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Probing Ethanol-Induced Phospholipid Phase Transitions by the Polarity Sensitive Fluorescence Probes Prodan and Patman

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Dedicated to Prof. Dr. Friedemann W. Schneider

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The emission behaviour of the two polarity sensitive probes Prodan and Patman in phospholipid vesicles was studied as a function of the concentration of ethanol. Comparing the spectral shifts in both the symmetric lipid 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) showing a phase transition from a normal to a fully interdigitated gel phase and the strongly asymmetric lipid 1-stearoyl-2-lauroyl-sn-glycero-3-phosphatidylcholine (C(18):C(12)-PC) favouring a mixed interdigitated gel phase we show that the huge red shifts of Prodan in presence of higher ethanol concentrations cannot be easily attributed to a specific lipid phase transition. Rather, probe relocation and a pronounced increase in solvent relaxation (SR) as monitored by time-resolved emission spectra (TRES) in presence of ethanol contribute to the large shifts observable in both lipid systems in case of Prodan. While Patman exhibits a red shift caused by increased SR due to the ethanol induced formation of a fully interdigitated phase in DPPC, hardly any shift occurs in C(18):C(12)-PC, which is supposed not to undergo an ethanol-induced phase transition.

1. Introduction

Beside the classic bilayer arrangement gel phase structures are possible in which the acyl chains of opposing monolayers interdigitate. Three types of bilayer interdigitation have been reported: fully, mixed and partially

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Much work has been done on the induction of this phase by ethanol [1–11], since Rowe reported the first observation that ethanol exerts a biphasic effect on the main phase transition temperature (T_m) of fully hydrated phosphatidylcholines [12]. For instance, in the absence of ethanol the Tm value of the fully hydrated 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) is 41.6 °C. It decreases linearly with increasing ethanol concentration up to a decrease of about 2 °C at 1.09 M ethanol. Above this threshold concentration, Tm increases with increasing ethanol concentration. This effect has been attributed by X-ray diffraction to the formation of a fully interdigitated $L_{\beta 1}$ gel phase in the DPPC bilayer [4]. Based on X-ray diffraction results, Adachi *et al.* [13] proposed a structural model for the equilibrium state of the fully interdigitated bilayer in the $L_{\beta 1}$ phase, considering a PC/ethanol molar ratio of 1 : 2. Specifically, the two linear all-trans acyl chains of each PC molecule (e.g., DPPC) penetrate through the hydrophobic core of the gel state bilayer with their terminal methyl groups located on the opposing side of the bilayer. Each of these methyl groups is proposed to be in line with the methyl group of an ethanol, and the hydroxyl group of the same ethanol is coplanar with the sn-1 carbonyl groups of neighbouring DPPC molecules, facing the ethanol containing aqueous medium.

The second type, called mixed interdigitation, is less common and results for phospholipids with a large difference in chain length. The overall symmetry/asymmetry of a molecular species of C(X):C(Y)-PC packed in the gel state bilayer can be described quantitatively by a structural parameter ΔC, defined as the effective chain length difference between the sn-1 and sn-2 acyl chains. The absolute value of ΔC, in C−C bond lengths, can be related to the X and Y values, C(X):C(Y)-PC as follows [14]: $\Delta C = |X - Y + 1.5|$, where X and Y are the numbers of carbons in the sn-1 and sn-2 acyl chains, respectively, and 1.5 is the effective chain length difference between the sn-1 and sn-2 chains, in C−C bond lengths, for identical chain PCs. This mixed interdigitation is characterised by the long acyl chain extending completely across the bilayer whereas the two shorter chains meet end to end in the bilayer midplane.

Finally, a partially interdigitated bilayer packing mode exists in which the shorter chain from one monolayer pairs with the longer from the opposing monolayer. This arrangement has been proposed for medium asymmetric lipids like 1-stearoyl-2-myristoyl-sn-glycero-3-phosphati-dylcho-line [15–17] and for asymmetric lipids in the fluid crystalline phase [18–20].

For quite long time, the investigation of the ethanol effect on the T_m of multilamellar vesicles (MLVs) has been confined to phosphatidylcholines with identical fatty acids esterified at the sn-1 and sn-2 positions of the glycerol backbone. This topic was addressed by Li *et al.* [21] by molecular mechanics computations, calculating the overall stabilisation energy difference between bilayers of C(X):C(Y)-PC with the $L_{\beta'}$ packing motif and those with the $L_{\beta 1}$ motif. The results of this study predict that MLVs composed of C(X):C(Y)-PC with modest chain asymmetry ($\Delta C < 4.2$) can be induced by ethanol to form

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the $L_{\beta 1}$ state bilayer at $T < T_m$, whereas highly asymmetric C(X):C(Y)-PCs ($\Delta C > 4.2$) cannot be converted into the $L_{\beta 1}$ state by ethanol. This view was supported, when calorimetrically determined T_m values were plotted against the ethanol concentration yielding a V-shaped biphasic curve for C(X):C(Y)-PCs with $\Delta C < 4.2$. In contrast, a linear curve with negative slope was detected for highly asymmetrical C(X):C(Y)-PCs with $\Delta C > 4.2$ [14].

Fluorescent probes with a high sensitivity to changes in viscosity and polarity in the close vicinity of the probe are especially useful for the study of phospholipid membranes. Such dyes described in recent publications were based on 2-amino-substituted naphthalene [22, 23] and anthracene-9-carboxylic acid [24]. Representatives for 2-amino-substituted naphthalenes are the dyes Prodan (6-propionyl-2-(dimethylamino)-naphthalene) and its long alkyl chain derivative Patman (6-palmitoyl-2-[[trimethylammonium-ethyl]methylamino]-naphthalene chloride). In previous studies by Chong [25] and Zeng and Chong [26, 27] Prodan has been used to monitor the ethanol-induced lipid interdigitation in DPPC multilamellar and unilamellar vesicles. It has been concluded that the abrupt spectral change of Prodan fluorescence at ethanol concentrations exceeding 1.1 M are due to a decrease in partitioning of Prodan in the fully interdigitated structure [26]. The authors suggested the intensity ratio of Prodan fluorescence at 435 nm to that at 515 nm (F_{435}/F_{515}) as an index for monitoring the ethanol-induced phase transition to a fully interdigitated structure, while the long-wavelength maximum was attributed principally to Prodan relocated into a higher polarity environment, *i.e.* the bulk solution.

Regarding the complex solvent relaxation (SR) behaviour of Prodan and Patman studied in detail during the last years, however [22, 23, 28], this conclusion might be an oversimplification. While the chromophore of Prodan is supposed to be located in the headgroup region of phospholipid bilayers close to the lipid/water interface the chromophore of Patman (similar to that of Laurdan) [29] has been shown to be embedded deeper in the bilayer, where it should be fixed tightly due to its long acyl chain [22].

If the pronounced red shift of Prodan during formation of the fully interdigitated phase in DPPC really reflects the change in bilayer structure a different behaviour should be expected when the dye is incorporated in a bilayer composed of the strongly asymmetric lipid C(18):C(12)-PC which adopts a mixed interdigitated bilayer structure below 17.5 °C. Because of its high ΔC value of 7.5, a transition from the mixed interdigitated to a fully interdigitated state should not be inducible by ethanol [30].

Therefore we investigated the behaviour of both Prodan and Patman in vesicles composed of either DPPC (C(16):C(16)-PC) and C(18):C(12)-PC in presence of different amounts of ethanol. In summary we provide evidence that the large red shift observed for Prodan is rather a general effect of high ethanol concentrations in vesicle samples than specifically attributable to the formation of a fully interdigitated phase. While care has to be taken in the interpretation of the shifts observed for Prodan, Patman seems to be the more reliable

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probe for the study of lipid interdigitation, being able to differentiate between the different ethanol-induced effects in both lipid systems.

2. Materials and methods

DPPC was obtained from Fluka and C(18):C(12)-PC from Avanti Polar Lipids. Prodan and Patman were obtained from Molecular Probes. Both lipids and dyes were used as received. The fluorophores were added to the respective lipid in CHCl3 from an ethanolic stock solution to a final lipid/dye ratio of 200 : 1. The total lipid concentration was 1.0 mM.

The solvent was removed under a stream of N_2 and the remaining lipid/dye mixture was kept under vacuum overnight. Tris buffer (20 mM Tris, 100 mM NaCl, pH 7.5) was added and the lipid film was allowed to swell for 1 h above the phase transition temperature with occasional vortexing to yield multilamellar vesicles (MLV). Large unilamellar vesicles (LUVET) were obtained by the extrusion method as described by MacDonald *et al.* [31]. The lipid/dye mixture was vortexed at a temperature above the phase transition and extruded through polycarbonate filters (Nucleopore, 100 nm pore size) using a Liposo-Fast extruder (Avestin). The vesicle suspensions were kept at 5.0 °C overnight to allow the formation of the mixed interdigitated gel phase for C(18):C(12)-PC or non-interdigitated gel phases (DPPC).

Fluorescence decays ($\lambda_{ex} = 358$ nm) were recorded in 10 nm steps across the entire emission spectrum with commercial single photon counting equipment (Edinburgh Instruments 199 S) and analysed using an iterative reconvolution technique, as described [32]. The time-resolved emission spectrum (TRES) at a given time t, $S(\lambda; t)$, is obtained by the fitted decays, $D(t; \lambda)$, by relative normalization to the steady-state spectrum, $S_0(\lambda)$, as follows:

$$S(\lambda; t) = \frac{(D(t; \lambda) \times S_0(\lambda))}{\int_0^\infty (D(t; \lambda))\, \text{TS}^{\text{b}}\, \mathrm{d}t}. \quad (1)$$

After conversion from a wavelength representation to one linear in frequency, the TRES are fit by the empirical 'log-normal function' [33]. Steady-state emission spectra were recorded with an Aminco Bowman II spectrometer using excitation at 360 nm. Ethanol was added to the sample to obtain the respective molar ethanol concentrations given. All spectra are corrected for instrument response and dilution. The temperature was controlled within 0.2 °C at 20 °C for DPPC and 10 °C for C(18):C(12)-PC.

3. Results

In this work we investigate the behaviour of the two well established polarity sensitive dyes Prodan and Patman which have been shown to be located

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in different depths in lipid bilayers [22], in response to ethanol-induced lipid interdigitation.

Steady-state emission spectra have been recorded as a function of ethanol concentration for both Prodan and Patman in the different lipid systems, at $T = 20\,^{\circ}\mathrm{C}$ for DPPC and $T = 10\,^{\circ}\mathrm{C}$ for C(18):C(12)-PC. Prodan in DPPC-MLV shows typical gel phase spectra at low concentrations of ethanol, *i.e.* an emission maximum near 435 nm. With increasing ethanol concentration this maximum decreases in intensity while a second maximum, considerably red-shifted to about 515 nm, becomes predominant at high ethanol concentrations (Fig. 1a). Compared to the typical red shift of Prodan during a bilayer transition from the gel to the liquid crystalline state the ethanol induced shift is even larger, suggesting an environment of high polarity (the emission maximum is

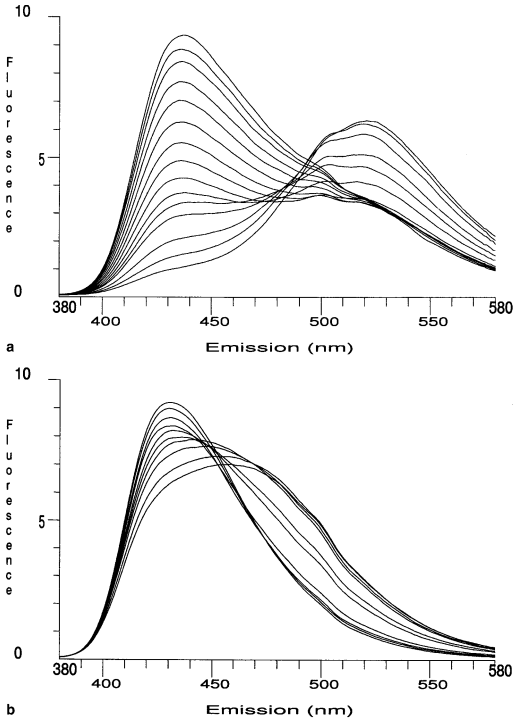

Fig. 1. (a) Steady-state emission spectra ($\lambda_{ex} = 358$ nm) for Prodan in LUVETs composed of DPPC (1 mM) at increasing concentrations of ethanol. The ethanol concentrations are, from top to bottom at 440 nm: 0; 0.34; 0.66; 0.97; 1.27; 1.42; 1.56; 1.70; 1.84; 1.97; 2.24; 2.49; 3.15; 3.43; 3.96 mol/l. The temperature was maintained at 20 °C. (b) Steady-state emission spectra ($\lambda_{ex} = 358$ nm) for Patman in LUVETs composed of DPPC′ (1 mM) at increasing concentrations of ethanol. The ethanol concentrations are, from top to bottom at 430 nm: 0; 0.82; 1.12; 1.42; 1.86; 1.97; 2.24; 2.49; 3.15; 3.96 mol/l. The temperature was maintained at 20 °C.

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even red-shifted compared to that in pure ethanol [34]) and fast solvent relaxation. However, the wavelength is still shorter than in pure bulk water and a shoulder in the blue wavelength region is still visible suggesting that most Prodan molecules are somehow membrane associated and not pushed out in the bulk environment where Prodan exhibits considerably reduced quantum yields.

The emission maximum of Patman is also red-shifted with increasing ethanol concentration (Fig. 1b); however, the shift is considerably smaller compared to Prodan. This is in agreement with the fact that the chromophore of Patman is located somewhat deeper in the membrane than that of Prodan [22], and it is fixed in the bilayer by its long acyl chain allowing less rearrangement. During lipid interdigitation Patman senses an environment of higher polarity and faster SR as will be further demonstrated by the time-resolved emission spectra discussed later. Very similar behaviour of both dyes was observed when the same experiments were performed with either LUVETs or MLVs (data not shown). From this first point of view both dyes seem of similar usefulness for the detection of ethanol-induced lipid interdigitation.

We therefore examined a second lipid system with different structural behaviour. The strongly asymmetric C(18):C(12)-PC is known to adopt a mixed interdigitated gel phase at low temperatures (10 °C). This phase allows better Van der Waals contact of the lipid acyl chains than a normal gel phase where large voids would result due to the different acyl chain lengths.

With increasing ethanol concentration Prodan in C(18):C(12)-PC shows a very similar behaviour to that observed in DPPC, *i.e.* a strong red shift to about 515 nm at high ethanol concentrations (Fig. 2a). Thus, it might be assumed that again some kind of phase transition might occur similar to the DPPC bilayer system. Interestingly, however, the behaviour of Patman in vesicles composed of C(18):C(12)-PC is completely different. Hardly any shift is observable, even at very high concentrations of ethanol (Fig. 2b). This behaviour strongly suggests that no major changes in both polarity and viscosity of the dye environment take place, as it has been shown extensively that Patman is quite sensitive to any such change [22, 23, 28]. The only plausible explanation is that Patman is tightly fixed in the mixed interdigitated lipid system and that this gel phase structure remains stable even in presence of higher ethanol concentrations. Prodan, in the opposite, which is located quite close to the headgroup region (which is also the region of the methyl terminus of the long acyl chain in a mixed interdigitated lipid system) is affected by ethanol which has been shown to be in contact with the sn-1 carbonyl group of the acyl chain (by its OH-group) and with the methyl termini (by its methyl group). The dye may be forced into a higher polarity region due to the competition of ethanol molecules and becomes effectively solvated in the outer headgroup region. Thus, the pronounced red shift of Prodan seems not to be associated with the lipid phase transition into a fully interdigitated phase but rather to be a general effect inducable by high concentrations of ethanol in a membrane system.

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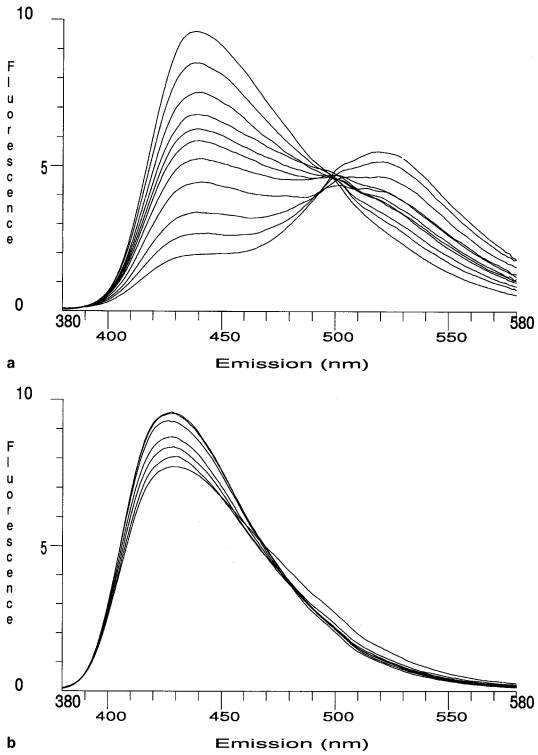

Fig. 2. (a) Steady-state emission spectra ($\lambda_{ex} = 358$ nm) for Prodan in LUVETs composed of C(18):C(12)-PC (1 mM) at increasing concentrations of ethanol. The ethanol concentrations are, from top to bottom at 440 nm: 0; 0.82; 1.12; 1.42; 1.86; 1.97; 2.24; 2.49; 2.86; 3.43; 3.96 mol/l. The temperature was maintained at 10 °C. (b) Steady-state emission spectra ($\lambda_{ex} = 358$ nm) for Patman in LUVETs composed of C(18):C(12)-PC (1 mM) at increasing concentrations of ethanol. The ethanol concentrations are, from top to bottom at 430 nm: 0; 0.82; 1.56; 1.91; 2.24; 2.86; 3.43; 3.96 mol/l. The temperature was maintained at 10 °C.

For the first time the effect of ethanol on the SR behaviour of Patman in gel phase bilayers has been examined by the reconstruction of time-resolved emission spectra. The validity of this approach has been demonstrated in several recent publications [22–24], and summarised in a few reviews [28, 35, 36]. The time-resolved spectra for Patman in DPPC at 20 °C have been reconstructed for $t = 0.2$ ns, 2.0 ns, 5.0 ns and 10.0 ns after excitation, in absence and presence of 2.25 M and 3.96 M ethanol, respectively. In absence of ethanol the SR in gel phase bilayers is very slow; thus, hardly any red shift with time is detectable (Fig. 3a). With 2.25 M ethanol (Fig. 3b), a concentration sufficient to induce the transition to the fully interdigitated phase, the red shift with time is considerably increased, suggesting a probe environment of

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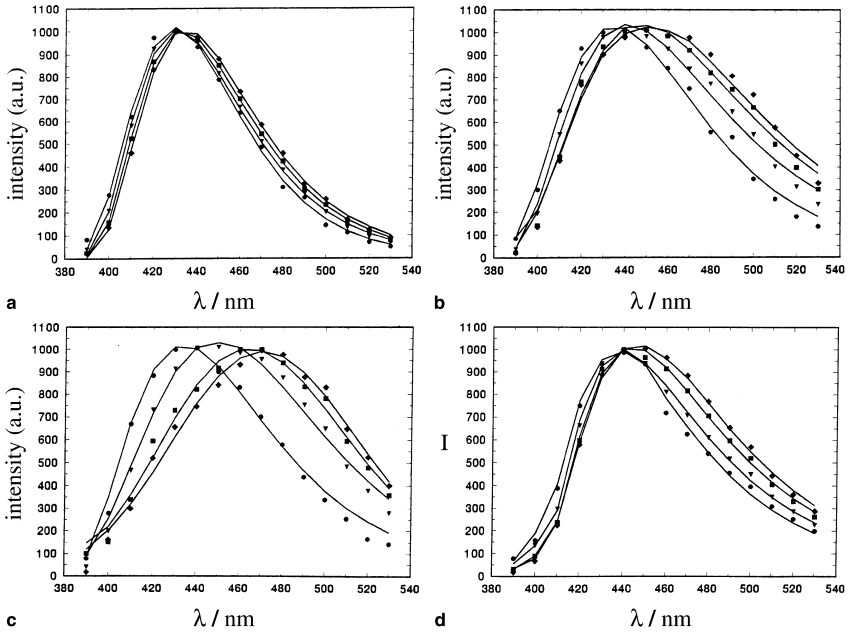

Fig. 3. Time-resolved emission spectra for Patman in LUVETs composed of DPPC in absence of ethanol (a), in presence of 2.25 mol/l ethanol (b) and in presence of 3.96 mol/l ethanol (c). The temperature was maintained at 20 °C. Spectra have been reconstructed for $t = 0.2$ ns (circles), 2.0 ns (triangles), 5.0 ns (boxes) and 10.0 ns (diamonds) after excitation and fitted by an empirical log-normal function according to [33].TS:C

higher polarity and/or reduced viscosity. In presence of 3.96 M ethanol the shift is further increased, and the SR is faster (Fig. 3c). Thus, the observed red shift in the steady-state spectra at higher concentrations of ethanol can be clearly attributed to increased SR which is nearly abolished in the normal gel phase in absence of ethanol (yielding the blue-shifted steady-state emission spectra), but is able to compete with fluorescence emission in presence of sufficient amounts of ethanol to induce the transition to the fully interdigitated phase.

In absence of ethanol the shift for Prodan is quite small, too. However, in contrast to Patman, no time-dependence of the TRES in presence of ethanol could be obtained due to the limited instrument time resolution. Obviously solvent relaxation was so fast that only the completely relaxed state could be observed. In Fig. 4 the emission maxima (cm^{-1}) in DPPC as function of time after excitation are summarized.

Embedded in a mixed interdigitated gel phase like that of C(18):C(12)-PC, however, Patman does hardly sense the presence of ethanol bound to the headgroup region. No lipid phase transition occurs, and SR remains very slow;

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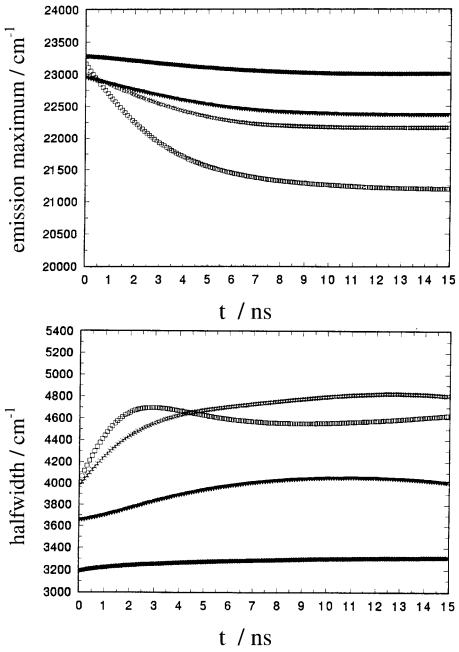

Fig. 4. Emission maxima (in cm^{-1}) as function of time after excitation in DPPC-LUVETS: from top to bottom at 10 ns: Patman; c (ethanol) = 0 mol/l (filled circles). Prodan; c (ethanol) = 0 mol/l (filled triangles). Patman; c (ethanol) = 2.25 mol/l (asterisk). Patman; c (ethanol) = 3.96 mol/l (open boxes). The temperature was maintained at 20 °C. TS[d]

thus, hardly any shift with time after excitation is detectable (time-resolved data not shown).

4. Discussion

This work provides evidence that much care has to be taken in interpreting the emission behaviour of the polarity sensitive membrane probe Prodan. In a previous work [27] Zeng and Chong investigated the effect of ethanol-induced lipid interdigitation on the membrane solubility of Prodan, Acdan (6-acetyl-2-(dimethylamino)naphthalene) and Laurdan (6-lauroyl-2-(dimethylamino)-naphthalene) and concluded that Prodan was the best probe of the three for monitoring ethanol-induced lipid interdigitation. Although conceding that the interpretation of Prodan fluorescence in membranes requires besides environmental polarity [25] and probe location [25, 28, 36] the consideration of solvent relaxation [22–24, 28, 38] their argumentation is principally focussed on a probe relocation mechanism. Thus, the red-shifted emission of Prodan around 515 nm, which appears during the ethanol-induced bilayer interdigitation, is

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attributed to increased partitioning of Prodan in the bulk water phase. While such a change in the partition coefficient will clearly contribute to the observed spectral change we are in doubt whether a percentage of Prodan in bulk water of 50% as postulated for an ethanol concentration of 1.8 M compared to 20% below 1.1 M ethanol [27] can explain the dramatic spectral changes, considering the low fluorescence quantum yield of Prodan in water. Reducing the DPPC-concentration from 1.17 mM to 0.48 mM Zeng and Chong observed a shift of the major peak from 518 to 521 nm. Their explanation that a decrease in DPPC-concentration should decrease probe partitioning from the bulk solution into the membrane seems reasonable; however, the concomitant increase in intensity of the 435 nm peak [27] remains completely unexplained and does not support their view.

As the work of Zeng and Chong is focussed on a single lipid system (DPPC-MLV) and its ethanol-induced transition to an interdigitated structure the dramatic spectral shift of Prodan seems to be an ideal indicator for this transition. Only by comparison with a structurally different lipid system like C(18):C(12)-PC which forms a mixed interdigitated gel phase in absence of ethanol and is very likely to remain in this phase even in presence of higher amounts of ethanol [21, 39] it becomes obvious that the spectral shift of Prodan in the DPPC-ethanol system is not really specific for the transition $L_{\beta'} \rightarrow L_{\beta 1}$. As we have shown in this work the behaviour of Prodan in the C(18):C(12)-PC in presence of increasing amounts of ethanol is very similar to that in DPPC. Thus, the observed red shift to about 515 nm in presence of higher amounts of ethanol seems to be a more general ethanol-induced effect on Prodan in phospholipid membranes, not related to a specific lipid phase transition to an interdigitated phase. Prodan may undergo some relocation in presence of ethanol to a position even closer to the bulk environment, but in any case it senses an environment of increased polarity and considerably reduced viscosity allowing much faster solvent relaxation. In our opinion, the occurrence of nearly complete SR is the main cause for the observed shift. This effect seems to be independent of the discrete lipid structure, *i.e.* the SR of Prodan is dramatically increased in both the fully interdigitated structure (DPPC or DMPC; data for DMPC not shown) and the mixed interdigitated structure (C(18):C(12)-PC) due to the presence of ethanol.

The tendency of asymmetric lipids to undergo an ethanol-induced transition to a fully interdigitated phase has been investigated by Li *et al.* in a molecular mechanics study [21]. In this study the normal gel phase of like-chain lipids like DPPC ($L_{\beta'}$) was termed partially interdigitated; thus, they calculated total stabilisation energies $\Delta E_{\mathrm{dif}}{}^{\mathrm{total}} = E_{\mathrm{f}}{}^{\mathrm{total}} - E_{\mathrm{p}}{}^{\mathrm{total}}$, where the subscripts f and p represent the fully interdigitated motif with ethanol and the partially interdigitated motif without ethanol, respectively, and the subscript dif refers to the difference between the two states. If the value of $\Delta E_{\mathrm{dif}}{}^{\mathrm{total}}$ is positive, then the bilayer with a partially interdigitated motif is more stable; otherwise the bilayer with ethanol characterised by a fully interdigitated

state ($L_{\beta 1}$) is more stable. The authors showed that PCs with $\Delta C < 4.2$ have negative ΔE_{dif}^{total} values, indicating an ethanol-induced transition to the fully interdigitated phase, while strongly asymmetric PCs ($\Delta C > 4.2$) like the examined C(12):C(20)-PC and C(18):C(14)-PC are not expected to undergo the phase transition [21]. Based on these results a transition of the even more asymmetric C(18):C(12)-PC (adopting a mixed interdigitated structure in absence of ethanol [30]) used in our study to the fully interdigitated state is even more unlikely.

This view is supported by the results obtained for Patman in both kinds of lipid systems. Patman has been shown to incorporate deeper in the bilayer [22]; its partition coefficient will be considerably higher than for Prodan due to its long acyl chain, in analogy to Laurdan [27]. As ethanol will predominantly accumulate in the headgroup region direct contact of the Patman chromophore with ethanol is relative improbable; instead, Patman senses the indirect effects on membrane polarity and viscosity induced by ethanol. As we do not expect a major relocation of Patman in the bilayer the observed red shift can be attributed mainly to increased SR. This can be demonstrated most directly by recording time-resolved emission spectra. The TRES and the time course of the emission maximum for Patman in DPPC in absence and presence of ethanol shown in Fig. 4 give direct evidence for increased SR in presence of ethanol.

As shown in Fig. 2a the SR is very slow in a normal gel phase in absence of ethanol, resulting in a relatively small spectral shift with time after excitation for both Patman and Prodan. This conclusion can be supported by an estimation of the emission maximum of the time-zero spectrum $\nu(0)$ for Prodan. It has been shown that the frequency of the $t = 0$ emission, $\nu(0)$, can be calculated quite accurately when the maxima of the absorption and fluorescence spectra in a non-polar solvent (ν_{np}(abs) and ν_{np}(em), respectively) as well as the absorption maximum in the system of interest (ν_p(abs)) are known [40]. Prodan is soluble in cyclohexane with an absorption maximum ν_{np}(abs) equal to 29 240 cm^{-1} and an emission maximum ν_{np}(em) equal to 24 938 cm^{-1} [41]. The absorption and the excitation spectra of Prodan in the gel phase of DPPC (data not shown) consist of two distinguished bands [42]. Assuming that the maximum of the more blue-shifted and intensive excitation band (27 778 cm^{-1}) [42] is equal to ν_p(abs), a $\nu(0)$ value of 23 476 cm^{-1} for Prodan in the DPPC gel phase is obtained. Since the maximum of the TRES of Prodan in the DPPC gel phase at 15 ns after excitation has been determined to be about 22 375 cm^{-1} the spectral shift with time after excitation $\Delta\nu = \nu(0) - \nu(\infty)$ for this system is rather small when compared to a total Stokes shift of about 3500 cm^{-1} of Prodan in phosphatidylcholine vesicles above the phase transition [35]. In other words we are confident that the averaged SR process is slower that the intrinsic fluorescence and that we would reach the same basic conclusions using an instrument providing ps or fs resolution.

On the other hand, the time-dependent red shift of Patman strongly increases in presence of 2.25 M and even more in presence of 3.96 M ethanol.

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For Prodan, SR in presence of these amounts of ethanol is too fast for the given instrument resolution. Independent of the lipid system and time (on the ns time scale) strongly red-shifted spectra are observed; thus, the behaviour of Prodan seems not to be a specific indicator for lipid interdigitation. In contrast to Prodan, Patman is able to differentiate between distinct lipid structures in presence of ethanol. This becomes obvious by comparison of the emission behaviour in DPPC and C(18):C(12)-PC: in the latter lipid system adopting the mixed interdigitated structure in both absence and presence of ethanol hardly any shift is observed even with high concentrations of ethanol supporting the view that Patman is not directly affected by the presence of ethanol but only by changes in lipid structure and dynamics. Consequently, only very small time-dependent shifts of the Patman emission can be observed in presence of ethanol in C(18):C(12)-PC. This behaviour is in clear contrast to that of Prodan and can be attributed to the different distinct locations of both probes.

In summary we want to point out that care must be taken in interpreting spectral shifts of Prodan, especially, if only a single system is examined. SR is very likely to be the most important parameter influencing the emission behaviour while direct conclusions from emission wavelength to lipid structure in presence of further polar molecules like alcohols can be misleading.

Another fluorescence method which gives direct evidence for interdigitated in contrast to non-interdigitated bilayers should not remain unmentioned. We have shown that steady-state anisotropy measurements employing a set of membrane probes with their chromophores located in different defined depths along the membrane normal can be used to construct anisotropy profiles which are characteristic for different gel phase structures, *i.e.* for non-interdigitated, fully and mixed interdigitated bilayers [36, 43]. The set of n-anthroyloxy fatty acids with the anthracene chromophore attached at different positions ($n = 2$, 6, 9, 12, 16) along a fatty acid chain constitute such a set of probes. The advantage of this method is that it allows a direct assessment of the respective lipid structure, similar to diffraction methods, and not only the detection of a change in bilayer structure. Of course, this method is also useful to monitor ethanol-induced lipid interdigitation as has been shown by the completely different anisotropy profiles of the set of n-anthroyloxy fatty acids in DPPC in absence and presence of 1.9 M ethanol [43].

5. Conclusion

While Prodan has been shown to be sensitive to the lipid main phase transition from the gel ($L_{\beta'}$) to the liquid crystalline phase (L_α) [22, 38], not every spectral shift of this probe may be attributed to a distinct lipid phase transition. Changes in its location or increased SR caused by other parameters like the presence of small polar molecules can induce pronounced shifts also in absence

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of a lipid phase transition as shown for an asymmetric lipid favoring a mixed interdigitated gel phase. Changes in SR may be considered the major parameter governing the spectral emission behaviour of strongly polarity sensitive probes like Prodan and Patman.

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