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Flavonols – new fluorescent membrane probes for studying the interdigitation of lipid bilayers

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Abstract

Two flavonols, 3-hydroxy-4′-dimethylaminoflavone (FME) and 3-hydroxy-4′-(15-azacrown-5) flavone (FRC) have been investigated as new fluorescence probes for studying the formation of the interdigitated gel phase in lipid bilayers. The formation of the interdigitated gel phase in the saturated symmetrical phosphatidylcholines (PCs) and phosphatidylethanol (Peth) in the presence of ethanol has been well studied. The present study examines the behavior of these new probes in PC–ethanol and Peth–ethanol systems, as well as in PC–cholesterol and Peth–cholesterol vesicles. The present results demonstrate that both flavonols give distinctively different spectra in interdigitated lipids compared to non-interdigitated lipids, when examined in lipids in which the interdigitation behavior is known. This makes them useful for determinations of the structural state of unknown lipids, and for following the transitions between interdigitated and non-interdigitated phases. However, in the presence of cholesterol, only FCR gave appropriate indications of interdigitation. The results with FME in the presence of cholesterol were not consistent with the known behavior of the lipids examined; instead, FME appears to be located preferentially in the cholesterol-rich non-interdigitated regions of the bilayer. © 1998 Elsevier Science B.V.

Keywords: Phospholipid; Interdigitated gel phase; Fluorescence; Flavonol

1. Introduction

Flavonols (3-hydroxyflavones) are natural dyes which are widespread in the plant world. In the past, mixtures of natural flavonols were used as yellow dyes. More recently, some of the flavonols have been used in the pharmacy, for example the P-vitamin containing drugs [1]. This class of chemical compounds attracted the attention of spectroscopists after the discovery of their dual fluorescence [2] and its explanation by Kasha [3].

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 1,3-DPPC, 1,3 dipalmitoyl-*sn*-glycero-2-phosphocholine; DPPeth, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol (sodium salt); FME, 4′-dimethylaminoflavonol; FCR, 4′-*N*-aza-15-crown-5-flavonol; DSC, differential scanning calorimetry; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; Pyr-PC, 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine; Peth, phosphatidylethanol; PC, phosphatidylcholine; MLV, multilamellar vesicles

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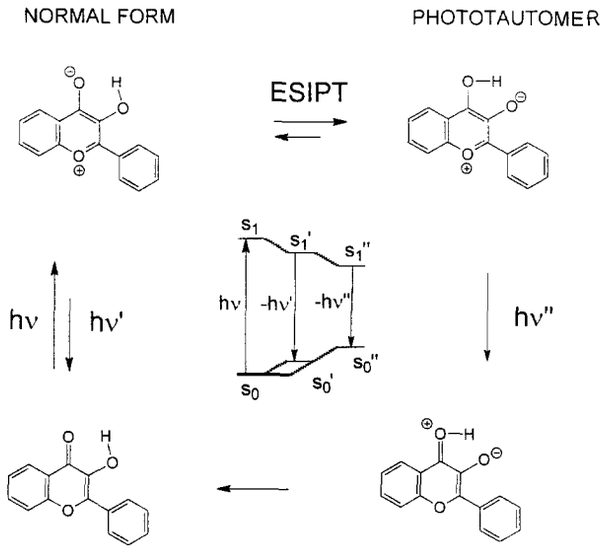

Fig. 1. Dual fluorescence in flavonols due to exited state intermolecular proton transfer effect (ESIPT).

As shown in Fig. 1, the phenomenon of flavonol dual fluorescence is due to the excited state intramolecular proton transfer (ESIPT), which results in the partial isomerization of the normal excited form of the flavonol to a phototautomer. The two excited isomers return to the ground state with the emission of protons of different energy. This is why the fluorescence emission spectrum of flavonols, in many solvents, consists of two distinct peaks.

The fluorescence emission spectra of 4′-dialkylaminoflavonols display high sensitivity to the nature of their microenvironment. The fluorescence maximum positions, as well as the ratio of intensity of the normal and tautomeric forms of flavonols, vary depending on solvent polarity [4] or solvent nucleophilicity [5–8]. These characteristics suggest that these chemical compounds may be effective fluorescence probes for investigating the structure and physical properties of biological membranes.

In the present study, we have used two flavonols, namely 3-hydroxy-4′-dimethylaminoflavone (FME) and 3-hydroxy-4′-(15-azacrown-5) flavone (FRC), as fluorescence probes to detect the transitions of phospholipid bilayers into the interdigitated gel phase structure. The interdigitated gel phase ($L_{\beta}I$), in which the acyl chains from opposing monolayers interpenetrate, is among the most recently characterized stable lipid phase states, and has been well demonstrated for the saturated symmetrical and asymmetrical phosphatidylcholines (PCs) [9–11,13]. The interdigitated gel phase can exist under a variety of conditions. For example, the saturated symmetrical PCs and phosphatidylglycerols (PGs) can form the interdigitated gel phase in the presence of numerous additives such as alcohols, glycerol, ethylene glycol, chlorpromazine, anisodamine, tetracaine and thiocyanate ion [9,10,12–15]. Polymyxin B and myelin basic protein can also induce the formation of $L_{\beta}I$ in PGs [16,17]. Some phospholipids, for example DHPC and 1,3 DPPC, exist in the interdigitated phase in water without any additives [18–21]. The negatively charged phospholipids PG and phosphatidylethanol (Peth) form the interdigitated gel phase in the presence of Tris cations [22,23]. Mixed-chain PCs can also exhibit the interdigitated phase if one acyl chain is approximately twice as long as the other [13,24].

The interdigitation of the gel phase has been directly detected by X-ray diffraction [9–12,23–25] and neutron diffraction [21] as well as by indirect methods such as differential scanning calorimetry and electron spin resonance [22,29–32]. Various fluorescence probes such as DPH, Pyr-PC and Prodan have successfully been used for detecting the interdigitated gel phase [18,22,26,33–35]. In our laboratory, we have been focusing on the formation of interdigitation in PCs, PGs and Peths caused by ethanol. In previous studies, we have shown that the ethanol-induced transition from the gel phase to the interdigitated gel phase is dependent on ethanol concentration, the lipid chain length, and also on temperature [26–28]. In the case of the charged phospholipids, PG and Peth, ethanol causes interdigitation in salt solutions and enhances the interdigitation in Tris-HCl [22,23].

In the present study, we have investigated the fluorescence spectra of both FME and FCR probes in several model membrane systems, which have already been established in our laboratory to form the interdigitated gel phase. These interdigitated systems include DPPC liposomes in the presence of different concentrations of ethanol, DPPeth liposomes, which interdigitate in Tris-HCl buffer without any additives, and also DPPC–cholesterol and DPPeth–cholesterol systems.

2. Materials and methods

2.1. Chemicals

DPPC and DPPeth were obtained from Avanti (Birmingham, AL), the ethanol was purchased from AAPER Alcohol and Chemical (Shelbyville, KY), and the cholesterol and all solvents were obtained from Sigma (St. Louis, MO). Di-*N*-methylaminobenzaldehyde and 2′-hydroxyacetophenone were purchased from Aldrich (Milwaukee, WI).

2.2. Sample preparation

Multilamellar vesicles (MLVs) were used in these studies. The stock solutions of lipids in chloroform and probes in ethanol were kept at −20°C. Lipid-probe or lipid-probe–cholesterol mixtures were evaporated under a stream of nitrogen gas and then kept overnight under vacuum to remove all residual organic solvents. DPPC–MLV samples were prepared in double distilled water by Bangham's method [36]. DPPeth–MLVs were prepared in 50 mM Tris-HCl buffer (pH 7.4). The lipids were hydrated at a temperature above the main phase transition for at least 30 min, in order to avoid the crystalline subgel phase. For experiments in a wide range of temperatures, the samples were then cooled to ca. 2°C and kept in the refrigerator at least 2 days. Ethanol was added to the samples at 2°C. The lipid concentration in all fluorescence experiments was 1.2–1.5 mM. Lipid concentrations were determined by Bartlett's method [37].

2.3. Synthesis of flavonols

The method described by Rekker [38,39] was used to calculate the lipophylicity fragment constants for various flavonol derivatives. Two derivatives which should exhibit approximately the same lipophylicity as Prodan, and which can be readily synthesized, are 4′-dimethylaminoflavonol (FME) [40] and 4′-*N*-aza-15-crown-5-flavonol (FCR) [7,8]. We have synthesized the FME and FCR compounds by the procedure of Smith et al. [41]. The corresponding di-*N*-methylaminobenzaldehyde [42] was dissolved in 2 ml of ethanol and then 1.2 mmol of 2′-hydroxyacetophenone was added to the solution. After this, 5 mmol of the hot solution of potassium hydroxide in 0.1 g of water was added in one step. This mixture was stirred during 4 days at 20°C, then cooled to −5°C, when 2.4 mmol of hydrogen peroxide was added. The stirring was continued at this temperature until the entire amount of intermediate product had been converted to the corresponding flavonol. TLC was run in chloroform– methanol (90 : 10 and 95 : 5) systems. Following this, the reaction mixture was collected by filtration, dried and recrystallized from ethanol three times.

The structures of both compounds have been confirmed by methods of quantitative elemental analysis, proton magnetic resonance (PMR), UV- and IR spectrometry. All PMR spectra were recorded on a Bruker WP-100 Fourier transform spectrometer and ordered as follows: signal position in ppm δ-scale, form of signal, (coupling constant in Hz), number of protons, and (their position in molecule). UV spectra were recorded on Specord UV Vis instrument for 2×10^{-5} M solutions of flavonols in ethanol or in 0.01 M Tris-buffer, pH 7.4. IR-spectra were recorded on Pye Unicam SP3-300 instrument in pellets of KBr. Data presented are in cm^{-1}.

2.3.1. For 3-hydroxy-4′-dimethylaminoflavone (FME)

PMR spectrum in DMSO 9.16s, 1H(OH-3); 8.08dd (J_1 = 8; J_2 = 2)1H(H-5); 7.66–7.77m, 2H(H-6,7); 7.43m, 1H(H-8); 8.13d(J = 9) 2H(H-2′,6′); 6.85d(J = 9)2H(H-3′,5′); 3.00c6H(NCH_3-4′). UV spectrum in ethanol: λ_{max} = 405 nm, (e = 39 000 $dm^3\,mol^{-1}\,cm^{-1}$).

2.3.2. For 3-hydroxy-4′-N-(15-azacrown-5)flavone (FCR)

PMR spectrum in $CDCl_3$ 8.98s, 1H(OH-3); 8.25m, 1H(H-5); 7.38, 1H(H-6); 7.63m, 1H(H-7); 7.55m, 1H(H-8); 8.17d(J = 9)2H(H-2′,6′); 6.80d (J = 9) 2H(H-3′,5′); 3.68m, 20H(CH_2–CH_2). IR Spectrum: 1090, 1120, 1170 – ν(C–O); 1580 – ν(C=O); 1505 – ν(C=C); 2860, 2920 - ν(CH_2). UV Spectrum in ethanol: λ_{max} = 408 nm (e = 39 900 $dm^3\,mol^{-1}\,cm^{-1}$).

2.4. Fluorescence measurements

Fluorescence studies for the FME and FCR probes were performed using the SLM 8300 spectrofluorometer. The probe : lipid molar ratio used was 1 : 500, unless specified otherwise.

The excitation wavelength was 405 nm for FME and 408 nm for FCR. The fluorescence emission spectra were measured in the 420–640 nm range. The excitation and emission slit widths were 8 and 16 nm, respectively. A control sample prepared without a fluoroprobe had < 1% scattering signal. The temperatures of the samples were monitored with an Instrulab Model 700 digital thermometer, with the thermistor placed in a parallel reference cuvette. The samples were magnetically stirred during the measurements.

3. Results

3.1. Fluorescence spectra of FME and FCR in various solvents

The solubility of both FME and FCR probes in solvents as different as hexane and water is sufficient for spectroscopic studies. Fig. 2(a) and (b) show the fluorescence spectra of FME and FCR in solvents with different polarities. The emission yield varies with the solvent, for example, the fluorescence intensity of both probes in water was several times less than that in the organic solvents under the same experimental conditions. FCR and FME each exhibit a single fluorescence peak at 535 nm in water and 518 nm in ethanol, which corresponds to the emission of the normal form of the flavonol molecules binding with molecules of solvent by intermolecular hydrogen bonds. The fluorescence spectra of both probes in hexane show a single maximum at 550 nm for FME and 555 nm for FCR, due to the emission band of the phototautomer form. In solvents of medium polarity, like acetonitrile, the probes exhibit two peaks. For both probes, the emission band of the normal form occurs at 500–505 nm, while the fluorescence maximum of the phototautomeric form undergoes a shift in the long wavelength direction to 570–573 nm. For FME in acetonitrile, the ratio of the spectral intensities of the phototautomeric and the normal form, PT/N, is approximately one but for FCR this ratio is greater than one.

We suggest that these flavonol compounds may be effective fluorescence probes for the investigation of the structure and physical properties of lipid bilayers. The peak positions and the PT/N intensity ratio can be used to estimate the degree of polarity and hydration of the probe environment.

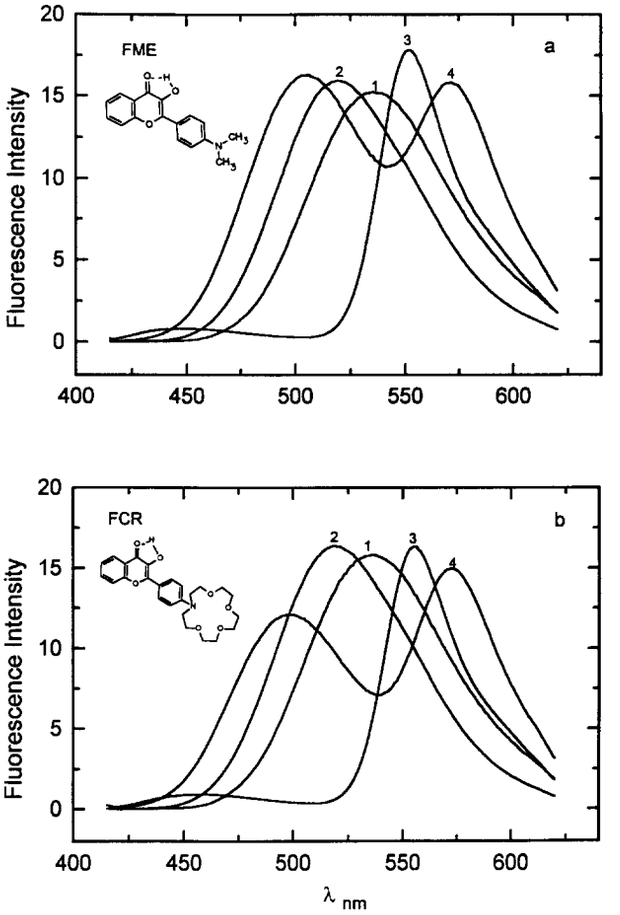

Fig. 2. The fluorescence emission spectra of (a) FME and (b) FCR in the various solvents: 1 – water; 2 – ethanol; 3 – hexane; and 4 – acetonitrile. The intensities do not reflect relative fluorescence yield.

3.2. Fluorescence study of the interdigitation of DPPC

Fig. 3 shows the fluorescence spectra of FME in DPPC in the presence and absence of ethanol. In the absence of ethanol, the fluorescence spectrum displays two maxima at 510 and 576 nm which correspond to the fluorescence of the normal (N) and phototautomer (PT) forms, respectively. In the presence of 30 mg/ml ethanol, which is below the threshold concentration for interdigitation, the fluorescence intensity decreases without changing the shape of the spectrum. The spectral shape changes significantly between 50 and 60 mg/ml ethanol,

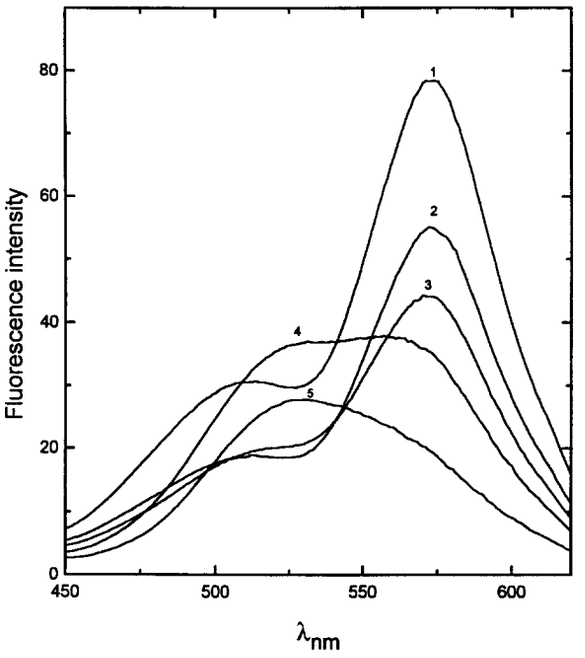

Fig. 3. Emission spectra of FME fluorescence in DPPC MLVs in water at 24°C in the presence of ethanol: 1 – 0 mg/ml ethanol; 2 –30 mg/ml ethanol; 3 – 50 mg/ml ethanol; 4 – 60 mg/ml ethanol; and 5 – 100 mg/ml ethanol.

which is the concentration range where the transition to the interdigitated phase is known to occur [26]. At 100 mg/ml ethanol, where the lipid is fully interdigitated, the spectrum shows a single peak at 525 nm.

Similar results are obtained for the FCR probe in DPPC in the presence and absence of ethanol, as shown in Fig. 4. Again, there is a shift in the spectral shape between 50 and 60 mg/ml ethanol, and again, the spectral shapes for interdigitated and non-interdigitated lipid are very distinctly different.

These data agree well with results which have been shown for DPPC using Prodan fluorescence [22,34,35,43–45]. As with Prodan, both of these flavonol probes are sensitive to the transition from the non-interdigitated to the interdigitated gel phase of DPPC. The change in the spectral shape during the phase transition from the non-interdigitated to the interdigitated phase can be conveniently followed using the intensity ratio of the two maxima, the PT/N ratio. Fig. 5 shows the PT/N ratio for FME in DPPC in the presence and absence of ethanol as a function of temperature. In the absence of ethanol, the PT/N ratio increases gradually up to $\approx$ 12°C, and then increases somewhat more rapidly to a maximum at 25°C. This indicates that the polarity of the probe environment decreases with increasing temperature. These changes suggest that the FME can move to the more hydrophobic region of the bilayer with increases in temperature as the bilayer goes through the pretransition to the rippled P_β phase. At the transition temperature for main transition to the liquid-crystalline phase (L_α), the PT/N ratio decreases very sharply, indicating increased polarity in the FME environment. Additional increase in temperature does not produce significant changes in the ratio values.

In the presence of 100 mg/ml of ethanol, the results for FME in DPPC are strikingly different. As shown in Fig. 5 (line 2), at low temperatures the polarity around the probe, as indicated by PT/N ratio, was somewhat higher than for pure DPPC but, beginning at 10–12°C which corresponds to the transition for the L'_β to the interdigitated $L_\beta I$ phase [18], the PT/N ratio decreases, indicating an increase in polarity. In the region of the existence of the interdigitated gel phase, 15°C–30°C, the polarity of the probe

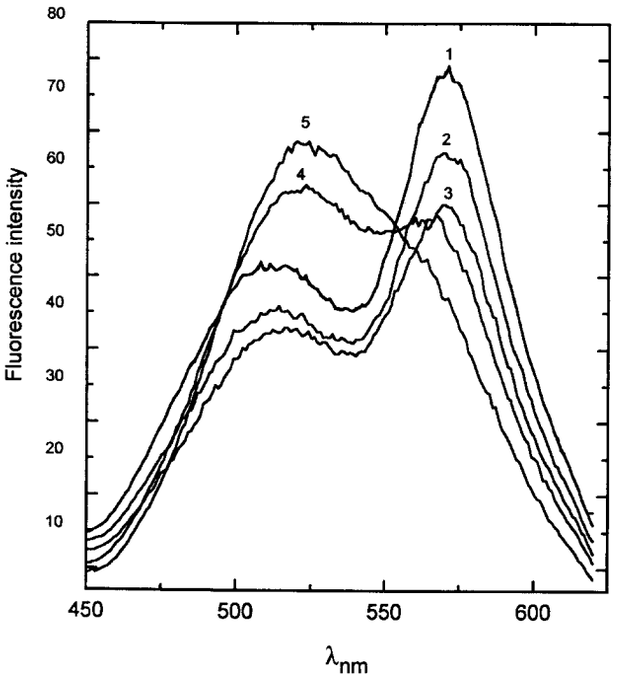

Fig. 4. Emission spectra of FCR fluorescence in DPPC–MLVs in water at 24°C in the presence of ethanol: 1 – 0 mg/ml ethanol; 2 – 20 mg/ml ethanol; 3 – 40 mg/ml ethanol; 4 – 60 mg/ml ethanol; and 5 – 100 mg/ml ethanol.

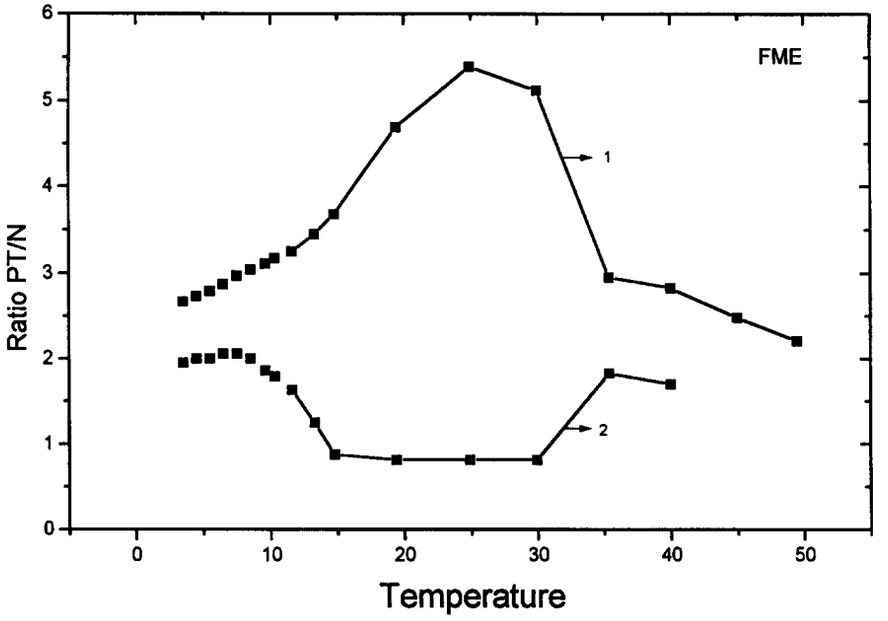

Fig. 5. PT/N ratio for FME as a function of temperature in DPPC–MLVs in water: 1 – heating sequence without ethanol; and 2 – heating sequence with 100 mg/ml ethanol.

environment does not change. Above 30°C, the PT/N ratio returned to the values found at the low temperatures.

The temperature dependences of the PT/N ratios for FME in DPPC show a discrepancy between the phase transition temperature as measured by differen-

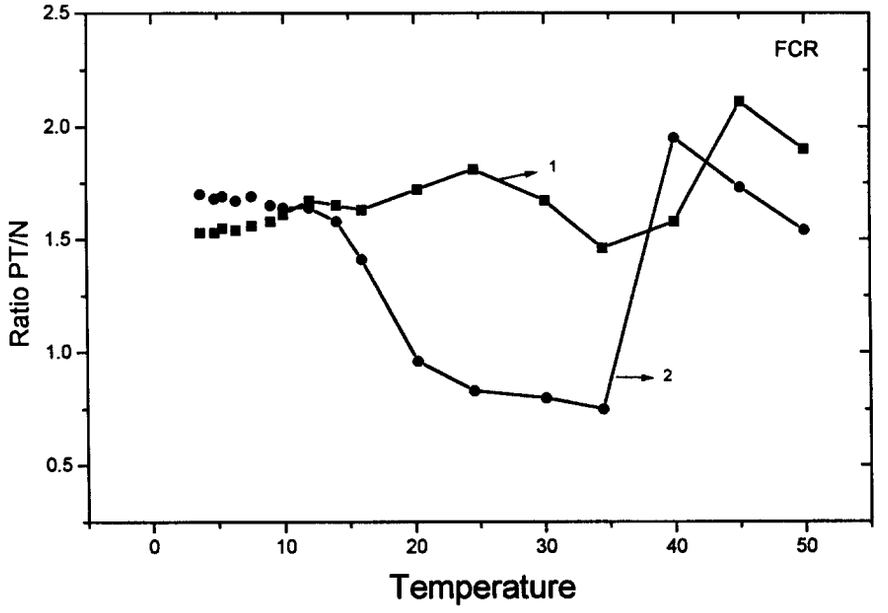

Fig. 6. PT/N ratio for FCR as a function of temperature in DPPC–MLVs in water: 1 – heating sequence without ethanol; and 2 – heating sequence with 100 mg/ml ethanol.

tial scanning calorimetry, 41.3°C, [43] and our fluorescence measurements, namely 35–38°C. Similar discrepancies were observed previously for temperature measurements using Pyr-PC fluorescence, and it has been suggested that this result reflects preferential interactions of the probe with the liquid-crystalline phase, i.e. the probe preferentially localizes in that part of the bilayers which have already melted [43,18].

The results which have been obtained for the FCR probe are somewhat different from those for FME. Fig. 6 shows the PT/N ratio vs. temperature for FCR in DPPC in the presence and absence of ethanol. The PT/N ratio for DPPC in the absence of ethanol is nearly constant with increase in temperature, with a small change in the region of the pretransition (35°C) and also at the temperature of the main phase transition (40°C). In the presence of 100 mg/ml ethanol, however, the PT/N ratio decreases abruptly at ca. 12°C, the temperature of the formation of the interdigitated gel phase. At ca. 35°C, it returns to the level obtained in the absence of ethanol, reflecting the presence of the L_α phase. Again, the transition observed here appears to occur below the true transition temperature of 41.3°C, suggesting that the probe preferentially locates itself in the melted regions of the bilayers.

The results reported here for both probes show that they can be used to monitor the non-interdigitated-to-interdigitated phase transition. One of the advantages of these probes is that both the FME and FCR measurements not only detect the presence of interdigitation but also gives a measure of the polarity of the environment around the fluorophore.

3.3. Fluorescence study of interdigitation of DPPeth

Our laboratory has previously shown that DPPeth in Tris-HCl buffer forms the interdigitated gel phase without any additives [22]. In the further pursuit of the possibility of using FME and FCR as probes for detecting the interdigitation of lipid bilayers, we investigated the fluorescence spectra of both probes in DPPeth.

Fig. 7(A) shows spectra of FME in DPPeth MLVs in 50 mM Tris-HCl in the presence, and absence of ethanol. The fluorescence spectrum in DPPeth in the absence of ethanol has a single broad maximum

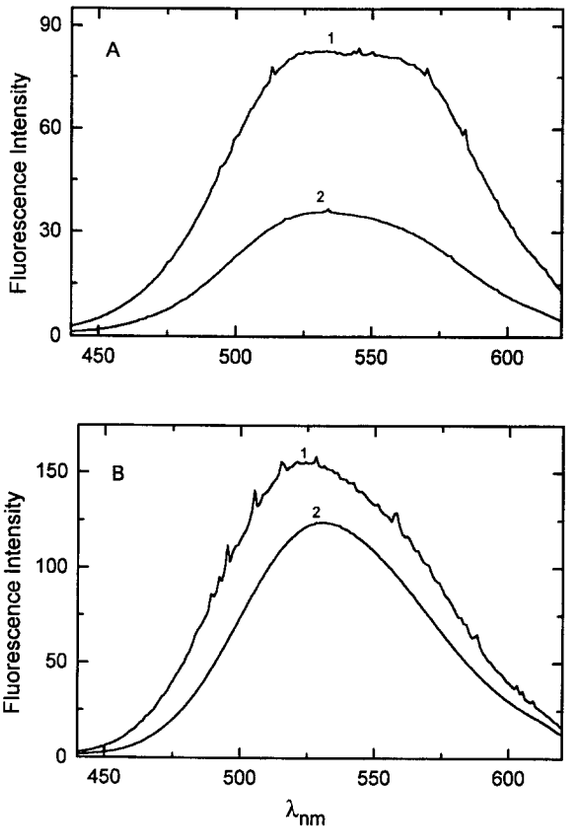

Fig. 7. (A) FME fluorescence spectra in DPPeth–MLVs in 50 mM Tris-HCl (pH 7.4) at 24°C: 1 – 0 mg/ml ethanol; and 2 – 100 mg/ml ethanol; (B) FCR fluorescence spectra in DPPeth MLVs in 50 mM Tris-HCl (pH 7.4) at 24°C: 1 – 0 mg/ml ethanol; and 2 – 100 mg/ml ethanol.

between 500 and 560 nm. The FME spectrum in pure DPPeth is similar to that of FME in DPPC in the presence of 60 mg/ml ethanol (Fig. 3), indicating that it is existing at least partially in an interdigitated gel phase. In the presence of 100 mg/ml ethanol, the fluorescence spectrum does not change significantly, although the peak is somewhat narrower. This suggests that ethanol enhances the formation of the interdigitated phase. These data are in good agreement with the results which were obtained for DPPeth using Prodan fluorescence and DSC techniques [22].

The corresponding data for FCR spectra in DPPeth are shown in Fig. 7(B). In the presence of 50 mM Tris-HCl, DPPeth has an FCR fluorescence spectrum with a maximum at 525 nm. Adding 100 mg/ml ethanol does not change the shape of the fluorescence

spectrum, but does shift the fluorescence maximum to 530 nm. As with DPPC, the fluorescence spectra of the two probes in DPPeth differ somewhat from one another, supporting our suggestion about the differing location of these two probes in the bilayer.

3.4. Fluorescence study in cholesterol-containing mixtures

It has been previously shown that increasing the concentration of cholesterol in PCs abolishes interdigitation of the gel phase, and more than 20 mol% of cholesterol prevents the induction of interdigitation by ethanol [18,46]. In the present study, both FME and FCR probes were used for the investigation of interdigitation in the presence of cholesterol under various conditions. Fig. 8(A) shows the fluorescence spectra of FME in DPPC–cholesterol MLVs in the presence and absence of ethanol. The fluorescence spectrum of FME in DPPC with 5 mol% of cholesterol (spectrum 1) exhibits two bands of fluorescence with the main maximum at 576 nm and a small peak at 480–500 nm, characteristic of the non-interdigitated structure. In the presence of 100 mg/ml ethanol (spectrum 2) the intensity of the 576 nm band decreased and the small shorter wavelength band shifted to 520–530 nm. In the case of DPPC with 20 mol% of cholesterol, the fluorescence spectrum of FME has a maximum at 578 nm and a weak shoulder at 486 nm (spectrum 3). Adding 100 mg/ml ethanol to the DPPC with 20 mol% cholesterol produces a spectrum nearly identical to that seen with 5 mol% cholesterol in the absence of ethanol (spectrum 4). These results are not consistent with the known behavior of the DPPC–cholesterol system. As has been shown by Komatsu and Rowe [18], ethanol restores interdigitation in DPPC with 5 mol% cholesterol. We would thus have expected that the FME spectrum of the DPPC–5 mol% cholesterol system in the presence of ethanol would change and reflect at least partial interdigitation of the bilayers. Instead, the observed spectrum continues to show the characteristic non-interdigitated spectrum.

Fig. 8(B) shows the fluorescence spectra of FCR in DPPC with two concentrations of cholesterol in the presence and absence of ethanol. The fluorescence spectrum of FCR in DPPC with 5 mol% cholesterol exhibits two maxima at 508 and 573 nm

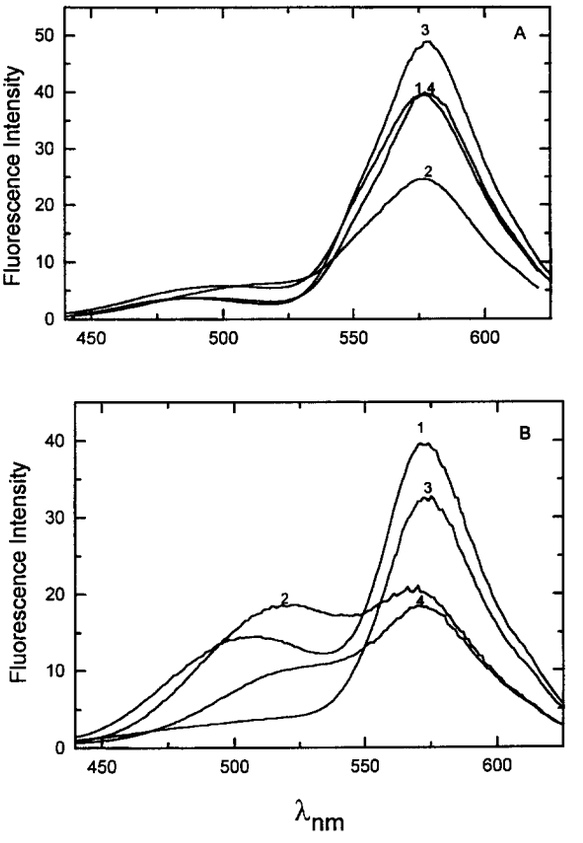

Fig. 8. Effect of cholesterol on the emission spectra of (A) FME fluorescence and (B) FCR fluorescence in DPPC–MLVs in water at 24°C: 1 – 5 mol% cholesterol; 2 – 5 mol% cholesterol in the presence 100 mg/ml ethanol; 3 – 20 mol% cholesterol; and 4 – 20 mol% cholesterol in the presence of 100 mg/ml ethanol.

(spectrum 1). Adding 100 mg/ml ethanol leads to a decrease in intensity of the longer wavelength maximum and shifts the shorter wavelength maximum to 520 nm, increasing its intensity (spectrum 2). The shape of this spectrum is similar to the DPPC spectrum in the presence of 60 mg/ml ethanol, and suggests a partially interdigitated gel phase. The FCR spectrum of DPPC with 20 mol% cholesterol shows one maximum at 573 nm, with disappearance of the shorter wavelength peak (spectrum 3). In the presence of 100 mg/ml ethanol (spectrum 4), the intensity of this maximum decreases and a slight shoulder reappears at 520 nm. This suggests that, in the presence of 20 mol% cholesterol, ethanol restores interdigitation in a smaller proportion of the membrane than in the presence of 5 mol% cholesterol. This is in agreement with previous results in which the fully

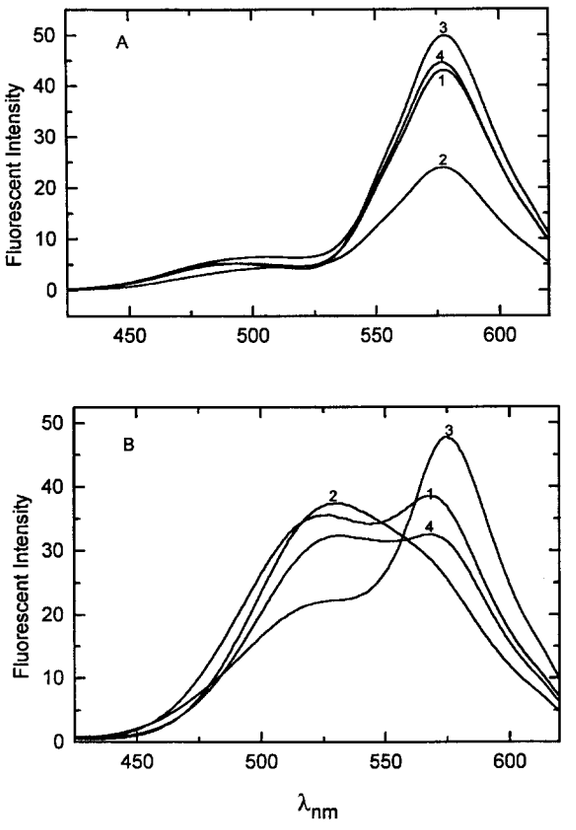

Fig. 9. Effect of cholesterol on the emission spectra of (A) FME fluorescence and (B) FCR fluorescence in DPPeth–MLVs in 50 mM Tris-HCl (pH 7.4) at 24°C: 1 – 5 mol% cholesterol; 2 – 5 mol% cholesterol in the presence 100 mg/ml ethanol; 3 – 20 mol% cholesterol; and 4 – 20 mol% cholesterol in the presence of 100 mg/ml ethanol.

interdigitated and non-interdigitated phases coexist in DPPC–MLVs at both, 5 mol% and 20 mol% cholesterol in the presence of ethanol [18,46].

In order to further investigate the behavior of the two flavonol probes in the lipid bilayer, we studied the DPPeth–cholesterol MLVs under various conditions. Fig. 9(A) shows the fluorescence spectra of FME in DPPeth with 5 mol% and 20 mol% cholesterol in Tris-HCl buffer in the presence, and absence of ethanol. The fluorescence spectra of FME in DPPeth–cholesterol mixtures exhibit one maximum at 576 nm, typical for the non-interdigitated spectrum. Neither an increased concentration of cholesterol, nor the presence of 100 mg/ml ethanol significantly changes the shape of the fluorescence spectrum, although ethanol does decrease the fluorescence intensity. The results of FME fluorescence in DPPeth–cholesterol mixtures are similar to the behavior of this probe in the DPPC–cholesterol system. It suggests that FME interacts preferentially with cholesterol, and remains in the lipid phase in the cholesterol rich regions, even in the presence of only 5 mol% of cholesterol.

Different results were obtained for FCR fluorescence in DPPeth–cholesterol MLVs in the presence, and absence of ethanol. As shown in Fig. 9(B), 5 mol% cholesterol only partially eliminates the interdigitated gel phase in DPPeth in Tris-HCl. The fluorescence spectrum of FCR has two maxima, at 525 and 570 nm, respectively (spectrum 1), and the shape of the fluorescence spectrum is similar to the fluorescence spectrum of FCR in DPPC in the presence of 60 mg/ml ethanol. This indicates that some parts of the lipid bilayer are still interdigitated in the presence of 5 mol% cholesterol. In the presence of 100 mg/ml ethanol, interdigitation is completely restored and the fluorescence spectrum exhibits one maximum at 525 nm (spectrum 2). The fluorescence spectrum of FCR in the DPPeth with 20 mol% cholesterol indicates a decrease in the area of interdigitated gel phase in the membrane and adding 100 mg/ml ethanol only partially restores the interdigitation. The spectrum is similar to that in DPPC in the presence of ≈ 60 mg/ml ethanol.

The foregoing results demonstrate that both, FME and FCR probes can successfully be used to investigate the interdigitated gel phase in pure lipid bilayers. FCR can also be used in mixtures which contain cholesterol; the data shown here indicate that it is still randomly distributed and reflects the bilayer structures. On the other hand, FME shows anomalous behavior in the presence of cholesterol, suggesting that it is not randomly distributed. Therefore, FME will be a useful probe for carefully known conditions, but should be used with caution in the presence of more complex mixtures such as biological membranes.

4. Discussion

Fluorescence techniques possess high sensitivity and relative simplicity in methodology. For this reason fluorescence spectroscopy has been widely uti-

lized to study the structure and dynamics of model and biological membranes. Widening the group of molecular probes, available for investigation of the structural properties of lipid membranes, can also be useful for our comprehension of the complicated mechanisms which take place in cell membranes. In the present study we employed two flavonols, FME and FCR, as new fluorescence probes for the study of interdigitation in the lipid bilayer in the presence, and absence of ethanol. This model of interdigitation has been well studied in our laboratory using several other methods, enabling us to assess the flavonols as probes of bilayer behavior.

The results obtained for DPPC in the absence of ethanol show two maxima in the fluorescence spectra of both, the FME and FCR probes. The peak at 570 nm corresponds to the fluorescence of the phototautomeric form of these probes, and is shifted to a longer wavelength region compared with its maximum in hexane; it coincides with the fluorescence maximum of FME in acetonitrile [6]. The shorter wavelength band of FME and FCR in DPPC comes from the fluorescence of the probes in normal form. The obtained spectra suggest that these probes are located in a region of the bilayer near the interfacial region and partially in the interfacial region, where they experience an intermediate polarity.

Adding 30–50 mg/ml ethanol leads to a decrease in the fluorescence intensity. This suggests that ethanol molecules displace water molecules at the lipid–aqueous interface and change the polarity around the head groups, without changing the distribution of FME or FCR in the membranes. Increasing the ethanol concentration to 60 mg/ml changes the fluorescence spectrum of both probes; this is the ethanol concentration at which interdigitation begins [26]. The fluorescence maximum at 513 nm shifts to 530 nm and the fluorescence intensity increases. At the same time, the fluorescence intensity at 570 nm decreases. These spectral changes indicate that interdigitation leads to the relocation of the probes in the bilayer. If we compare the FME fluorescence peak at 530 nm with its position in water (535 nm) or ethanol (520 nm), we can suggest that most of the FME molecules are located in the region of the phosphatidylcholine head groups. The presence of the peak at 570 nm suggests that some of the molecules of FME are still located in the hydrophobic acyl chain region of the membrane. In the case of fully interdigitated gel phase at 80 mg/ml ethanol, the fluorescence maximum at 570 nm completely disappears, indicating that the relocation of the probes to the interfacial region is complete. Additional increases in ethanol concentration only decrease the fluorescence intensity at 530 nm, which can be explained by a decrease in viscosity around the probe in the bilayer.

The overall behavior of both probes in the DPPC membrane in the presence and absence of ethanol was similar. However, there were some significant differences in the fluorescence spectra of FME and FCR. The PT/N ratios for FCR and FME in the pure gel phase DPPC were different. For FME this ratio was 2.55, and for FCR it was 1.52. This predominance of the phototautomer for FME suggests that the FME molecules are located more deeply in the hydrophobic region of the membrane than FCR. The lower PT/N ratio for FCR indicates that its location is more polar, i.e. more shifted toward the polar interfacial region.

Differences in the behavior of the probes were also observed in the presence of ethanol. The PT/N ratio for FME was higher than for FCR in the presence of low concentrations of ethanol. This again indicates that FME is situated in the less hydrated area compared with FCR. The shift of the short wavelength maximum of FME took place at 50 mg/ml ethanol, whereas for FCR it began at 20 mg/ml. The relocation of FCR into the more polar environment is, thus, more sensitive to the alterations in the membrane caused by the presence of ethanol. In the presence of high concentrations of ethanol, the ratios of intensities for both probes were equal, suggesting the same location in the bilayer after full interdigitation.

In order to discuss the differences in the behavior of these two probes, we have to examine the chemical structural of these molecules. A main factor which can influence the behavior of FME and FCR is the difference in the sizes and hydrophilicity of the methyl groups of FME compared with the macrocyclic fragment of FCR. On the one hand, the azacrown group of FCR does not allow this part of the FCR molecules to easily penetrate into the region of the lipid acyl chains. On the other hand, the polar molecules of solvent cannot easily reach the nitrogen atom of FCR, which has the positive charge when FCR is in the

exited state. This means that the solvation of FME by ethanol is more effective than for FCR.

The differences in the chemical structure also suggest an explanation for the differing results which were obtained for the DPPC–cholesterol and DP-Peth–cholesterol systems with the two probes. We have found that the spectra of FME do not change as expected for the known transitions of cholesterol–DPPC system to the partially and fully interdigitated phases, whereas the FCR spectra do reflect these phase transitions which have already been established by other means. The FME spectrum in the non-interdigitated bilayer with cholesterol is slightly more non-polar than in the absence of cholesterol. The FME spectrum in the presence of cholesterol with ethanol reflects the more polar environment of the non-interdigitated phase even in the presence of only 5 mol% cholesterol, where it is known that the bilayer is nearly completely interdigitated [18,46]. This suggests that the FME has a strong preference for the non-interdigitated phase in the presence of cholesterol, so that it remains distributed in this portion of the bilayer even when it is a very small portion of the lipids. Hence the conclusion that the FME has specific interactions with cholesterol, because it does not show any preference for the non-interdigitated gel in the absence of cholesterol. On the other hand, the FCR probe behaves as if it is randomly distributed in the bilayers, reflecting the same proportion of interdigitated and non-interdigitated phases as expected from previous studies. The structure of FME suggests that it is more hydrophobic than FCR, as discussed here and, thus, its relocation to the interfacial region due to interdigitation is less favorable than for FCR. This property of FME of preferentially interacting with cholesterol may be useful in particular situations, but it does limit the usefulness of FME for studying the structure of membranes with complex compositions including biological membranes.

The present study clearly shows that both flavonols, FME and FCR, can be useful for investigation of the transition of the non-interdigitated to the interdigitated gel phase. These probes have some advantages over other fluorescence probes that have been used to monitor this transition. The spectral change which accompanies the transition is large, making it relatively easy to measure accurately. In contrast to some other fluorescence methods, for example the DPH method [26], the detection of interdigitation does not depend on observing changes in fluorescence at the transition itself, but is based on the spectral shape itself. Prodan is another probe which has a similar behavior to these flavonols [22,44,45]. We have found that FME behaves anomalously in the presence of cholesterol, apparently interacting preferentially with it, so that this probe will not detect interdigitated phases in the presence of cholesterol. A behavior similar to that of FME in the presence of cholesterol was observed with Prodan (Bondar and Rowe, unpublished).

These new probes should also be useful in studying other aspects of membrane structure and dynamics. The spectral parameters which are sensitive to the probe environments are both ratios of phototautomer to normal forms, and also the spectral shift of the maxima for each form. Both of these properties are reflections of the microenvironemnt of the probes, and it can be expected that these probes will be found to be useful in studying other subtle lipid structural and environmental changes. The behavior of FME in the presence of cholesterol is anomalous and, therefore, this probe will not reflect the total lipid behavior in membranes containing cholesterol. However, now that it is recognized, there may be some ways to exploit this property in other membrane systems.

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