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Ground- and excited-state proton transfer and antioxidant activity of 3-hydroxyflavone in egg yolk phosphatidylcholine liposomes: absorption and fluorescence spectroscopic studies

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ABSTRACT: Excited-state intramolecular proton transfer (ESIPT) and dual luminescence behaviour of 3-hydroxyflavone (3-HF) have been utilized to monitor its binding to liposomal membranes prepared from egg yolk phosphatydilcholine (EYPC). Additionally, absorption spectrophotometric assay has been performed to evaluate the antioxidant activity of 3-HF against lipid peroxidation in this membrane system. When 3-HF molecules are partitioned into EYPC liposomes, a weak long-wavelength absorption band with λ_{abs}^{max} ~410 nm appears in addition to the principal absorption at ~λ_{abs}^{max} = 345 nm. Selective excitation of the 410 nm band produces the characteristic emission (λ_{em}^{max} ~460 nm) of the ground-state anionic species, whereas excitation at the higher energy absorption band leads to dual emission with predominatly ESIPT tautomer fluorescence (λ_{em}^{max} = 528 nm). Both ESIPT tautomer and the anionic species exhibit fairly high fluorescence anisotropy (*r*) values (*r* = 0.122 and 0.180, respectively). Biexponential fluorescence decay kinetics are observed for the ESIPT tautomer as well as the ground-state anionic forms, indicating heterogeneity in the microenvironments of the corresponding emitting species. Furthermore, we demonstrate that lipid peroxidation of EYPC liposomes is significantly inhibited upon 3-HF binding, suggesting that 3-HF can be potentially useful as an inhibitor of peroxidative damage of cell membranes. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: 3-hydroxyflavone; luminescence spectroscopy; excited-state intramolecular proton transfer; egg yolk phosphatydilcholine liposome; lipid peroxidation

Introduction

3-hydroxyflavone (3-HF), the basic structural moiety of an important group of naturally occurring, biologically active plant flavonoids, is one of the best-known prototype molecules exhibiting excited-state intramolecular proton transfer (ESIPT) and dual fluorescence characteristics (1, 2). The proton transfer proceeds via an internal hydrogen bond (between adjacent proton donor and acceptor sites located within the molecule; Scheme 1). The dual emission behaviour and ESIPT of 3-HF in membrane systems have been extensively investigated in a wide range of model biomembranes, including red blood cell ghost membranes (3–5). In previous studies from our laboratory, the ESIPT and dual fluorescence behaviour of 3-HF were used as highly sensitive probes for exploring its microenvironments in dipalmitoyl phosphatydilcholine (DPPC) and dimyristoyl phosphatydilcholine (DMPC) liposomes as well as in erythrocyte ghosts (4, 5).

As plant flavonoids are emerging as potent therapeutic drugs effective against free radical-mediated diseases, their interactions with cell membranes (which generally serve as targets for lipid peroxidation) are of enormous interest. Polyunsaturated fatty acids (PUFA) in cellular membranes are especially prone to damage by lipid peroxidation. Previous literature data reported the antioxidant properties of different natural flavonoids in various model membrane systems, such as microsomes, liposomes, low-density lipoprotein (LDL), etc. (6–8). Recently, we have demonstrated the binding and antioxidant activity of the simple synthetic prototype flavonoid 3-HF in red blood cell ghost membranes (5). However, applications of flavonoids as viable alternatives to conventional therapeutic drugs are often limited by their poor solubility in aqueous systems. Consequently, there is much current interest in using suitable drug delivery vehicles which are capable of ensuring increased hydrosolubility and bioavailability of these drugs. Liposomes have gained importance as vehicles for delivery of drugs and biomolecules to a desired site in the living system (9–11). Egg lecithin is the most important ingredient used for these purposes, since it is immunologically inert and biodegradable when used in liposome form (12). Furthermore, owing to the presence of polyunsaturated fatty acids, the use of egg lecithin liposomes as a model membrane system in the study of lipid peroxidation provides distinct advantages (13). To date, there is no clear picture regarding the

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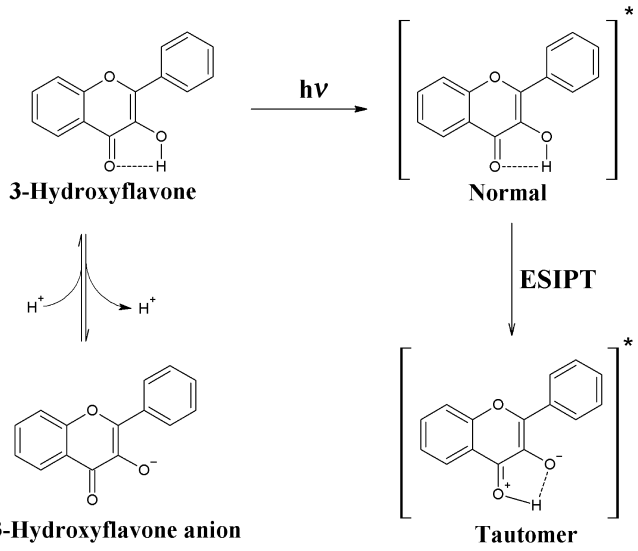

Scheme 1. ESIPT and anion formation in 3-HF.

antioxidant properties and use of the fluorescence emission characteristics of 3-HF for probing the microenvironments of the binding sites of liposomes prepared from egg yolk phosphatydilcholine (EYPC), which contains a mixture of natural, polyunsaturated fatty acids.

As an extension of the previous studies (4), we demonstrate here the novel uses of the absorption and the exquisitely sensitive intrinsic fluorescence behaviour of 3-HF to characterize its binding and location in small unilamellar EYPC vesicles. Furthermore, we have examined the antioxidant activity of 3-HF in liposomal membranes against 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced lipid peroxidation. Such studies reveal that 3-HF is a significant inhibitor of lipid peroxidation. The present work should provide the basis for further investigation regarding the binding and antioxidant activities of 3-HF in more complicated natural biomembranes.

Materials and methods

Reagents

3-HF and AAPH were obtained from Sigma-Aldrich and used without further purification after confirming their purity by comparing their electronic absorption and emission spectra with published data (14). Egg yolk phosphatidylcholine (EYPC) was obtained from Sigma. Trichloroacetic acid (TCA; SRL, India) and thiobarbituric acid (TBA; BDH, UK) were both 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 3-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.

Liposome preparation

Small unilamellar liposomes of EYPC phospholipids (in the absence and presence of 3-HF) were prepared by a method described in the literature (15). Briefly, appropriate quantities of 3-HF and EYPC were co-solubilized in chloroform:methanol (2:1 v/v), from which thin films were deposited on the inner walls of a round-bottomed flask using a stream of dry nitrogen. The films were dried under 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 and then sonicated.

Labelling of liposomes for partition experiments

A stock solution of 3-HF was prepared in methanol. Partition experiments were performed using the titration method, by adding the methanolic solution of 3-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 2–80. After adding the probe, the solutions were allowed to equilibrate for 1 h at 25°C (above the phase transition temperature of the lipid, where EYPC liposomes exist in a liquid crystalline phase) before the experiment. EYPC solutions containing the same concentrations of lipid components and the same volume of MeOH, but devoid of 3-HF, were prepared under identical conditions to serve as blank solutions.

Spectroscopic measurements

Steady-state absorption and fluorescence spectra were recorded using a Cecil model 7500 spectrophotometer and a Perkin-Elmer LS 55 spectrofluorimeter, respectively. The background spectra (from unlabelled liposomes treated in the same way) were subtracted from those of the labelled liposomes. 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 (16). Each intensity value used in this expression represents the computer-averaged values of 10 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 set-up with a 370 nm nanosecond diode laser excitation source (nanoLED-03, IBH, UK) having a pulse FWHM of ~1.2–1.4 ns. Data analysis was carried out by a deconvolution method, using a non-linear least square-fitting program 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.

Lipid peroxidation in liposomal membranes

AAPH is an azo-compound that generates peroxide radicals after thermal homolysis in both aqueous and lipid phases (17). Lipid peroxidation of liposomal membranes was performed using this radical generator, AAPH. The reaction mixture contained liposomal suspensions in water (pH 6.9) and 25 mmol/L AAPH and the final volume was 1.0 ml. This was incubated for 1 h with shaking in contact with air at 37°C.

Detection of oxidation in liposomes

Polyunsaturated fatty acids in the egg lecithin liposomal membranes are prone to peroxidative damage by free radicals. The

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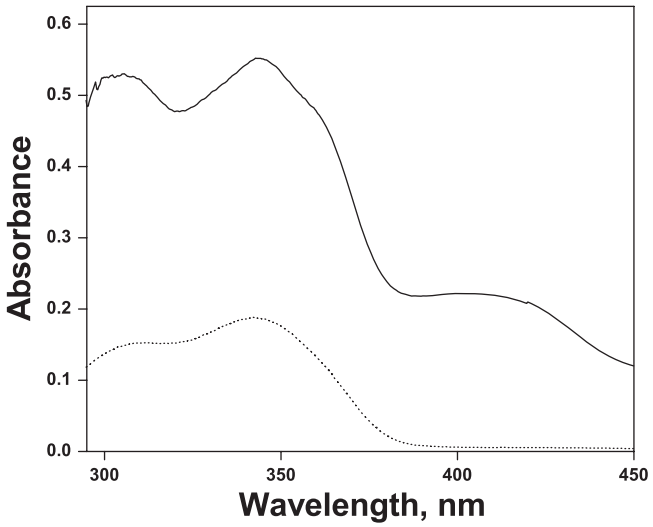

Figure 1. Absorption spectrum of 2.5×10^{-5} mol/L 3-HF in 2.0 mmol/L EYPC liposomes (—) and in water (....) at 25°C.

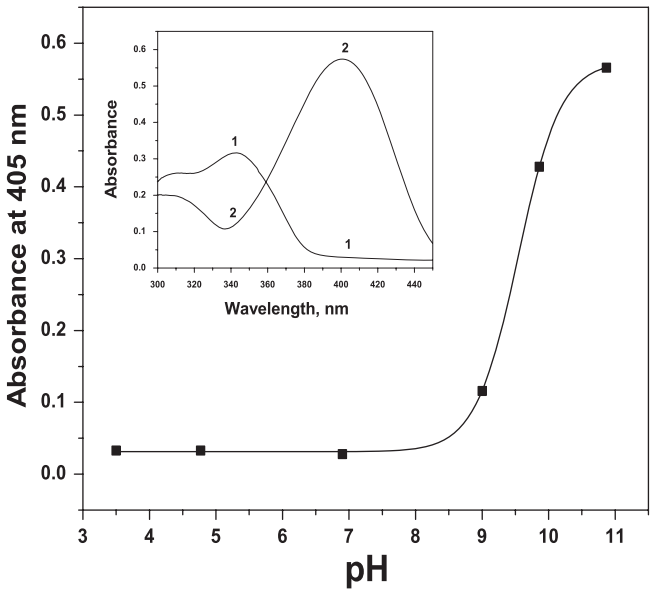

Figure 2. pH-Dependent absorption of the anionic species of 2.5×10^{-5} mol/L 3-HF at 25°C. (Inset) Representative absorption spectra of 2.5×10^{-5} mol/L 3-HF at neutral (1; pH 6.9) and alkaline (2; pH 10.87) pH values at 25°C.

essential stages in the peroxidation process include the formation of conjugated diene followed by hydroperoxide formation. Malondialdehyde (MDA) is only formed from fatty acids with three or more methylene-interrupted double bonds (18). Monitoring such reactions involves the absorption spectrophotometric measurements of MDA equivalents formed, with thiobarbituric acid (TBA) and trichloroacetic acid (TCA) (13, 19), at the absorption maximum of MDA (535 nm). For measuring MDA in peroxidized liposomes (in both the presence and the absence of 3-HF), 1.0 mL liposomal suspension was mixed with 2.5 mL 20% TCA and 1.0 mL 0.67% aqueous solution of TBA. After heating for 10 min in a boiling water bath, the pink pigment was extracted using 4.0 mL *n*-butanol and the absorbance was checked at 535 nm (for the MDA–TBA adduct). The malondialdehyde concentrations of the samples were calculated using an extinction coefficient (ε) of 1.56×10^5 mol/L/cm (20). All values are expressed as mean ± standard deviation (SD; $n = 3$).

Results and discussion

Steady-state absorption and emission behaviours of 3-HF in EYPC liposomal membranes

Figure 1 presents the electronic absorption spectra of 3-HF in egg PC liposomes along with the reference spectrum in water. Upon incorporation in liposomes, a long-wavelength absorption band with λ_{abs}^{max} ~410 nm was observed that had not been detected in the previous study of 3-HF in DPPC and DMPC liposomes (4). From the spectral position, we attributed this band to the anionic species of 3-HF (3, 21–22). Thus, the apparent ionization process of 3-HF should be reflected when its absorbance is monitored as a function of pH. As the pH was increased, the absorption spectrum of 3-HF showed a significant increase in absorbance at 405 nm with a concomitant decrease in absorbance at 340 nm. In Fig. 2 the absorbance change at 405 nm is plotted as a function of pH. The ground-state pK_a value derived from this curve was ~9.4, which was in excellent agreement with that reported by Wolfbeis *et al.* (21). Figure 2 (inset) shows the representative absorption spectra of 3-HF at near-neutral (pH 6.9) and alkaline (pH 10.87) pH values. Thus, it is evident that in EYPC liposomes, both the neutral ($\lambda_{abs}^{max} = 345$ nm) and anionic ($\lambda_{abs}^{max} = 410$ nm) forms contribute to the probe (3-HF) absorption. Because the long-wavelength absorption can be observed only in EYPC liposomes but not in water, the anionic form is generated by EYPC-mediated deprotonation of the 3-hydroxy group of the molecule in the ground state. Because 3-HF contains an electron-withdrawing carbonyl group adjacent to the hydroxyl group, it can be expected to be a weak acid, making the generation of the anion possible in the EYPC liposomal environment. The presence of a large spectral shift between the absorption maxima of these two species of 3-HF allows selective excitation of each species and separate study of their characteristic emission behaviours as well as binding properties with EYPC liposomes.

Figure 3 displays the fluorescence excitation and emission spectra of the neutral form ($\lambda_{ex} = 340$ nm) and the anionic form ($\lambda_{ex} = 410$ nm) of 3-HF (2.5×10^{-5} mol/L) incorporated in EYPC liposome suspension (2.0 mmol/L) along with the reference spectra in water (pH 6.9). In water the emission spectra of the neutral form of 3-HF consists of well-resolved dual emission bands, consisting of a blue-violet normal fluorescence with $\lambda_{em}^{max} = 408$ nm along with a green proton-transferred tautomer fluorescence band with $\lambda_{em}^{max} = 512$ nm (note that the weak band at shorter wavelengths is attributed to Raman scattering from the aqueous solvent medium). In EYPC liposomes this dual emission profile shows dramatic changes with significant enhancement in the emission intensity of the green fluorescence band, accompanied by a red-shift in the emission maximum ($\lambda_{em}^{max} = 528$ nm). However, the blue-violet fluorescence undergoes very little increase in intensity, and its emission maximum remains almost unchanged (Table 1). This emission behaviour of 3-HF in EYPC liposomes is consistent with that reported previously in DPPC liposomes as well as erythrocyte ghost membranes (4, 5). The significantly enhanced fluorescence quantum yield of the green emission band can be explained in terms of ESIPT tautomer emission, which predominates in liposomal membrane environments. It appears

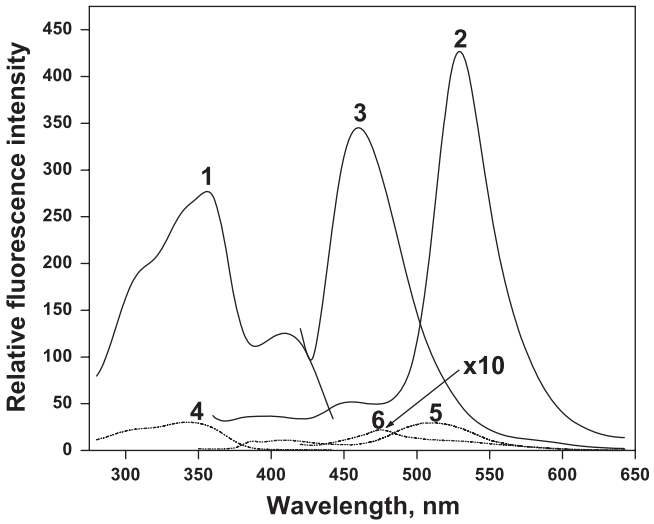

Figure 3. Fluorescence excitation (at λ_{em} = 530 nm; 1) and emission spectra of the neutral (at λ_{ex} = 340 nm; 2) and anionic species (at λ_{ex} = 410 nm; 3) of 2.5 × 10^{-5} mol/L 3-HF in 2.0 mmol/L EYPC (—) and in water (4, 5, 6; ---) at 25°C.

that in EYPC liposomes 3-HF molecules are mainly solubilized in the hydrophobic environments of the lipid bilayer, where external H-bonding perturbation is minimized, resulting in an efficient ESIPT process and high quantum yield of the green fluorescence band. The ratio of intensities of the green (I_1) and blue-violet (I_2) fluorescence bands is strongly dependent on solvent effects and related environmental parameters (23). As is evident from Fig. 3, the I_1/I_2 of 3-HF in EYPC liposomes is ~20, as compared with I_1/I_2 ~3 in water. Interestingly, the I_1/I_2 value obtained here is significantly lower than the value we reported in DPPC and DMPC liposomes (where I_1/I_2 ~30) in previous studies (4). This difference could be due to the fact that the unsaturated fatty acid chains present in EYPC increase the polarity of the hydrophobic locations of the lipid bilayer (24).

Figure 3 also shows that in EYPC liposomes, upon using λ_{ex} = 410 nm, the anionic species is selectively excited, resulting in a fluorescence emission band of significant intensity with emission maximum (λ_{em}) at 460 nm. It is noteworthy that in water, this anionic emission occurs with extremely low intensity. Thus, it appears that a significant population of 3-HF molecules is in an environment, probably containing acidic polar head phosphate groups, which facilitates proton abstraction at near pH neutrality. In a previous study Samanta *et al.* (25) reported that the anionic emission band disappears almost completely in presence of trace amounts of water. Thus, it appears that in EYPC vesicles, this population of 3-HF molecules (which are involved in anion formation) occurs in a relatively polar aprotic environment, binding to the polar head-group region rather than to the non-polar acyl side-chain region.

The inference regarding the existence of the anionic species of 3-HF in EYPC liposome derives additional credence from the spectral characteristics of the excitation profile (Fig. 3), which clearly shows a long-wavelength band with a maximum near 410 nm.

The fluorescence intensity enhancement of 3-HF (neutral form) with increasing EYPC concentration (shown in Fig. 4a) exhibits a saturating tendency. Figure 4b shows the variation of the ESIPT tautomer fluorescence intensity (F) of 3-HF (neutral form) with lipid concentration ([*EYPC*]). 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/[*EYPC*], according to the equation $1/F = 55.6/(K_p F_{max}[EYPC]) + 1/F_{max}$, where F_{max} is maximum fluorescence resulting from total probe incorporation into the membrane (3, 26, 27). The corresponding double-reciprocal plot of 1/F against 1/[*EYPC*] (Fig. 5) shows good linearity, as predicted by the equation. From the slope and 1/F intercept of this linear plot the partition coefficient K_p was found to be 4.2 × 10^4 at 25°C, where EYPC exists in liquid crystal phase. The partition coefficient value was found to be of the same order of magnitude to that observed by Mishra *et al.* for 3-HF in the solid gel phase of DMPC liposomes (3).

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 (16, 28). r is very low in fluid solution, where the fluorophore can freely rotate, and high for a rigid environment. For 3-HF the anisotropy value increases considerably (r = 0.124 for the neutral species monitored at the tautomer emission, whereas r = 0.180 for the anionic species) when incorporated into EYPC liposomes (Table 1). Such high values of the fluorescence anisotropy of 3-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.* (in DMPC liposomes), respectively (3–5). The relatively high anisotropy values suggest that both the species of 3-HF are rigidly bound in the membrane matrix.

Time-resolved fluorescence studies

The fluorescence decay kinetics of both the ESIPT tautomer and the ground-state anionic form of 3-HF in liquid crystalline phase of EYPC liposomes are found to be biexponential. The lifetime values are given in Table 2. The observation of biexponential

Table 1. Fluorescence emission properties of 3-HF in water and EYPC liposomes

Species	Environment	λ_{em}^{max} (nm)		Fluorescence anisotropy (r)
		Normal	Tautomer	
Neutral species of 3-HF	Water	408	512	0.042
	Egg PC liposomes	408	528	0.124
Anionic species of 3-HF	Water	ca. 475*		0.048
	Egg PC liposomes	460		0.180

*Very low intensity.

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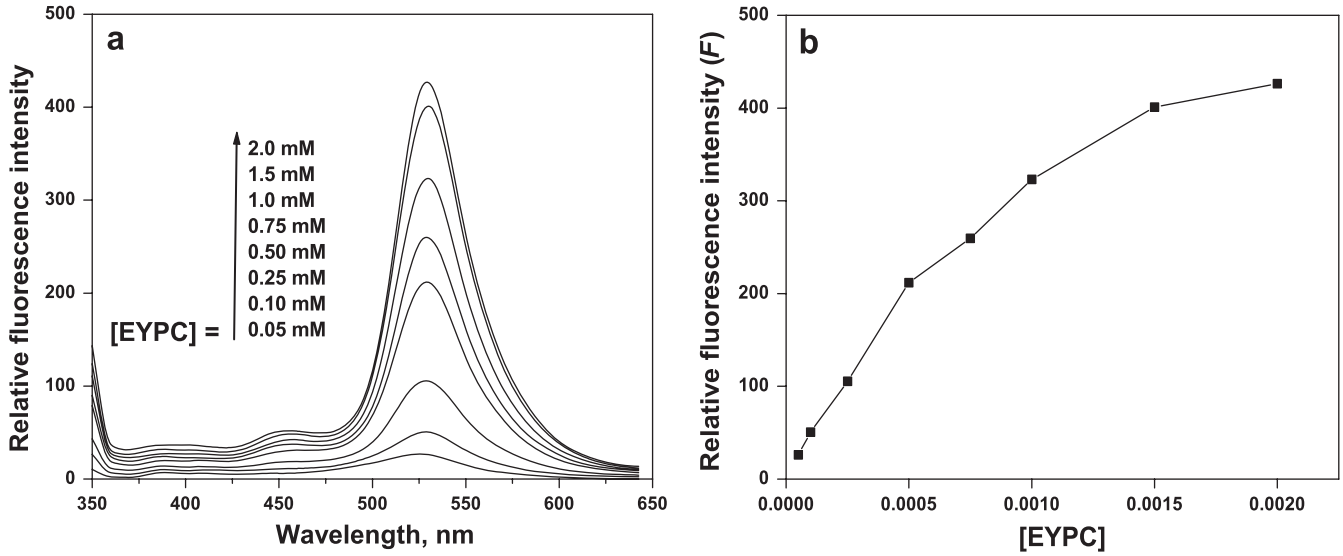

Figure 4. (a) Set of fluorescence emission spectra of 2.5×10^{-5} mol/L 3-HF in EYPC liposome for a concentration of lipid in the range 0.05–2.0 mmol/L at 25°C, λ_{ex} = 340 nm. (b) Plots of variation of ESIPT tautomeric fluorescence intensities of 3-HF with varying EYPC concentrations.

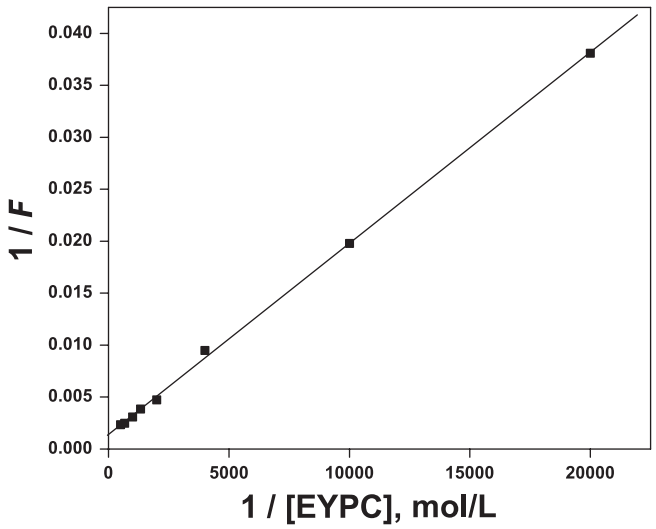

Figure 5. The double-reciprocal plot of 1/*F* against 1/[*EYPC*].

Table 2. Fluorescence decay parameters of the ESIPT tautomer and the ground-state anionic form of 3-HF in EYPC liposomes at 25°C

Species	τ_1 (ns)	A_1	τ_2 (ns)	A_2	χ^2
Phototautomer	1.074	0.79	2.82	0.21	1.20
Anion	0.792	0.495	5.34	0.505	1.06

λ_{ex} = 370 nm; λ_{em} = 570 nm for the tautomer and 440 nm for the anion. 3-HF concentration was 2.5×10^{-5} mol/L and that of EYPC was 2.0 mmol/L.

decays for the ESIPT tautomer as well as for the ground-state can be attributed to heterogeneity in distribution of 3-HF molecules in at least four different regions of the liposome. This is consistent with previous studies by Mishra *et al.* for 3-HF in DMPC liposomes.

The steady-state and time-resolved fluorescence emission characteristics suggest that the neutral and anionic forms of 3-HF molecules are distributed in the EYPC liposomes in different sites, characterized by different polarity and hydration properties. Binding to the hydrophobic region of the lipid bilayer minimizes external H-bonding perturbation effects, resulting in an efficient ESIPT process (1, 4). On the other hand, binding to the polar head-group region presumably leads to proton abstraction from 3-HF molecules, resulting in formation of the anionic species, which is stabilized by the electrostatic interaction with the positively charged nitrogen centre of the choline group (3). 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 (24). Such examples of the heterogeneous distribution in lipid vesicles are available in the literature for other types of neutral fluorescent probes as well (29, 30).

Effects of flavonoids on the production of TBARS

From the fluorescence emission studies, it is evident that the 3-HF molecules bind to the EYPC 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 3-HF in EYPC liposomes by measuring lipid peroxidation as thiobarbituric acid-reactive substances (TBARS) in the presence of the free radical generator AAPH. Figure 6 shows that 3-HF inhibits lipid peroxidation induced by AAPH in a dose-dependent manner. In order to assess the antioxidant potential of 3-HF, 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

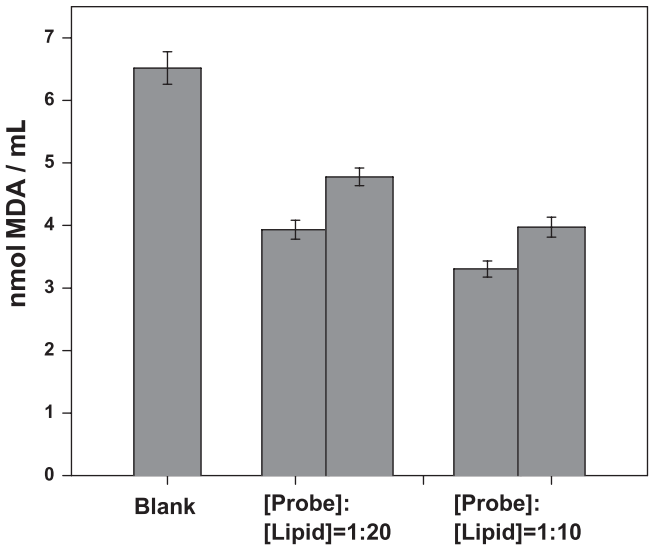

Figure 6. Effect of 3-HF on TBARS formation induced by AAPH in EYPC liposomes. 3-HF:phospholipid molar ratios were kept at 1:20 (100 μmol/L 3-HF, 2 mmol/L lipid) and 1:10 (200 μmol/L 3-HF, 2 mmol/L lipid). Values are expressed as mean ± SD ($n = 3$). For each [Probe]:[Lipid] molar ratio the right and left hand side bars show the values for 3-HF and Quercetin respectively.

activities of quercetin and 3-HF, determined by inhibition of the production of MDA in AAPH-induced lipid perixidation (LP), is in the ratio ~1:0.8. Since quercetin is nearly four times as potent as an antioxidant in relation to Trolox (31), it turns out that the antioxidant potential of 3-HF is nearly three 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 the 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 (28). Thus, the antioxidant activity of 3-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 (5). In this context the steady-state fluorescence anisotropy data are relevant, since the high anisotropy values observed clearly indicate that both (i.e. neutral as well as anion) forms of 3-HF are rigidly bound inside the membrane matrix, thus facilitating the antioxidant activity of 3-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 an electron transfer process (7, 32–34).

In conclusion, we have explored the binding of 3-HF in EYPC liposomal membranes, exploiting the excited-state intramolecular proton transfer (ESIPT) and dual luminescence behaviour of 3-HF. The present study demonstrates that apart from various naturally occurring flavonoids, their basic chromophoric moiety 3-HF exhibits significant antioxidant and radical scavenging activity in this membrane system. Since EYPC liposomes prepared from natural unsaturated phospholipids offer distinct advantages over saturated phospholipid vesicles (e.g. DPPC and DMPC 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.

Acknowledgements

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References

1. Kasha M. Proton-transfer spectroscopy. Perturbation of the tautomerization potential. *J. Chem. Soc. Faraday Trans. 2* 1986; **82**: 2379–2392.
2. Sengupta PK, Kasha M. Excited state proton transfer spectroscopy of 3-hydroxyflavone and quercetin. *Chem. Phys. Lett*. 1979; **68**: 382–385.
3. Shyamala T, Mishra AK. Ground- and excited-state proton transfer reaction of 3-hydroxyflavone in dimyristoylphosphatidylcholine liposome membrane. *Photochem. Photobiol*. 2004; **80**: 309–315.
4. Guharay J, Chaudhuri R, Chakrabarti A, Sengupta PK. Excited state proton transfer fluorescence of 3-hydroxyflavone in model membranes. *Spectrochim. Acta A* 1997; **53**: 457–462.
5. Chaudhuri S, Banerjee A, Basu K, Sengupta B, Sengupta PK. Interaction of flavonoids with red blood cell membrane lipids and proteins: antioxidant and antihemolytic effects. *Int. J. Biol. Macromol*. 2007; **41**: 42–48.
6. Ozgova S, Hermanek J, Gut I. Different antioxidant effects of polyphenols on lipid peroxidation and hydroxyl radicals in the NADPH-, Fe-ascorbate- and Fe-microsomal systems. *Biochem. Pharmacol*. 2003; **66**: 1127–1137.
7. van Acker FAA, Schouten O, Haenen GRMM, van der Vijgh WJF, Bast A. Flavonoids can replace α-tocopherol as an antioxidant. *FEBS Lett*. 2000; **473**: 145–148.
8. Gordon MH, Roedig-Penman A. Antioxidant activity of quercetin and myricetin in liposomes. *Chem. Phys. Lipids* 1998; **97**: 79–85.
9. Gregoriadis G, Neerunjun DE. Control of the rate of hepatic uptake and catabolism of liposome-entrapped proteins injected into rats. Possible therapeutic applications. *Eur. J. Biochem*. 1974; **47**: 179–185.
10. Weber SR, Lopez-Berestein G. Treatment of invasive aspergillus sinusitis with liposomal amphotericin B. *Laryngoscope* 1987; **97**: 937–941.
11. Sculier JP, Coune A, Meunier F, Brassinne C, Laduron C, Hollaert C, Collette N, Heymans C, Klastersky J. Pilot study of amphotericin B entrapped in sonicated liposomes in cancer patients with fungal infections. *Eur. J. Cancer. Clin. Oncol*. 1988; **24**: 527–538.
12. van Rooijen N, van Nieuwmegan R. Liposomes in immunology: multilamellar phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without any immunogenic activity of its own. *Immunol. Commun*. 1980; **9**: 243–256.
13. Chatterjee SN, Agarwal S. Liposomes as a membrane model for study of lipid peroxidation. *Free Rad. Biol. Med*. 1988; **4**: 51–72 [and references cited therein].
14. Mabry TJ, Markham KR, Thomas MB. *The Systematic Identification of Flavonoids*. Springer-Verlag: Heidelberg, New York, Berlin, 1970.
15. Huang CH. Phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry* 1969; **8**: 344–352.
16. Lakowicz JR. *Principles of Fluorescence Spectroscopy*, 3rd edn. Springer-Verlag: New York, 2006.
17. Niki E. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Methods Enzymol*. 1990; **186**: 100–108.
18. Murray RK, Granner DK, Mayes PA, Rodwell VW. *Harper's Illustrated Biochemistry*, 26th edn. McGraw-Hill: New York, 2003; 111–121.
19. Klein RA. The large-scale preparation of unsaturated phosphatidyl cholines from egg yolk. *Biochim. Biophys. Acta*. 1970; **210**: 486–489 [and references cited therein].
20. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol*. 1978; **52**: 302–306.
21. Wolfbeis OS, Knierzinger AJ, Schipfer R. pH-Dependent fluorescence spectroscopy. XVII. First excited singlet state dissociation constants, phototautomerism and dual fluorescence of flavonol. *J. Photochem*. 1981; **21**: 67–69.
22. Wolfbeis OS, Leiner M, Hochmuth P. Absorption and fluorescence spectra, pK_a values and fluorescence lifetimes of monohydroxy flavones

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and monomethoxy flavones. *Ber. Bunsenges Phys. Chem*. 1984; **88**: 759–767.
23. Strandjard ASG, Courtney SH, Friedrich DM, Barbara PF. Excited state dynamics of 3-hydroxyflavone. *J. Phys. Chem*. 1983; **87**: 1125–1133.
24. Klymchenko AS, Duportail G, Demchenko AP, Mely Y. Bimodal distribution and fluorescence response of environment-sensitive probes in lipid bilayers. *Biophys. J*. 2004; **86**: 2929–2941.
25. Mandal PK, Samanta A. Evidence of ground-state proton-transfer reaction of 3-hydroxyflavone in neutral alcoholic solvents. *J. Phys. Chem. A*. 2003; **107**: 6334–6339.
26. Bondar OP, Rowe ES. Preferential interactions of fluorescent probe Prodan with cholesterol. *Biophys. J*. 1999; **76**: 956–962.
27. Huang Z, Haugland RP. Partition coefficients of fluorescent probes with phospholipid membranes. *Biochem. Biophys. Res. Commun*. 1991; **181**: 166–171.
28. Sengupta B, Banerjee A, Sengupta PK. Investigations on the binding and antioxidant properties of the plant flavonoid fisetin in model biomembranes. *FEBS Lett*. 2004; **570**: 77–81.
29. Chong PL. Effects of hydrostatic pressure on the location of Prodan in lipid bilayers and cellular membranes. *Biochemistry* 1988; **27**: 399–404.
30. Alakoskela JMI, Kinnunen PKJ. Probing phospholipids main phase transition by fluorescence spectroscopy and a surface redox reaction. *J. Phys. Chem. B*. 2001; **105**: 11294–11301.
31. Naguib YMA. A fluorometric method for measurement of oxygen radical-scavenging activity of water-soluble antioxidants. *Anal. Biochem*. 2000; **284**: 93–98.
32. Nicoli MC, Toniolo R, Anese M. Relationship between redox potential and chain-breaking activity of model systems and foods. *Food Chem*. 2004; **88**: 79–83.
33. Cotelle N, Hapiot P, Pinson J, Rolando C, Vézin H. Polyphenols deriving from chalcones: investigations of redox activities. *J. Phys. Chem. B* 2005; **109**: 23720–23729.
34. Buettner GR. The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol and ascorbate. *Arch. Biochem. Biophys*. 1993; **300**: 535–543.

