Photophysical behaviour of ground state anion and phototautomer of 3-hydroxyflavone in liposome membrane†

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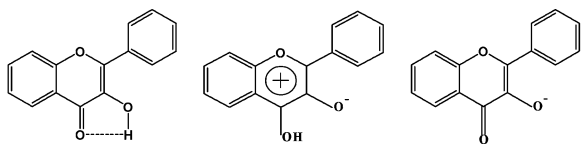
A detailed account of the photophysical behaviour of the phototautomer (PT) and the ground state anion (A^-) of 3-hydroxyflavone in liposome membrane at various membrane conditions is presented. A quenching study with a hydrophilic quencher Ag^+ suggests that the phototautomeric emission generates from the fraction of 3HF that is located at the inner hydrophobic core, whereas the ground state anionic emission is from the fraction that resides near the water-accessible surface site. However, the biexponential nature of fluorescence decays of both the forms indicates that there is local heterogeneity in the distribution. Temperature dependence studies and experiments in the presence of ethanol reveal that, as the membrane becomes more fluid, redistribution of 3HF takes place between the two sites leading to increase in A^- population. The temperature dependence of the fluorescence anisotropy change of PT shows good correlation with the phase change and shows a sharp drop at the transition temperature, whereas the corresponding change in the case of A^- is gradual.

Introduction

3-Hydroxyflavone (3HF) contains the basic structural moiety of naturally occurring flavonoids such as chrysin, fisetin, quercetin *etc.* It is considered as one of the best models for the study of excited state intramolecular proton transfer (ESIPT); consequently its solution-phase photophysics has been a topic of great interest.[1–5] In nonpolar, noninteracting solvents such as hydrocarbons, 3HF mainly shows emission from the excited state intramolecular proton-transferred species known as the phototautomer (PT; λ_{ex} = 345 nm, λ_{em} = 535 nm); in protic solvents such as alcohols both intra- and inter-molecular (3HF–solvent) hydrogen bonding are possible resulting in dual emission from the neutral species (λ_{ex} = 345 nm, λ_{em} = 410 nm) and phototautomer. In water at neutral pH, 3HF exists in its neutral form in the ground state and exhibits emission at two wavelengths, 410 nm and 510 nm. The 410 nm emission is assigned to the neutral form, however the emission at 510 nm has been a subject of discussion. The broad emission region at 510 nm (512 nm in neutral water solution) has been assigned to the phototautomer luminescence[6–8] and the same 510 nm region emission is also suggested to be due to the photoanion,[1,9,10] taking into consideration the hydrogen-bonding characteristics of water and its ability to accept free protons. There are also literature reports on the formation of ground state anion (A^-) of 3HF in solvents such as formamide,[11] neat alcohol,[12] acetonitrile–methanol mixture[12] and DMSO.[5] Fluorescence of the photoanion and the ground state anion can easily be differentiated by their excitation wavelengths: for the photoanion it is at 345 nm, whereas for the ground state anion it is at 417 nm (Scheme 1).



3HF Neutral form
λ_{ex} = 345 nm,
λ_{em} = 410 nm

3HF Tautomer form
λ_{ex} = 345 nm,
λ_{em} = 535 nm

3HF Anionic form
In water (excited state anion):
λ_{ex} = 345 nm; λ_{em} = 510 nm
In liposome (Ground state anion):
λ_{ex} = 417 nm; λ_{em} = 490 nm

Scheme 1 Different forms of 3-hydroxyflavone and their excitation and emission maxima.

3HF has found application as a probe in the study of micelles, reverse micelles, liposomes and proteins.[13,14] In a liposome medium the predominant emission is due to the phototautomer.[15] Shyamala *et al.*[9] observed the existence of ground state anion of 3HF in dimyristoylphosphatidylcholine (DMPC) liposome membrane even at neutral pH. Recently the ground state anion (λ_{ex} = 410 nm, λ_{em} = 460 nm) formation has also been observed in egg yolk phosphatydilcholine (EYPC) liposomes.[16] In order to use 3HF as a probe for liposomal studies it was felt necessary to acquire a more detailed understanding of the photophysical behaviour of both phototautomer and ground state anion under different membrane conditions. This is the objective of the present work.

Experimental

Materials

3HF (Sigma–Aldrich, Bangalore, India) was recrystallized twice from cyclohexane. DMPC and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma Chemical Co. (Bangalore, India) and used as received. All solvents were distilled before use. Water, distilled twice from alkaline permanganate solution, was used for the experiments.

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† Electronic supplementary information (ESI) available: Emission spectra of PT and A^- with temperature from 15 °C to 35 °C in methanol (Fig. S1); emission spectra of PT and A^- with temperature from 28 °C to 55 °C in DPPC vesicles (Fig. S2).. See DOI: 10.1039/b905906g

Liposome preparation

For this work, small unilamellar vesicles (SUV) were prepared by the ethanol injection method.[17] The stock solution of the lipid was prepared in ethanol. The desired amount of ethanolic solution of lipid was injected rapidly into the aqueous solution of 3HF equilibrated for 30 min at 45 °C and 55 °C for DMPC and DPPC, respectively. The percentage of ethanol in the solution was less than 1% (v/v). Throughout the experiments, the 3HF-to-lipid molar ratio was kept constant at 1 : 120 (5 μM 3HF, 0.6 mM lipid). At this concentration, maximum partitioning of 3HF into the membrane is expected.[9] All experiments were performed with freshly prepared solutions of both 3HF and liposome.

Solutions for quenching studies

For the quenching studies, a fixed volume of Ag^+ solution of appropriate concentration was added to the experimental liposome solution under dark condition and fluorescence measurements were performed immediately.

Fluorescence measurements

Fluorescence measurements were carried out with a Hitachi F-4500 spectrofluorometer. The emission spectra were recorded with slit widths of 5/5 nm, by fixing excitation wavelengths at 345 nm for the PT and 417 nm for the A^-. Temperature was controlled by circulating water through a jacketed cuvette holder from a refrigerated bath (JULABO, Germany). The steady state fluorescence anisotropy (r_{ss}) values were obtained by using the expression

$$r_{ss} = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + 2GI_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities when the emission polarizer is parallel and perpendicular, respectively, to the direction of polarization of the excitation beam and G is the factor that corrects for unequal transmission by the diffraction gratings of the instrument for vertically and horizontally polarized light.

Fluorescence lifetime measurements

Fluorescence lifetime measurements for A^- (λ_{ex} = 413 nm, λ_{em} = 490 nm) were carried out using an IBH single-photon counting fluorimeter in a time-correlated single photon counting arrangement based on ps/fs Ti–Sapphire Laser system (Tsunami Spectra Physics, Bangalore, India) for excitation. The pulse repetition rate was 82 MHz and the full width half maximum was less than 2 ps. The emission was collected at magic angle polarization (54.7°) to avoid any polarization in the emission decay. The instrument response time is approximately 50 ps. For PT (λ_{ex} = 340 nm, λ_{em} = 535 nm) lifetime measurements were carried out using Horiba Jobin Yvon TCSPC lifetime instrument in a time-correlated single-photon counting arrangement. A 340 nm nano-LED was used as the light source. The pulse repetition rate was set to 1 MHz and the instrumental full width half maximum of the 340 nm LED, including the detector response is ~800 ps. The instrument response function was collected using a scatterer (Ludox AS40 colloidal silica). The decay data were analyzed using IBH software. A value of $0.99 \leq \chi^2 \leq 1.4$ was considered as a good fit, which was further judged by the symmetrical distribution of the residuals. The average fluorescence lifetime values were obtained by the following equation[18]

$$\tau_{avg} = \sum_{i=1}^{n} \alpha_i \tau_i \Big/ \sum_{i=1}^{n} \alpha_i$$

where τ_i is the individual lifetime with corresponding amplitude α_i.

Results and discussion

Earlier studies on 3HF in liposomes suggest that the phototautomer emission arises from the 3HF molecules deeply buried in the hydrophobic acyl chains region of the bilayer; the bi-exponential nature of its fluorescence decay however indicated heterogeneity in the distribution.[15] The unusually higher value of steady state anisotropy of A^- in liposome membrane suggested it is located in a rigid environment. Based on the experiments with charged lipids, it was proposed that A^- forms in the perturbed water region of the lipid bilayer membrane close to the PC head group.[9] A clear confirmation about the locations of the two species can be obtained from fluorescence quenching studies.

Fluorescence quenching studies

In the present study Ag^+ ion has been taken as a quencher. Being a hydrophilic cationic quencher it is expected to quench only the water-accessible form of 3HF near the interfacial region. Fig. 1A and B show the emission spectra of PT (λ_{ex} = 345 nm,

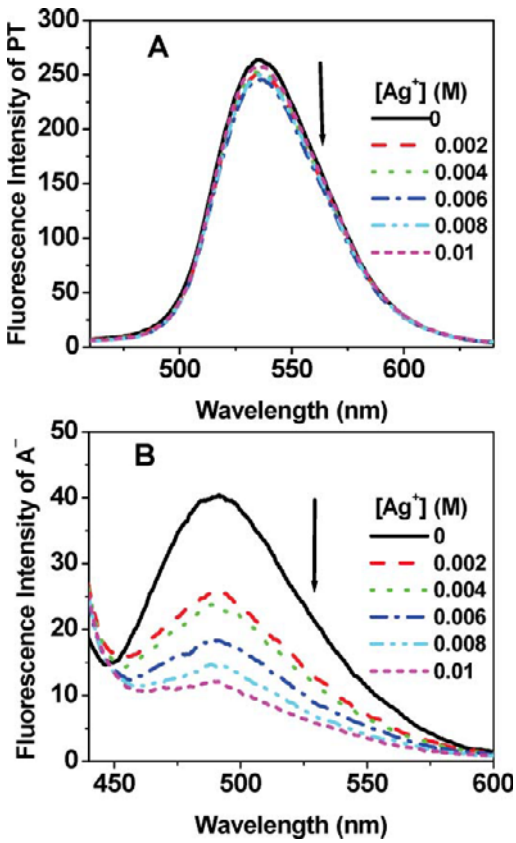

Fig. 1 Emission spectra of (A) PT (λ_{ex} = 345 nm), (B) A^- (λ_{ex} = 417 nm) with increasing Ag^+ concentration (0 M to 0.01 M) at 35 °C. [DMPC] = 0.6 mM, [3HF] = 5 μM.

λ_{em} = 535 nm) and A^- (λ_{ex} = 417 nm, λ_{em}= 490 nm) in DMPC liposomes with increasing Ag^+ concentration (0 M to 0.01 M) at 35 °C. This shows a significant quenching of A^- intensity on addition of just 0.002 M of Ag^+, whereas the PT intensity remains almost unquenched even at the highest concentration of the quencher used (0.01 M). Quenching efficiency was calculated from the Stern–Volmer plot of $((F_0/F) - 1)$ against Ag^+ concentration for the quenching of the ground state anion in DMPC liposome as shown in Fig. 2.

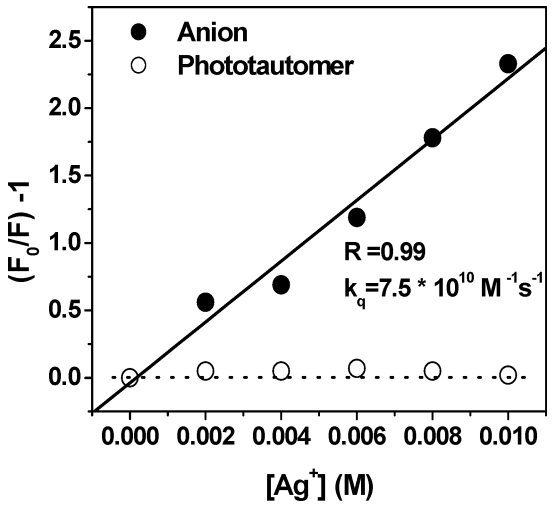

Fig. 2 Stern–Volmer plot for the quenching of fluorescence intensity of A^- and PT by Ag^+ as quencher in DMPC liposome.

The quenching constant was calculated from the slope of the linear plot based on the following equation

$$F_0/F = 1 + \tau_0 k_q[Q]$$

where F_0 and F are fluorescence intensities in absence and in presence of quencher, respectively, [Q] is the quencher concentration, τ_0 is the average lifetime in absence of quencher at 35 °C (3 ns for A^-) and k_q is the bimolecular quenching constant. The k_q value for A^- is found to be 7.5×10^{10} M^{-1} s^{-1}. The plot of $((F_0/F) - 1)$ against Ag^+ concentration for the PT is a straight line parallel to the x axis, which indicates there is almost no quenching for PT. Thus the quenching study provides strong evidence that the preferential locations of phototautomer is near the hydrophobic core, which is not accessible to the hydrophilic quencher and the ground state anion is located at the water-accessible surface site of liposome.

Temperature dependence studies

Phospholipid bilayers are known to change their phase state from a highly ordered solid gel (SG) phase at lower temperature to a fluid liquid crystalline (LC) phase at higher temperature.[17] This phase change in biological membranes is responsible for numerous vital membrane functions. Hence, it is important to know how the temperature-induced phase change affects the photophysical behaviour of the two forms of 3HF in the membrane. The phototautomer anisotropy is known to be sensitive to the phase change and from the temperature dependent anisotropy profile phase transition temperatures for DMPC and DPPC vesicles have been estimated.[15] In order to know the effect of phase change on the fluorescence behaviour of PT and A^- in greater detail, temperature dependence steady state fluorescence anisotropy and emission studies were carried out.

Fluorescence anisotropy studies

Fig. 3 shows the variation of steady state fluorescence anisotropy of PT and A^- with temperature from 15 °C to 35 °C in DMPC vesicles. Both the forms show remarkable increase in anisotropy upon partitioning into liposome medium. At 15 °C in methanol medium, PT and A^- show anisotropy values of 0.015 and 0.010, respectively; but at the same temperature PT exhibits anisotropy of 0.17 and A^- shows a value of 0.34 in DMPC liposome. The unusually high fluorescence anisotropy value 0.34 of A^- in liposome, which is close to its fundamental anisotropy (0.35 measured in glycerol at −10 °C), is attributed to the specific interaction of this form with the PC head group of lipid bilayer.[9] However for the PT form the r_{ss} value in liposome (0.17) is smaller than its fundamental anisotropy (0.26 measured in glycerol at −10 °C) indicating its location to be in a relatively less rigid environment. Temperature dependence studies show a decrease in anisotropy for both the forms as the temperature increases and the membrane fluidity increases. However, unlike the PT, where the anisotropy change follows nicely the phase change in membrane, the effect of temperature on the anisotropy of A^- is rather gradual. Since the PT is located at the core and the A^- at the surface site, the present study indicates that the phase change induced modifications at the core as sensed by the PT is sharper as compared to that at the surface site as sensed by A^-.

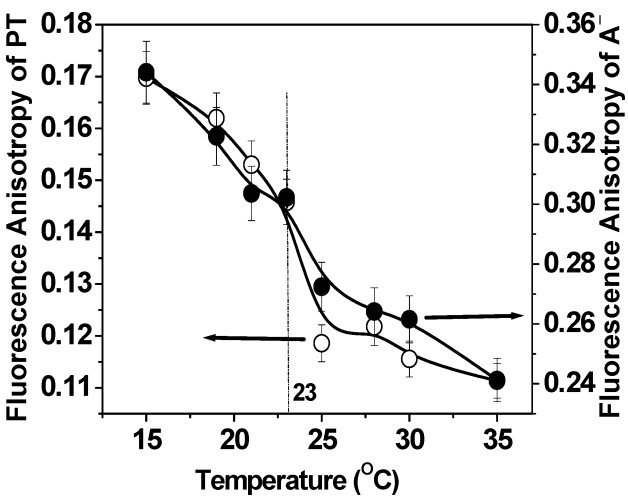

Fig. 3 A plot of variation of steady state fluorescence anisotropy of PT (λ_{ex} = 345 nm, λ_{em} = 535 nm) and A^- (λ_{ex} = 417 nm, λ_{em} = 490 nm) with temperature from 15 °C to 35 °C in DMPC vesicles. [DMPC] = 0.6 mM, [3HF] = 5 μM.

Fluorescence intensity studies

Fig. 4A and B represent the emission spectra of PT (λ_{ex} = 345 nm, λ_{em} = 535 nm) and A^- (λ_{ex} = 417 nm, λ_{em} = 490 nm) with increase in temperature from 15 °C to 35 °C in DMPC liposome. Fluorescence intensites of the two forms, *i.e.* PT and A^-, show

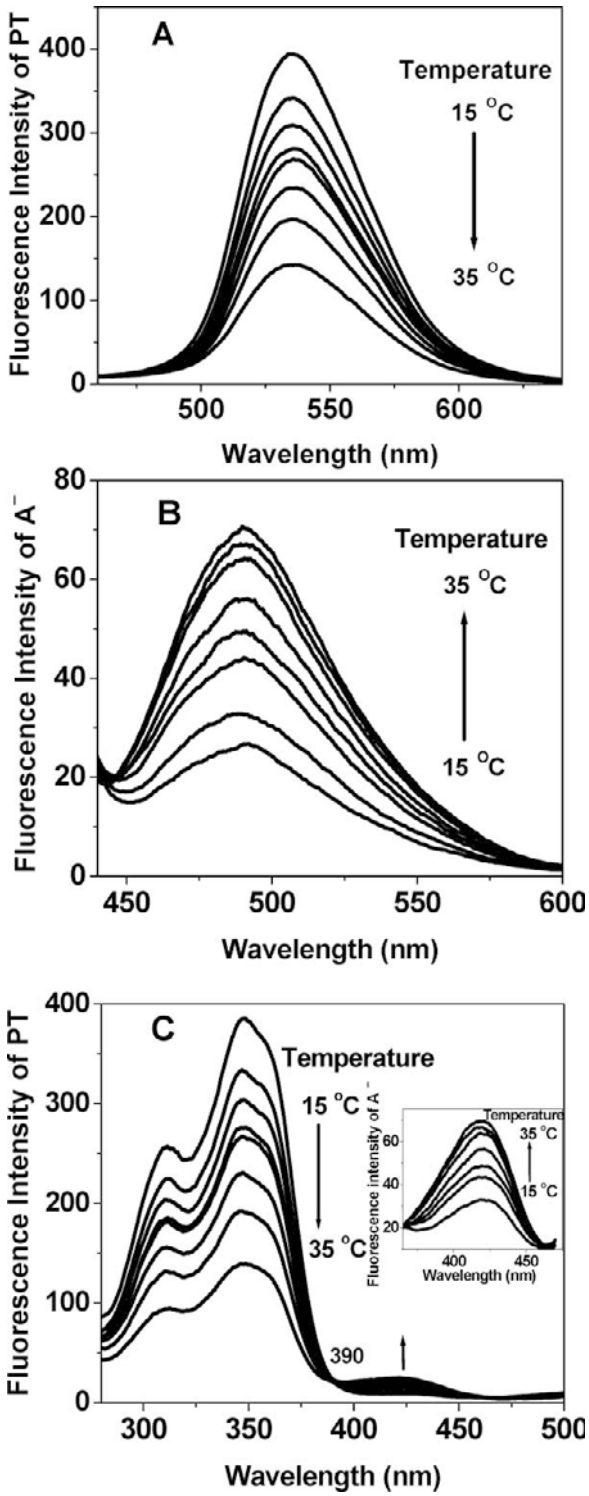

Fig. 4 (A) Emission spectra of PT ($\lambda_{ex} = 345$ nm), (B) emission spectra of A^- ($\lambda_{ex} = 417$ nm), (C) excitation spectra of PT ($\lambda_{em} = 535$ nm) and (inset) excitation spectra of A^- ($\lambda_{em} = 490$ nm) with increasing temperature from 15 °C to 35 °C in DMPC vesicles. [DMPC] = 0.6 mM, [3HF] = 5 μM.

opposite trends with increase in temperature. Fig. 4C represents the excitation spectra of PT ($\lambda_{em} = 535$ nm) and the inset shows the excitation spectra of A^- ($\lambda_{em} = 490$ nm) with temperature in liposome medium. The excitation spectra monitored at 535 nm show a decrease in intensity around 345 nm with corresponding increase in intensity of 417 nm band, which is further supported by the excitation spectra monitored at 490 nm (inset).

Fig. 5 shows the corresponding plot of variation of fluorescence intensity of PT and A^- with temperature in DMPC liposome. There is a decrease in the fluorescence intensity of phototautomer with a concomitant increase in the intensity of the ground state anion with temperature. For the same temperature range in neat methanol, intensity of both PT and A^- decreases with increase in temperature, which can be attributed to the increase in rates of non-radiative (k_{nr}) processes with increase in temperature (Fig. S1 in ESI†). In neat alcohol the trend is similar for both PT and A^-, whereas it is in opposite direction in the liposome medium. The observed spectral change in liposome medium with increasing temperature can be attributed to the redistribution of 3HF between the two sites of occupancy in the lipid matrix. This is supported by the excitation spectra (Fig. 4C), the presence of an isoemissive point at 390 nm clearly indicates the presence of a two component equilibrium. As the membrane becomes more fluid, more of the 3HF molecules from the inner hydrophobic site migrate towards the surface site resulting in depletion in the PT population with simultaneous increase in the A^- population. Similar observations have also been reported for 1-naphthol and several other fluorophores where distribution happens between the two sites, *i.e.* the inner site and surface site of liposome.[9–21] Hence, the decrease in intensity of PT with increase in temperature is due to decrease in 3HF population at the non-polar site and increase in the k_{nr}. But in case of A^- increase in population dominates over the increased k_{nr} effect showing an overall increase in intensity.

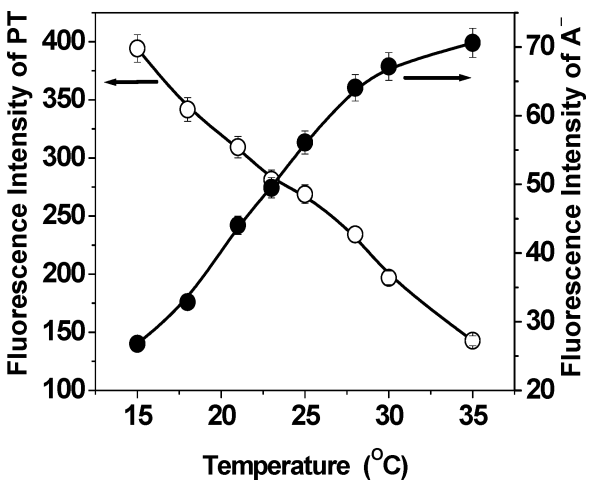

Fig. 5 Plot of variation of PT ($\lambda_{ex} = 345$ nm) and A^- ($\lambda_{ex} = 417$ nm) fluorescence intensity with temperature in DMPC vesicle from 15 °C to 35 °C.

Fluorescence lifetime studies

The effect of fluidity of membrane is clearly seen from the average fluorescence lifetime of both the forms in liposome medium. The value of τ_{avg} is found to decrease for both the phototautomer and the ground state anion as the membrane goes from rigid solid gel phase at lower temperature to fluid liquid crystalline phase at higher temperature (Table 1).

Both the forms show biexponential decays in the membrane. This suggests that although the preferred location for PT is at the

Table 1 Fluorescence lifetime data of PT (λ_{ex} = 340 nm, λ_{em} = 535 nm) and A^- (λ_{ex} = 413 nm, λ_{em} = 490 nm) in DMPC vesicles. [DMPC] = 0.6 mM, [3HF] = 5 μM

	Species	τ_1/ns, (α_1)	τ_2/ns, (α_2)	τ_{avg}/ns	χ^2
Solid gel phase (15 °C)	PT	3.5 ± 0.1, (53.0)	1.4 ± 0.1, (46.9)	2.5	1.1
	A^-	4.5 ± 0.2, (85.9)	1.4 ± 0.1, (14.1)	4.1	1.0
Liquid crystalline phase (35 °C)	PT	1.6 ± 0.1, (62.7)	0.8 ± 0.02, (37.3)	1.3	1.0
	A^-	3.9 ± 0.2, (82.3)	1.8 ± 0.1, (17.7)	3.5	1.0

PT: phototautomer, A^-: ground state anion, τ: Fluorescence lifetime, τ_{avg}: average fluorescence lifetime, α: relative amplitude in %, χ^2: fitting parameter.

hydrophobic core and that for A^- is at the interfacial region, there still remains a local heterogeneity in the distribution at the two sites of occupancy.

Since the relative intensities for PT and A^- in liposome medium are dependent on the redistribution of 3HF between the two sites of occupancy, any additive to membrane that can alter the distribution should also bring about a change in the intensities of the two forms. In order to verify this proposition, experiments were carried out by adding ethanol to DMPC vesicles.

Effect of ethanol

Ethanol, being amphiphilic in nature, stays near the head group region of liposome. This location near the headgroup region disturbs the natural microstructure of the lipid membrane and hence induces significant changes in the membrane properties resulting in increased membrane fluidity and disorderliness.[22] The effect of ethanol on membrane properties is dependent on the concentration of ethanol and the formation of interdigitated phase is an extreme example of the effect that alcohol can have on the structure of the membrane (≥0.8 M of ethanol). At low ethanol concentration (<0.8 M), *i.e.* before the onset of interdigitation,[23,24] ethanol is known to impart fluidity to the membrane. The concentration of ethanol used for the present study was well below 0.8 M to avoid interdigitation, *i.e.* 0.2 M and 0.4 M.

Fig. 6 shows the emission spectra of phototautomer and ground state anion in DMPC liposome at various ethanol concentrations at 10 °C. There is a decrease in intensity of the PT with corresponding increase in the A^- intensity as a function of ethanol concentration. For the same concentration of ethanol addition in water there was no ground state anion formation. So the observed changes in the fluorescence intensities of PT and A^- in liposome are due to the fluidizing effect of ethanol on membrane which leads to redistribution of 3HF between the two sites. This experiment also supports the bimodal distribution of 3HF in the membrane.

Study of mixed lipid system

In order to verify whether similar behaviour of 3HF could be seen for liposomes constructed from different lipids, studies were carried out with liposomes consisting of varying ratio of DMPC and DPPC lipids.

The spectral behaviour in the case of the mixed lipid systems was found to be similar to that observed in pure DMPC and DPPC liposomes. In all the cases the PT intensity decreases and the A^- intensity increases as the liposome goes from solid gel to liquid crystalline phase, supporting the redistribution of 3HF between the two sites of occupancy. Fig. 7A represents a plot of variation of ground state anion fluorescence intensity with

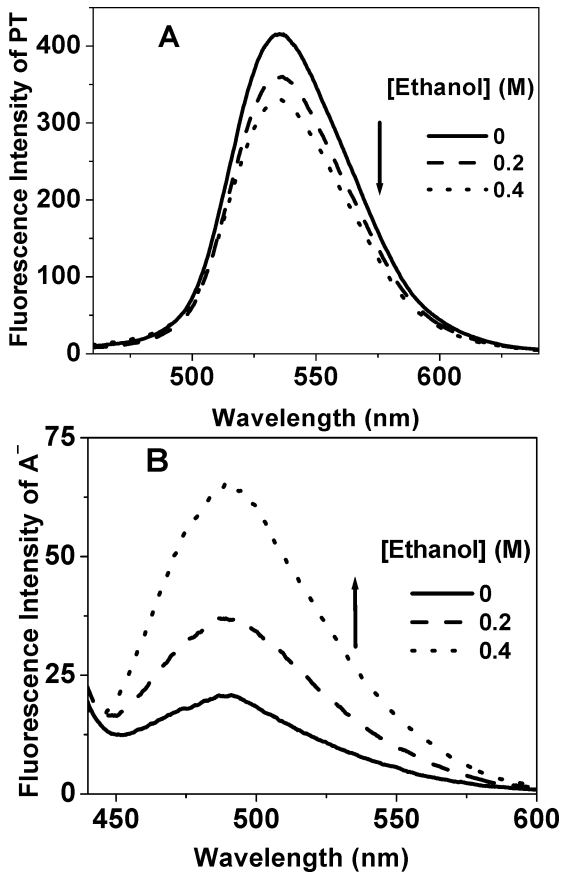

Fig. 6 Emission spectra of (A) PT (λ_{ex} = 345 nm), (B) A^- (λ_{ex} = 417 nm) with ethanol concentration (0 M, 0.2 M, and 0.4 M) at 10 °C.

temperature from 15 °C to 55 °C in mixed lipid systems. It is interesting to observe that the sigmoidal plots keep shifting to higher temperatures as the DPPC mole percentage in the liposome increases. Fig. 7B represents the plot of fluorescence anisotropy of PT with temperature for liposomes of different compositions. As the DPPC mole percent increases the phase transition temperature shifts towards higher temperature. Similar behaviour of shift in phase transition temperature with increasing DPPC mole fraction have been reported in the literature.[19,25,26]

Conclusion

The detailed photophysical behaviour of phototautomer and the ground state anion of 3HF in DMPC liposome membrane have been investigated. A quenching study using a hydrophilic quencher Ag^+ provides strong evidence that the preferential

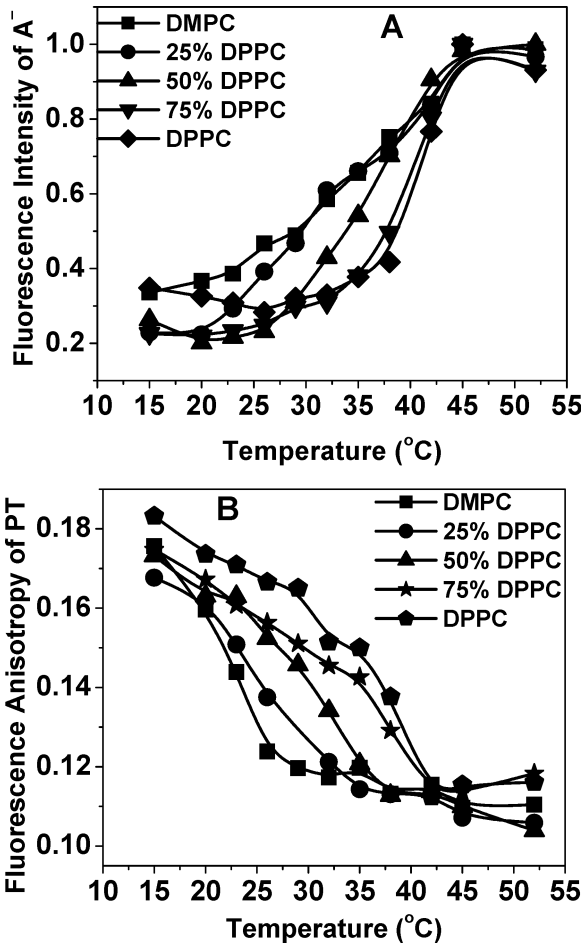

Fig. 7 A plot of variation of (A) A^- (λ_{ex} = 417 nm, λ_{em} = 490 nm) fluorescence intensity, (B) PT (λ_{ex} = 345 nm, λ_{em} = 535 nm) anisotropy with temperature from 15°C to 55 °C in liposomes constructed from DMPC and DPPC mixed lipids.

location of PT and A^- is at the water-inaccessible core and the water-accessible interface, respectively. However, the biexponential nature of the fluorescence decays for both the forms suggest that there still exists certain degree of local heterogeneity in their distribution at respective locations. From the temperature dependence fluorescence studies and effect of ethanol experiments, it is clear that there is a redistribution of 3HF between these two locations as the membrane becomes more fluid. The 3HFs occupying the inner site migrate to the outer site with increasing fluidity of the membrane resulting in a decrease in PT intensity and a concomitant increase in the ground state anion intensity. The fluorescence anisotropy for both the forms decreases with increase in temperature. Though the response of the anisotropy change to phase transition for PT is fairly prompt, the corresponding change for the A^- form is gradual, which shows a continuous drop with increasing temperature. This spectral behaviour is not limited to just pure DMPC or DPPC liposomes but also observed for liposomes constructed from mixed lipids of DMPC and DPPC. Experiments with mixed lipid systems clearly indicate that the distribution of 3HF between the two sites of occupancy is strongly dependent on the membrane fluidity.

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