 to 1,30 ns (see Table 1) results from the emission of PRODAN surrounded by water molecules. Such decay components appear in the emission of neat Tris buffer and its DPPC solution at 25 °C. The τ_3 value decreases with increasing DPPC concentration. We suppose that it is a result of self-quenching being a consequence of the presence of water molecules in the phospholipd bilayer. Their amount decreases by simultaneous increase of the PRODAN concentration in the DPPC bilayer. The changes of the pre-exponential coefficient A_3 and the fluorescence spectrum give additional arguments for the existence of such a process.

• In the liquid crystalline phase of the DPPC bilayer (50°), the PRODAN excited molecules are in a fully equilibrated state, e.g., vibrational, configurational, and thermal, $S_1(CT)_{EQ}$. As follows from Table 2, A_2 changes with increase of lipid concentration, and it suggests that about 54 – 80% of the molecules take part in this emission. Only about 5% of the molecules undergo the TICT phenomena from the $S_1(LE)$ to the $S_1(CT)$ state (see Table 2; A_3 is negative). Our fluorescence decay data show that in the gel phase (25 °C) such a dynamic process does not exist, since the PRODAN molecules incorporated in the lipid bilayer possess different space conformational structures. Its decay describes a component with $\tau_1 = 0,4$ ns and $A_1 = 32\%$, resulting from the emission of vibrationally (but not thermally) equilibrated space conformers, which are in part created by the radiationless transition $S_1(LE) \rightarrow S_1(CT)_{nEQ}$. This fluorescence decay component possesses a corresponding τ_1 decay component in the emission, detected at $\lambda_{em} = 440$ nm starting from the $S_1(LE)_{nEQ}$ state. The participation of that component to the total radiation is about 70%.

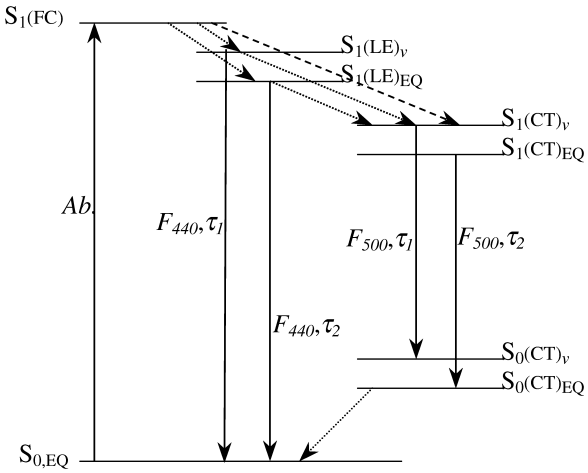

Fig. 6. Electronic energy diagram of PRODAN and LAURDAN in inhomogeneous media with the indicated radiation modes. The dashed line corresponds to radiationless transition in polar solution (Tris buffer) and the dotted lines correspond to radiationless transition in membrane environment (DPPC vesicles).

Table 3 gives the fluorescence decay data of LAURDAN determined for Tris buffer and DPPC bilayer at 25 and 50 °C. In the used solutions, the experimental decay data can be fitted using biexponential functions. Decay times detected at the maximum of the LE emission band, $\lambda_{LE} = 450$ nm, show two emission modes joined from the $S_1(LE)_{nEQ}$ and $S_1(LE)_{EQ}$ states. The participation coefficient (A) and the decay time (τ) of the named modes depend on the phase state (temperature) of the DPPC lipid bilayer. In the gel phase, most of the LE fluorescence ($\sim 90\%$) results from the vibrationally and configurationally relaxed singlet state $S_1(LE)_{EQ}$. In the liquid crystalline phase of the DPPC membrane, the emission from both states is equally probable; it causes obviously a shorting of the decay time τ_1. The rules for these radiationless transitions are reflected in the obtained τ_1, τ_2 and A_1, A_2 data determined for the CT fluorescence band ($\lambda_{CT} = 520$ nm). The fluorescence decay data for LAURDAN in Tris buffer as well as in DPPC bilayer (two exponential decays) coincide with results of other authors [5, 8, 14, 22]. Figure 6 shows the schematic illustration of the electronic energy scheme which explains the appearance of the above noted radiation modes. Directly after the absorption, the excited molecules in the Franck-Condon S_1 state are not in equilibrium with their environment; they undergo a two step equilibration process: the fast one causing a vibrational relax-

ation to the $v = 0$ level, and the slower one bonded with reorientation relaxations of the solvent molecules in solvates (luminescence centers). Both processes populate the S_1(LE) state without changing the conformer form of the excited molecules. As a result of the above population mechanisms in the radiation processes from the S_1(LE) as well as from the S_1(CT) states, two radiation modes are observed. One starting from only the vibrationally relaxed excited state $S_1(LE)_v$ ($S_1(CT)_v$), and the other from a totally thermally equilibrated state $S_1(LE)_{EQ}$ ($S_1(CT)_{EQ}$). Additionally, in the long wavelength CT fluorescence band a third component with a τ_3 decay time and negative preexponential coefficient participates in the depopulating process of the S_1(LE) state [5, 22]. Its influence in the population of S_1(CT) state is noted for both molecules under study at 50 °C only and has a minor meaning for PRODAN solution.

4. Summary

The spectroscopic photophysical data determined in the performed studies of PRODAN and LAURDAN in a DPPC bilayer for different lipid concentrations and phase states can be explained using an extended energy scheme as shown in Figure 6. As has been mentioned earlier (see also Fig. 2) the fluorescence spectrum of each compound consists of two bands: blue (LE) and green (CT). The fluorescence decay of separated bands possesses two or three decay components connected with the radiation of molecules in an inhomogeneous solvent. The fluorescence time studies point also to the fact that in the phospholipid bilayer both molecules possess different space conformers. Their existence is related to the twisting motions of the dimethyl amino group around the C-N bond in PRODAN and LAURDAN, occurring differently in phospholipid vesicles and bulk solution water. The transformation of the plane LE molecule to the perpendicular CT molecule is accompanied by an internal energy change described by the energy distribution functions $\rho_g(\varphi)$ and $\rho_{ex}(\varphi)$. It is easy to show, see Fig. 4, that the space conformer distribution function $\rho(\varphi)$ is a Boltzman type function, which depends on temperature, but is independent of the phospholipid concentration.

The thermal equilibrium between the LE and CT components depends on the relaxation time τ_{rel}. The above process, connected with the heterogeneity of the solution and characterized by the τ_{rel} value, has a static and dynamic character. The value of τ_{rel} depends on the physical properties of the environment, mainly viscosity, polarity and temperature. In accordance with the above model, the fluorescence quanta are emitted from a vibrationally and thermally (configurationally) equilibrated state. The pre-exponential coefficients are a measure of those radiationless dynamic relaxation processes. Their efficiency depends on the relation between τ_{rel} and τ_{fl}. In the gel phase, the molecules are tightly connected to the membrane and $\tau_{rel} > \tau_{fl}$, whereas in the liquid crystalline phase $\tau_{rel} \cong \tau_{fl}$. For the Tris buffer solution the opposite inequality $\tau_{rel} < \tau_{fl}$ holds, causing that TICT state emission is noted only. In accordance with the above time dependencies, the fluorescence emission from non-equilibrated excited states consists of a dominant or minor radiation process in the LE or CT band emission. This information can be obtained by analyzing pre-exponential coefficient values (see Table 1, 2 and 3).

The proposed model of static and dynamic changes of the solvent heterogeneity causing the appearance of thermally relaxed and unrelaxed excited states of solvated fluorescent probes needs further studies, in particular studies of the emission anisotropy decay curves and time resolved fluorescence spectra obtained by exciting the luminophor in the red region of the long wavelength band.

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