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Ground and excited state proton transfer and antioxidant activity of 7-hydroxyflavone in model membranes: Absorption and fluorescence spectroscopic studies

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ABSTRACT

Steady state and time resolved fluorescence spectroscopy have been used to probe microenvironments of the therapeutically active intrinsically fluorescent flavonoid, 7-hydroxyflavone (7-HF), in model membranes consisting of multilamellar phosphatidylcholine liposomes. Additionally, the antioxidant effects of 7-HF against lipid peroxidation have been evaluated using spectrophotometric assay. Large Stokes shifted emissions with distinct spectroscopic signatures, are observed from the excited state proton transfer (ESPT) tautomer (which is generated by a solvent mediated mechanism) and the ground state anion of 7-HF. The neutral (7-HFN) and anionic (7-HFA) species' appear to be located in the non-polar acyl chain and the polar head group regions of the lipid vesicles respectively. The partition coefficients of 7-HFN and 7-HFA in these vesicles have also been estimated using their intrinsic fluorescence. Anisotropy (r) versus temperature (T) measurements reveal the utility of the tautomer fluorescence anisotropy as a sensitive parameter for exploring structural changes in the membranes. Fluorescence decay kinetics studies indicate heterogeneity in the microenvironments of both 7-HFN and 7-HFA. Furthermore, we demonstrate that lipid peroxidation of the model membranes is partially arrested upon 7-HF binding, suggesting its potential usefulness as an inhibitor of peroxidative damage of cell membranes.

1. Introduction

Plant flavonoids are emerging as potent therapeutic drugs effective against free radical mediated diseases [1–3] and hence their interactions with cell membranes (which generally serve as targets for lipid peroxidation) are of enormous interest. Flavones, along with flavonols and anthocyanidins are the most commonly occurring classes of flavonoids found in nature [4]. 7-Hydroxyflavone (7-HF) (Scheme 1) is of particular interest from two different perspectives. First, it can serve as a simple representative for naturally occurring flavones of therapeutic importance. Secondly, it undergoes photo-induced excited state proton transfer (ESPT) fluorescence with a large Stokes shift. Regarding its therapeutic importance, vasorelaxing properties of 7-HF has been reported in particular [5]. Excited state proton transfer (ESPT) fluorescence of 7-HF has been previously investigated in considerable detail [6–9]. It is noteworthy that unlike the more widely studied cases of 3-hydroxyflavone (3-HF, a prototype model flavonol) and its derivatives, where the ESPT is intrinsic (i.e. proceeding across an internal H-bond of the molecule) and barrier free [10,11], the ESPT in 7-HF (where proton donor and acceptor sites are not located adjacent to each other) is solvent assisted and consequently strongly depends on the nature of the solvent medium. In previous studies from our laboratory we have shown that solvent dipolar relaxation around the excited 7-HF molecules is an important relaxation mechanism of the excited state of 7-HF for both its anionic [12] and neutral forms [13]. We also demonstrated the usefulness of the ESPT fluorescence of 7-HF as a highly sensitive probe for exploring its microenvironments in reverse micelles and proteins [14–16].

Polyunsaturated fatty acids (PUFA) in cellular membranes are especially prone to damage by lipid peroxidation. The antioxidant properties of different natural flavonoids in various model membrane systems like microsomes, liposomes, low density lipoprotein (LDL) etc. have been reported [17–19]. Recently, we demonstrated the binding and antioxidant activity of the simple synthetic prototype flavonoid 3-HF along with some of its naturally occurring derivatives in red blood cell ghost membranes [20]. Unfortunately, applications of flavonoids as viable alternatives to conventional therapeutic drugs are often limited by their poor solubility in aqueous system. Consequently, there is much current interest in using suitable drug delivery vehicles which are

Abbreviations: 7-HF, 7-hydroxyflavone; ESPT, excited state proton transfer; EYPC, egg yolk phosphatidylcholine; DMPC, dimyristoyl phosphatydilcholine; DPPC, dipalmitoyl phosphatydilcholine; LP, lipid peroxidation; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid.

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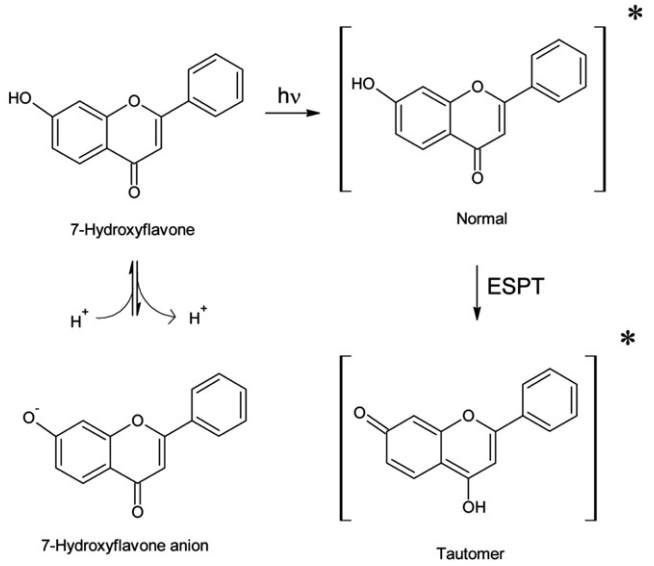

Scheme 1. ESPT and anion formation in 7-HF.

capable of ensuring increased hydrosolubility and bioavailability of these drugs. In this context, liposomes have gained importance as vehicles for delivery of drugs and biomolecules to desired sites in the living system [21–23]. Egg lecithin is a commonly used ingredient used for such purposes, since it is immunologically inert and biodegradable when used in liposome form [24]. Furthermore, owing to the presence of polyunsaturated fatty acids, the use of egg lecithin liposomes as model membrane system provides distinct advantages for the study of lipid peroxidation [25]. Till date, not much is known regarding the antioxidant properties of 7-HF as well as its potential as a fluorescence probe for monitoring the microenvironments of the binding sites of liposomes.

As an extension of previous studies from our laboratory [14,15], we demonstrate here the novel uses of the environmentally sensitive intrinsic fluorescence behavior of 7-HF together with relevant absorption studies, to characterize its binding and location in multilamellar egg yolk phosphatydilcholine (EYPC) and dimyristoyl phosphatydilcholine (DMPC) vesicles. We also examined the antioxidant activity of 7-HF in EYPC liposomal membranes against 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) induced lipid peroxidation. Such studies reveal that 7-HF is a significant inhibitor of lipid peroxidation. The present work should provide the basis for further investigations regarding the binding and antioxidant activities of 7-HF and other flavones of related interest in more complicated natural biomembranes.

2. Materials and methods

2.1. Chemicals

7-HF was obtained from Sigma-Aldrich and used without further purification after confirming its purity by comparing its electronic absorption and emission spectra with published data [26]. Egg yolk phosphatidylcholine (EYPC), dimyristoyl phosphatidylcholine (DMPC) and AAPH were obtained from Sigma. Trichloroacetic acid (TCA) (SRL, India) and thiobarbituric acid (TBA) (BDH, UK) were all reagent grade products. All solvents were of spectroscopic quality and were used without further purification, after confirming the absence of absorbing or fluorescent impurities. Concentrated stock solutions of 7-HF were prepared in methanol and the final concentrations of methanol were kept <1% (by volume) in all samples. Triple distilled water was used for all liposomal experiments.

2.2. Liposome preparation

Multilamellar liposomes of EYPC and DMPC phospholipids were prepared by a method described in the literature [27]. Briefly, appropriate quantities of phosphatydilcholine were solubilized in chloroform/methanol (2:1 ratio by volume), from which thin films were deposited on the inner walls of round-bottom flask using a stream of dry nitrogen. The films were dried in vacuum and stored at 4 °C for 48 h. The films were then hydrated in water above the phase transition temperature of the lipid and the lipid was dispersed by vortexing.

2.3. Labelling of liposomes for partition experiments

A stock solution of 7-HF was prepared in methanol. Partition experiments were performed using the titration method by adding the methanolic solution of 7-HF, to 1 ml of various concentrations of liposome in water (pH 7.0), to yield solutions of different lipid/probe (L/P) ratios in the range 10–100. After adding the probe, the solutions were allowed to equilibrate for 1 h at 30 °C (above the phase transition temperature of the lipid, where EYPC and DMPC liposomes exist in a liquid crystalline phase) before the experiment. EYPC and DMPC suspensions containing the same concentration of lipid component and the same volume of MeOH but devoid of 7-HF were used to prepare liposomes under identical conditions to serve as blank solution.

2.4. Spectroscopic measurements

Steady state absorption and fluorescence spectra were recorded with a Cecil model 7500-spectrophotometer and Perkin Elmer LS 55 spectrofluorimeter respectively. Fluorescence anisotropy measurements were performed using a Varian Cary Eclipse spectrofluorimeter. The background spectra (from unlabeled liposomes prepared in the same way) were subtracted from that of the labeled liposomes. Gaussian deconvolution of emission spectra was performed using the Origin 6.1 software. The fluorescence anisotropy (r) values were obtained using the expression $r=(I_{VV}-GI_{VH})/(I_{VV}+2GI_{VH})$, where I_{VV} and I_{VH} are the vertically and horizontally polarized components of probe emission with excitation by vertically polarized light at the respective wavelength and G is the sensitivity factor of the detection system [28]. Each intensity value used in this expression represents the computer-averaged values of ten successive measurements. All spectral measurements were carried out at room temperature (298 K) on freshly prepared solutions.

Fluorescence decay measurements were performed using an Edinburgh Instruments nanosecond time correlated single photon counting setup with 295 nm and 370 nm nanosecond diode laser excitation sources (IBH, UK, nanoLED) having pulse FWHM ~1.2–1.4 ns. Emission was observed through an emission monochromator set at 475 nm and 525 nm wavelengths for 7-HFN and 7-HFA respectively with a nominal spectral bandwidth of 24 nm to block scattered light and isolate the emissions of 7-HF. Data analysis was carried out by a deconvolution method using a non-linear least square fitting programme and fitted with a multi exponential decay function, $F(t)=\sum_i A_i \exp(-t/\tau_i)$, $\sum_i A_i=1$, where A_i and τ_i represent the amplitudes and time constants, respectively, of the individual components in multiexponential decay profiles. The goodness of fit was estimated by using χ^2 values.

2.5. Lipid peroxidation in liposomal membranes

AAPH is an azo-compound that generates peroxide radicals after thermal homolysis in both aqueous and lipid phase [29]. Lipid peroxidation of liposomal membranes was performed using this radical generator, AAPH. The reaction mixture contained liposomal

suspensions in water (pH 7.0), and 25 mM AAPH and the final volume was 1.0 ml. This was incubated for 1 h with shaking in contact of air at 37 °C.

2.6. Detection of oxidation in liposomes

Polyunsaturated fatty acids in the egg lecithin liposomal membranes are prone to peroxidative damages by free radicals. The essential stages in the peroxidation process include formation of conjugated diene followed by hydroperoxide formation. Malondialdehyde (MDA) is formed only from fatty acids with three or more methylene-interrupted double bonds [30]. Monitoring such reactions involve the absorption spectrophotometric measurements of MDA equivalents formed, with thiobarbituric acid (TBA) and trichloroacetic acid (TCA) [25,31], at the absorption maximum of MDA (535 nm). For measuring MDA in peroxidized liposomes (both in presence and absence of 3-HF), 1.0 ml of liposomal suspension is mixed with 2.5 ml of 20% TCA and 1.0 ml of 0.67% aqueous solution of TBA. After heating for 10 min in a boiling water bath, the pink pigment is extracted with 4.0 ml of n-butanol and the absorbance was checked at 535 nm (for MDA-TBA adduct). The malondialdehyde concentrations of the samples are calculated using an extinction coefficient (ε) of 1.56×10^5 M^{-1} cm^{-1} [32]. All values are expressed as mean ± standard deviation ($n=3$).

3. Results and discussion

3.1. Steady state absorption and emission behaviors of 7-HF in EYPC and DMPC liposomal membranes

Fig. 1 presents the electronic absorption spectra of 2.5×10^{-5} M 7-HF in liposomes along with the reference spectrum in water (pH 7.0). In water the onset of the absorption is at around 420 nm and peaks are observed at 375 nm and 310 nm. The values of molar extinction coefficients at 375 nm and 310 nm are of comparable magnitudes. This spectral profile of 7-HF in water is consistent with that reported previously in neutral organic solvent, according to which the absorption band at 310 nm is due to the neutral form (7-HFN) while the 375 nm band originates from the corresponding anion of 7-HF (7-HFA) formed in the ground state ($pK_a=7.39$) [9,14–16]. Upon incorporation in liposomes, the absorbance of the higher energy absorption band (at λ_{abs}^{max} ~310 nm) increases significantly, which can be understood in terms of substantial ground state interaction of 7-HF with lipid vesicles. If there were no ground state interaction between 7-HF and phosphatydilcholine liposomes, the absorption spectra would be unchanged. It is also noteworthy, that in EYPC vesicles the increase in absorbance of the higher energy band is much more pronounced in comparison to rather weak absorbance enhancement in DMPC vesicles. These data clearly indicates that the interaction of 7-HF with EYPC vesicles is significantly different in nature than with DMPC vesicles.

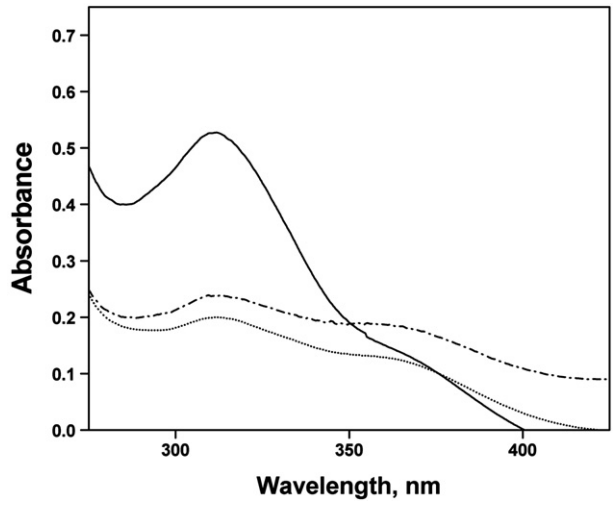

Fig. 1. Absorption spectra of 2.5×10^{-5} M 7-HF in 1.0 mM EYPC (—) and DMPC (-·-·-) liposomes and in water (…) at 30 °C.

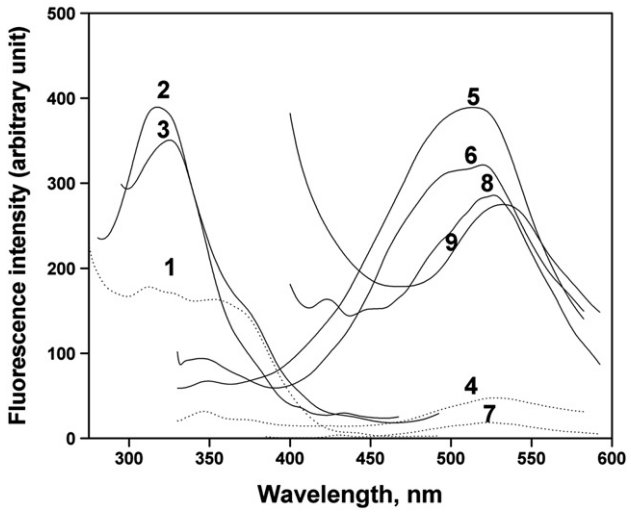

Fig. 2. Fluorescence excitation (monitored at $\lambda_{em}=530$ nm) (2 and 3) and emission spectra of 7-HFN ($\lambda_{ex}=310$ nm) (5 and 6) and of 7-HFA ($\lambda_{ex}=375$ nm) (8 and 9) in EYPC and DMPC liposomes respectively (—) as well as in water (1, 4, 7) (….) at 30° C. [7-HF]= 2.5×10^{-5} M, [EYPC]/[DMPC]=2.5 mM.

Fig. 2 displays the fluorescence emission spectra of 7-HFN and 7-HFA (obtained using $\lambda_{ex}=310$ nm and 375 nm respectively) in EYPC and DMPC liposomes. The corresponding excitation spectra are shown in the same figure. The reference spectra in water (pH 7.0) are also included for comparison. In water, broad emission bands of low fluorescence intensities are observed between 510 nm and 535 nm for 7-HFN and 7-HFA. In liposomal membranes intense emission bands with large Stokes shifts and distinct spectroscopic signatures, are observed for the excited state proton transfer (ESPT) tautomer and the ground state anion. Excitation with $\lambda_{ex}=310$ nm results in a broad emission profile (clearly arising from strong overlap of two distinct fluorescence bands) showing λ_{em}^{max} ~500 nm, 520 nm for EYPC and ~495 nm, 525 nm for DMPC vesicles (Table 1). Apart from the fact that the emission spectrum is relatively better resolved in DMPC vesicles, the spectral behaviour of 7-HF is very similar in EYPC and DMPC liposomes. On the other hand, excitation at the anionic absorption maxima (375 nm) gives fluorescence emission with $\lambda_{em}^{max}=527$ nm and 532 nm for EYPC and DMPC vesicles respectively (Table 1). The fact that the fluorescence of 7-HF changes on addition of phosphatydilcholine confirms its incorporation in the liposomes. This conclusion derives

Table 1
Fluorescence emission properties of the neutral (7-HFN) and the ground-state anionic species (7-HFA) of 7-HF in water and phosphatydilcholine liposomes

Species	Environment	λ_{em}^{max}		Limiting fluorescence anisotropy
		I_1	I_2	(r)
Neutral Species of 7-HF (7-HFN)	Water	510–535		0.05
	EYPC Liposomes	500	520	0.13
	DMPC Liposomes	495	525	0.12
Anionic Species of 7-HF (7HFA)	Water	510–535		0.05
	EYPC Liposomes	527		0.25
	DMPC Liposomes	532		0.25

additional credence from the spectral characteristics of the excitation profile (Fig. 2) which clearly shows dramatic enhancement in fluorescence intensities at ~310 nm in liposomes.

It appears that in case of 7-HFN (λ_{ex}=310 nm) the higher energy fluorescence emission (λ_{em}^{max}~500 nm for EYPC and ~495 nm for DMPC) occurs from the solvent assisted ESPT tautomer of 7-HFN, whereas the lower energy one (λ_{em}^{max} ~520 nm for EYPC and ~525 nm for DMPC) (Table 1) originates from the ground state anion (7-HFA) [9,14–16]. The supporting evidence for these assignments comes from the study of excitation wavelength dependence of the emission spectra (Fig. 3). In EYPC liposomes, as the excitation wavelength is shifted from 300 nm to 400 nm, the ratio of the emission intensity of the band at ca. 500 nm band to that at ca. 525 nm band is gradually decreased (Fig. 3a) (the corresponding spectra in DMPC liposomes are not shown as they look similar). Fig. 3b depicts the deconvoluted spectra with excitation wavelengths near two extremes of the absorption band (310 nm and 380 nm) into the two components arising from the ESPT tautomer and anion which clearly shows that the ratio of the emission intensity of the band at ca. 500 nm band to that at ca. 525 nm band is significantly lower with excitation at 380 nm than with excitation at 310 nm. To test whether the apparent red shift originates from red edge excitation shift (REES) effect, dependence of excitation spectra on emission wavelength was examined (Fig. 4). When the excitation spectra are monitored with emission in the range 500 nm–550 nm (where the emission is predominantly from 7-HFA), the usual band corresponding to the anionic species having a maximum at 375 nm is seen. However, the excitation spectra monitored at ~480 nm produced a single band with a maximum at ~310 nm. This observation clearly indicates that the apparent red shift is not due to REES but due to selective excitation of two distinct species, namely 7-HFN and 7-HFA. The ESPT tautomer emission maxima of 7-HFN (λ_{ex}=310 nm) in liposomes (Fig. 2) is noticeably (by >15 nm) blue-shifted relative to that obtained previously in case of 7-HF in reverse micelles [15]. Presumably, in both EYPC and DMPC vesicles the neutral population of 7-HF molecules is solubilized in the hydrophobic environments (non-polar acyl-chain region) of the lipid bilayer. The emission maxima of 7-HFA (for λ_{ex}=375 nm) in liposomes (Fig. 2) is significantly red-shifted relative to that obtained in case of 7-HF in human serum albumin [16]. Since the energy of 7-HFA strongly depends upon dipolar relaxation of the excited fluorophore molecules (owing to substantial change in the dipole moment of 7-HFA upon excitation), we infer that the polarity of the 7-HFA binding site on EYPC and DMPC vesicles is significantly more polar than in HSA. It appears that in liposomal membranes, this population of 7-HF molecules (7-HFA) is located in a relatively polar environment, binding to the polar head-group region rather than to the non-polar acyl side-chain region. Presumably, in this environment this anionic species is stabilized by the electrostatic interaction with the positively charged nitrogen center of the choline group. This is reminiscent of a similar observation by Mishra et al for 3-HF incorporation in DMPC vesicles [33].

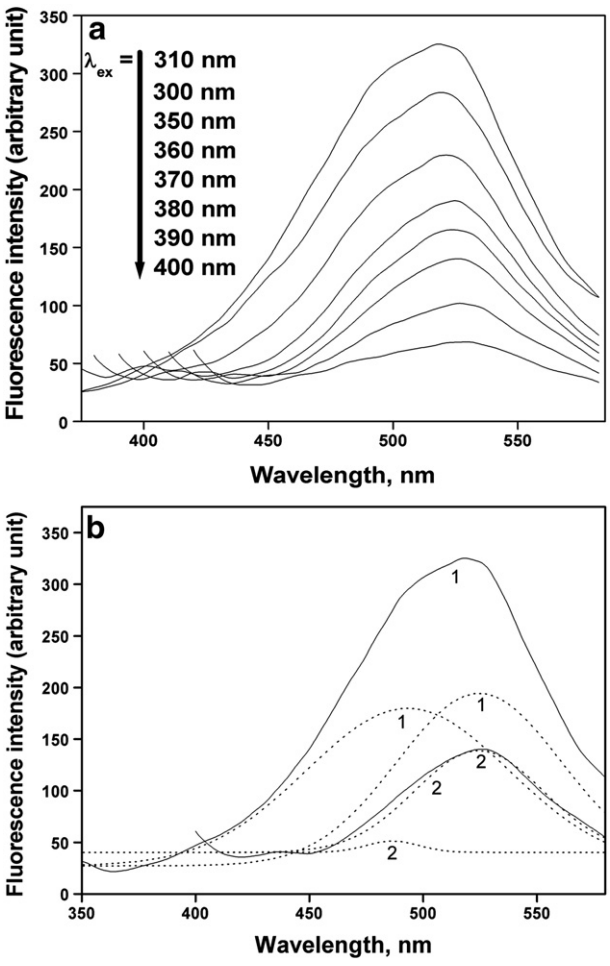

Fig. 3. (a) Emission spectra of 7-HF in EYPC liposome for varying excitation wavelengths (λ_{ex}=300, 310, 350, 360, 370, 380, 390, and 400 nm). (b) Component analysis by deconvolution of 7-HF fluorescence excited at 310 nm (1) and 380 nm (2). (—), total fluorescence of 7-HF, (…) the ESPT and anionic emission of 7-HF.

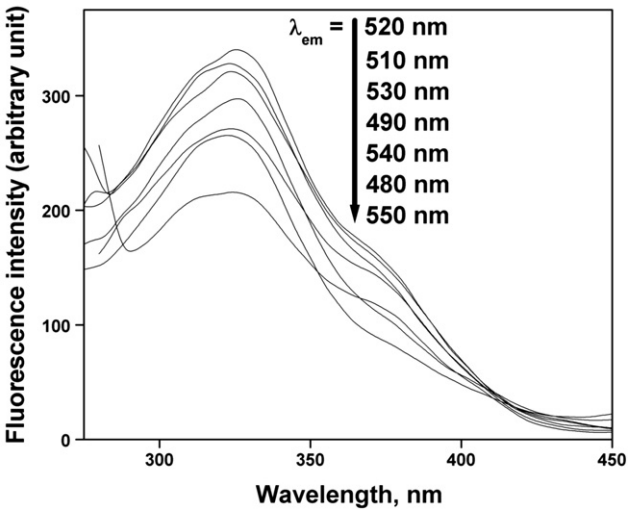

Fig. 4. Excitation spectrum of 7-HF in EYPC liposome for varying emission wavelengths. (λ_{em}=480, 490, 510, 520, 530, 540, 550 nm). [7-HF]=25 μM.

Fig. 5 shows the variation of ratio of the ESPT to anionic emission intensity (I_1/I_2) with increasing lipid concentration. In both EYPC and DMPC vesicles, the I_1/I_2 ratio increases progressively with lipid concentration. Since the neutral population of 7-HF molecules is solubilized in the hydrophobic environments of the lipid bilayer, the consequence of the dramatically lower dielectric constant of the hydrocarbon tails of the membrane bilayer is that the energy of singly charged 7-HF anion (E_S) in the lipid bilayer is significantly higher than that in aqueous solution. This energy can be treated as a perturbation to the dissociation constant by $\Delta pK_a = \frac{\Delta E_S}{2.303 k_B T}$ [34]. Consequently, the pK_a for a 7-HF buried in the hydrophobic core of the lipid bilayer is much higher than that in aqueous solution (where pK_a=7.39). It appears that with increasing concentration of phosphatydilcholine the equilibrium of the proton dissociation reaction (Scheme 1) in aqueous environment shifts readily from 7-HFA to 7-HFN because the subsequent incorporation of 7-HFN in liposomes remove this species from the aqueous environments.

The representative set of emission spectra of 7-HFN (2.5×10^{-5} M) (λ_{ex}=310 nm) showing the variation of fluorescence intensity with

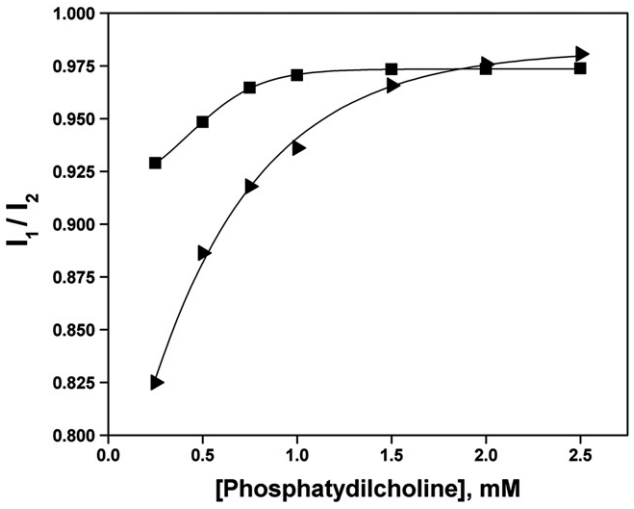

Fig. 5. The ratio of ESPT to anionic emission intensity (I_1/I_2) of 7-HF as a function of [Phosphatydilcholine] in EYPC (►) and DMPC (■) liposomes. ([7-HF]=2.5×10^{-5} M; λ_{ex}=310 nm for I_1 and 375 nm for I_2).

lipid (EYPC / DMPC) concentration is shown in Fig. 6 (The corresponding spectra for 7-HFA are not shown). The fluorescence intensity enhancement of 7-HF (for both 7-HFN and 7-HFA forms) with increasing lipid concentration exhibits a saturating tendency for EYPC and DMPC vesicles. Fig. 7a shows the variation of the

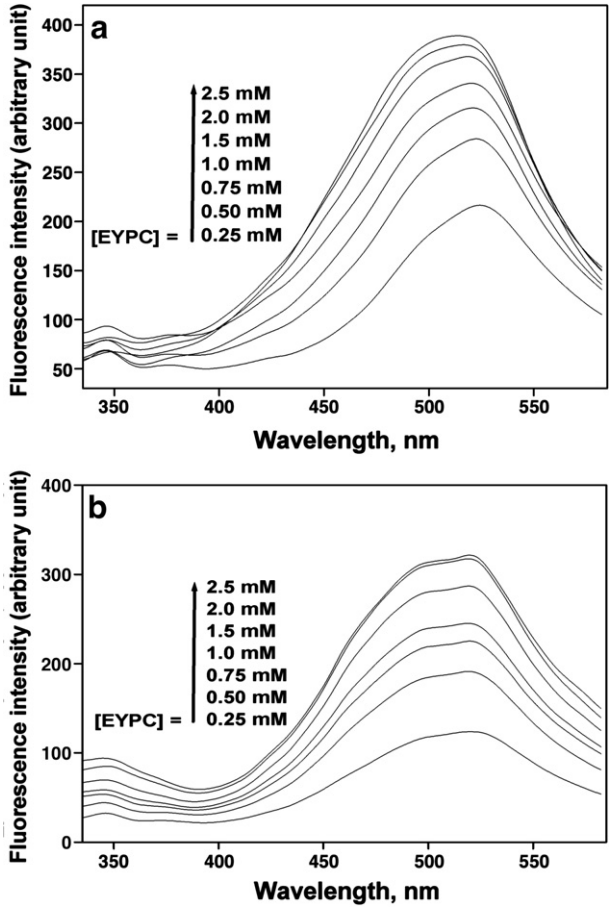

Fig. 6. Set of fluorescence emission spectra of 2.5×10^{-5} M 7-HFN (neutral form) in (a) EYPC liposome (b) DMPC liposome, for phosphatydilcholine concentration in the range of 0.25–2.5 mM at 30° C. λ_{ex}=310 nm.

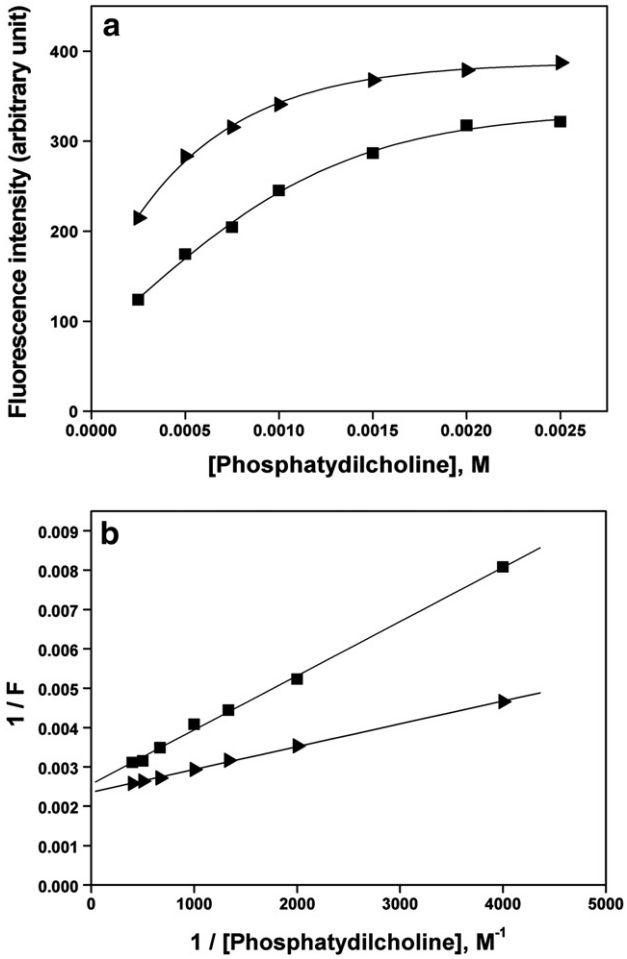

Fig. 7. (a) Plots of variation of fluorescence intensities of 7-HFN and 7-HFA with varying phosphatydilcholine concentrations. (b) the respective double-reciprocal plots of 1/*F* against 1/[Phosphatydilcholine], (►) for EYPC and (■) for DMPC.

fluorescence intensity (F) of 7-HFN with lipid concentration. The partition coefficient (K_p) is calculated from the slope and 1/F intercept of the double-reciprocal linear plot of 1/F against 1/[Lipid], according to the equation,

$$\frac{1}{F} = \frac{55.6}{K_p.F_{max}} \times \frac{1}{[\text{Lipid}]} + \frac{1}{F_{max}}$$

where F_{max} is maximum fluorescence resulting from total probe incorporation into the membrane [33,35,36]. The corresponding double-reciprocal plots of 1/*F* against 1/[Lipid] (Fig. 7b) shows a good linearity as predicted by the equation. From the slope and 1/*F* intercept of these linear plots the partition coefficients K_ps were determined at 30 °C where both EYPC and DMPC exist in liquid crystal phase (Table 2). It is evident that 7-HFN and 7-HFA partition to the lipid bilayer with reasonably large partition coefficients. In case of 7-HFN the slightly lower K_p for the DMPC liposomes is probably due to its tight lipid packing which makes the partitioning of 7-HFN molecules difficult. On the other hand, the presence of a number of

Table 2
Calculated values of partition coefficient of the neutral (7-HFN) and the ground-state anionic species (7-HFA) of 7-HF in the EYPC and DMPC vesicles at 30 °C and pH 7.0

Vesicles	K_p for 7-HFN	K_p for 7-HFA
EYPC	10.32×10^4	22.66×10^4
DMPC	4.68×10^4	20.58×10^4

unsaturated acyl chains create more 'dead space' (i.e., space not occupied by lipids) in EYPC liposomes through which probe molecules can easily partition into the membrane [37]. However, in case of 7-HFA the K_p values in EYPC and DMPC vesicles are of comparable magnitudes which may be attributed to its binding in similar locations (near the polar head group region) in both types of vesicles. These partition coefficient values are of the same order of magnitude as reported by Mishra et al for 3-HF in the solid gel phase of DMPC liposomes [33].

3.2. Fluorescence anisotropy measurements

Fluorescence anisotropy (r) measurements were also performed since this parameter serves as a sensitive indicator for monitoring fluorophore binding to motionally constrained regions of biological membranes [28,38]. The anisotropy value (r) is expected to be very low in fluid solution where the fluorophore can freely rotate, and high for rigid environment. Fig. 8a presents the variation of the fluorescence anisotropy values for 7-HF with increase in phosphatydilcholine concentration. The anisotropy of the neutral form of 7-HF (7-HFN) increases from 0.05 ([Phosphatydilcholine]=0 mM) to 0.13 for EYPC and 0.12 for DMPC ([Phosphatydilcholine]=2.5 mM) (Table 1). The gradual increase in anisotropy of 7-HFN emission with lipid concentration is consistent with the picture that more and more 7-HF molecules are getting bound to liposomes. The slightly higher limiting fluorescence anisotropy value of 7-HFN obtained here in case of EYPC liposomes is in agreement with the slightly higher K_p value estimated in this liposomal membrane. The pronounced increase in fluorescence anisotropy of the ground state anion of 7-HF (7-HFA) (Fig. 7a) is also gradual (anisotropy increases from 0.08 at [Phosphatydilcholine]=0 mM to 0.25 at ([Phosphatydilcholine]=2.5 mM for both EYPC and DMPC vesicles) (Table 1). Presumably, the relatively higher fluorescence anisotropy value associated with 7-HFA is due to its restricted motion as a result of its stabilizing electrostatic interaction with the positively charged nitrogen center of the choline group. High values of the limiting fluorescence anisotropy of 7-HF observed for the neutral as well as the anionic species are consistent with previous studies by us (for 3-HF in unilamellar DPPC and DMPC liposomes as well as erythrocyte ghost membranes) and by Mishra et al (for 3-HF in DMPC liposomes) [20,33,39]. Anisotropy values suggest that both the species of 7-HF are rigidly bound in the membrane matrix. We also examined the temperature dependence of the ESPT fluorescence anisotropy (r) of 7-HFN in DMPC vesicles, to explore the potentiality and usefulness of this emission parameter for probing structural changes in liposomes. The 'r' vs temperature plots (Fig. 8b) show characteristic sigmoidal shape, revealing the thermotropic phase transition of the phospholipids from gel to liquid crystalline states. Phase transition temperature (T_m=21–22 °C) value estimated from the midpoints of the sigmoidal shaped curves, are in fairly good agreement with existing literature data based on different methods [38–42]. Hence 7-HF can serve as a fluorescent probe for monitoring the changes in the properties of liposomes due to phase transition.

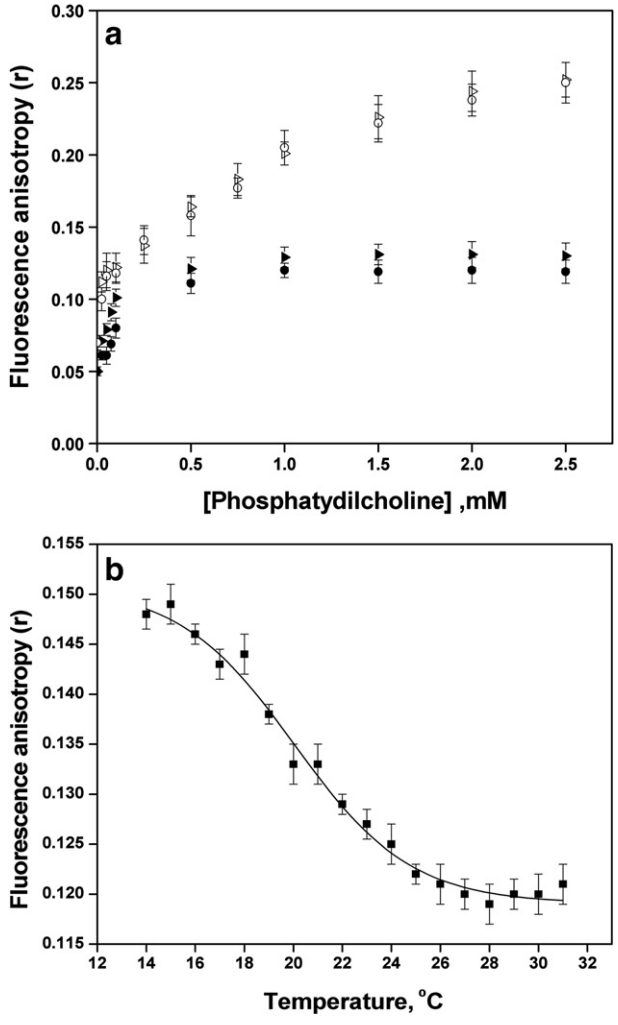

Fig. 8. (a) Variation of fluorescence anisotropy (r) of 7-HFN (λ_{ex}=310 nm) and 7-HFA (λ_{ex}=375 nm) with phosphatydilcholine concentration and or the neutral and anionic form respectively (λ_{em}=530 nm); (►) and (●) for 7-HFN in EYPC and DMPC respectively, whereas (Δ) and (○) for 7-HFA in EYPC and DMPC respectively. (b) Variation of steady-state fluorescence anisotropy (r) of ESPT fluorescence of 7-HF (neutral form) in DMPC liposomes as a function of temperature. λ_{ex}=310 nm; λ_{em}=530 nm. Each data point indicates the average of three determinations where error bars indicate the standard deviation.

Table 3
Fluorescence decay parameters of the neutral (7-HFN) and the ground-state anionic form (7-HFA) of 7-HF in EYPC and DMPC liposomes

Species	λ_{ex}	λ_{em}	A_1	τ_1 (ns)	A_2	τ_2 (ns)	A_3	τ_3 (ns)	χ^2
7-HFN	295	475	0.925	0.442	0.075	2.06	–	–	1.08
7-HFA	370	525	0.012	1.45	0.986	0.054	0.002	5.26	1.05

7-HF concentration was 2.5×10^{-5} M and that of EYPC was 2.5 mM.

3.3. Time resolved fluorescence studies

Representative fluorescence decay parameters of the 7-HF molecules in the liquid crystalline phase of EYPC liposomes are listed in Table 3. The fluorescence decay of 7-HFN exhibits a bi-exponential behavior in EYPC vesicles with predominant contribution from a sub-nanosecond component with decay time 442 ps (A_1=0.925) together with a relatively minor contribution from a 2.06 ns component (A_2=0.075). Although the fluorescence decay for the anionic species (7-HFA) could be best fitted to a triple exponential function, a bi-exponential decay fit seems to be a reasonable approximation of the data, since the very long decay time component (τ_3=5.26 ns) in the triple exponential fit makes only a negligible contribution of about 0.2%. The decay, in this case, is essentially composed of a predominant contribution from a rather short component of 54 ps (A_2=0.986), which is accompanied by a minor component with a relatively longer decay time (τ_1=1.45 ns, A_1=0.012). Observations of heterogeneous fluorescence decays for the ESPT tautomer as well as for the ground-state anion suggest distribution of 7-HF molecules in different sites of the liposomal membranes.

The steady state and time resolved fluorescence emission data suggest that the 7-HF molecules are distributed in the EYPC and DMPC liposomes in different sites, which presumably differ in their polarity

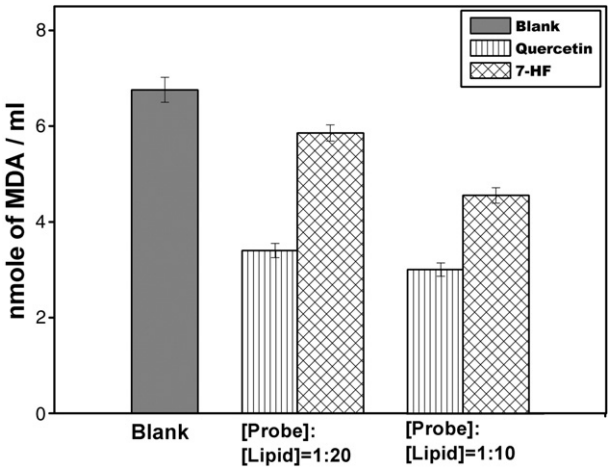

Fig. 9. Effect of 7-HF on TBARS formation induced by AAPH in EYPC liposomes. 7-HF: phospholipids molar ratios were kept at 1:20 (100 μM 3-HF, 2 mM lipid) and 1:10 (200 μM 7-HF, 2 mM lipid). Values are expressed as mean ± standard deviation (n=3).

and hydration properties. The neutral (7-HFN) and anionic (7-HFA) forms appear to be located in the non-polar acyl chain region, and near the polar head groups of the lipid vesicles respectively. Binding of 7-HFA to the polar head-group region is presumably stabilized by the electrostatic interaction with the positively charged nitrogen center of the choline group [33]. The motional constraint imposed by such specific interaction explains the high fluorescence anisotropy value obtained for the anionic species. The present findings are consistent with a previous study by Demchenko et al where it was pointed out that the structurally heterogeneous EYPC vesicles allow the distribution of 4-dimethylamino-3-hydroxyflavone between different sites in the bilayer membrane [43]. Such examples of the heterogeneous probe distribution in lipid vesicles are available in the literature for other types of neutral fluorescent probes as well [37,44].

3.4. Effects of flavonoids on the production of TBARS

From the fluorescence emission studies, it is evident that the 7-HF molecules bind to the liposomal membranes in motionally constrained regions, where they are expected to be adequately accessible to the incoming free radicals. We evaluated the antioxidant activity of 7-HF in EYPC liposomes by measuring lipid peroxidation as TBARS in the presence of the free radical generator AAPH. Fig. 9 shows that 7-HF inhibits lipid peroxidation induced by AAPH in a dose dependent manner. We made a comparison with respect to quercetin (3,3′,4′,5,7-pentahydroxyflavone) for which the relative antioxidant potential was previously established in terms of the known standard Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid, an water-soluble derivative of vitamin E). From the present study it is evident that the relative antioxidant activities of quercetin and 7-HF, determined by inhibition of the production of MDA in AAPH induced LP, is in the ratio ~1:0.65. Since quercetin is nearly four times potent as an antioxidant in relation to Trolox [45], it turns out that the antioxidant potential of 7-HF is nearly 2.5 times that of Trolox. The hydroxyl groups of flavonoids are known to be important for their antioxidant activity. Our earlier findings based on calculated bond dissociation energy (BDE) values indicate that in case of the natural flavonoid fisetin (3,3′,4′,7-tetrahydroxyflavone) the 3- hydroxyl group is the most effective in scavenging free radicals followed by hydroxyl groups in the 3′, 4′, and 7 positions respectively [38]. Thus the antioxidant activity of 7-HF can largely be attributed to its capacity to interact with free radicals and inhibit the propagation of lipid peroxidation. Moreover, the binding site(s) of the flavonoids in the membrane matrix are expected to be important in determining the relative efficiencies as antioxidants [20]. In this context the steady state fluorescence anisotropy data are relevant since the high anisotropy values observed clearly indicate that 7-HF molecules are rigidly bound inside the membrane matrix, thus facilitating the antioxidant activity of 7-HF against LP. Furthermore, it is noteworthy that besides these two factors redox potential values of flavonoids influence their action as antioxidants with their ability to scavenge free radicals by electron transfer process [18,46–48].

4. Conclusions

We have explored the binding of 7-HF in EYPC and DMPC liposomal membranes, exploiting the intrinsic fluorescence emission behavior of 7-HF. The fluorescence properties of 7-HF were also used to determine quantitatively its partition coefficients in these vesicles at pH 7.0 Temperature dependent fluorescence anisotropy (r versus T) measurements reveal the novel application of the ESPT tautomer fluorescence of 7-HF for monitoring the structural changes in phosphatidylcholine liposomes. The present study also demonstrates that 7-HF exhibits significant antioxidant and radical scavenging activity in EYPC liposomes. Since EYPC liposomes prepared from natural unsaturated phospholipids offer the distinct advantages over saturated phospholipid vesicles (e. g. DPPC of synthetic origin) of being biodegradable as well as immunologically inert, the promising new findings may open the door to new avenues for delivery of drugs in physiological systems. Further extension of the present research to encompass comparative studies on different membrane systems, should be of considerable interest in relation to studies on biological activities of 7-HF, including its therapeutic applications.

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