Available online at www.sciencedirect.com
ScienceDirect
Journal of Photochemistry and Photobiology A: Chemistry 184 (2006) 113–124
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

3-Hydroxy-4′-[di-(2-hydroxyethyl)amino]flavone as a new step in search of an ideal membrane ratiometric fluorescent probe

Gora M'Baye [a], Oleksandr V. Martyloga [b], Guy Duportail [a], Vasyl G. Pivovarenko [b,*]

[a] *Département de Pharmacologie et Physicochimie, Equipe Photophysique des Interactions Biomoléculaires, UMR 7175, Institut Gilbert Laustriat, Université Louis Pasteur, BP 60024, 67401 Illkirch, France*
[b] *Chemistry Department, National Taras Shevchenko University, Kyiv 01033, Ukraine*

Received 21 February 2006; received in revised form 29 March 2006; accepted 30 March 2006
Available online 18 April 2006

Abstract

We report on the synthesis and fluorescence properties, in solvents and lipid vesicles, of a new 3-hydroxyflavone derivative: 2-[4-di(2-hydroxyethyl)aminophenyl]-3-hydroxy-4*H*-4-chromenone (FHE). Like others 3-hydroxyflavones, this probe is characterized by an excited-state intramolecular proton transfer reaction displaying two tautomeric excited states, which results in two emission bands well separated on the wavelength scale. Their relative intensities are highly environment-sensitive, allowing a two-band ratiometric analysis. The novel and original structural characteristics of FHE consist in the presence of two hydroxyethyl groups able to form H-bonds with phospholipid ester groups, thus conferring it anchoring properties at the membrane interface without any electrostatic interactions. FHE was tested in a series of organic solvents and demonstrates a good response to solvent polarity, with a peculiar sensitivity to solvents with strong H-bonding ability. In lipid vesicles, FHE displays a good sensitivity to membrane dipole potential, as shown by the electrochromism of both the excitation and the tautomer T^* emission spectra. FHE is also sensitive to changes in membrane fluidity and to the binding of amphiphilic polyanions of biological importance as adenosine triphosphate (ATP). Finally, a tentative comparison of spectral parameters was made between the data obtained in solvents and in vesicles by using as "criterion coordinates" the logarithm of the two bands intensity ratio versus the difference in wavenumbers of their maxima.

Keywords: Ratiometric fluorescent probe; 3-Hydroxyflavone derivatives; Solvent polarity; Phospholipid vesicles

1. Introduction

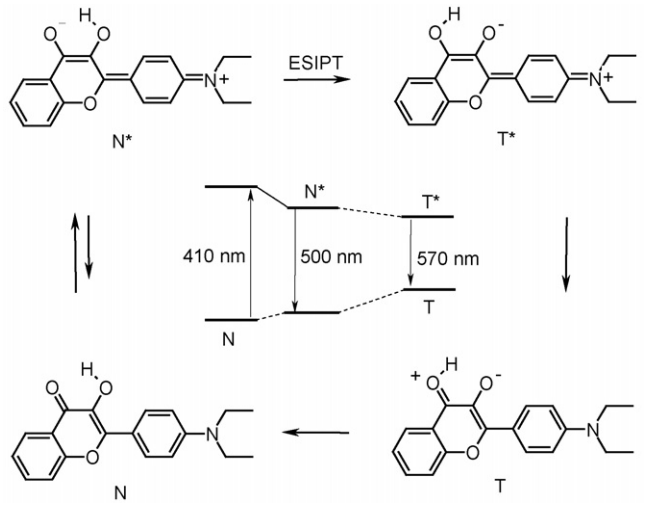
3-Hydroxyflavone (3-HF) dyes present a considerable interest in the design of multi-parametric fluorescence probes for the study of physicochemical processes in solutions as well as in biological systems. This interest proceeds from their excited-state intramolecular proton transfer (ESIPT) reaction which results in two excited state forms: a normal (N^*) and a tautomer (ESIPT product, T^*) forms, which are both highly emissive and exhibit well separated emission bands [1] (Scheme 1). The relative intensities and the positions of these two emission bands can report the physicochemical properties of the microenvironment of the dye. Moreover, the addition at 4′ position of the 3-HF moiety of an electron–donor dialkylamino group renders these dyes more strongly solvatochromic [2–4], so that the interactions with the environment are producing stronger perturbations not only in the equilibrium between the excited-state tautomeric forms, but also in the positions of the emission bands [3]. In some specific conditions, the hydroxyl group in position 3 can dissociate to yield the anionic form of the molecule, both in its ground and excited-state, but this occurrence has to be avoided to take advantage of the ESIPT reaction [5,6].

Due to their extreme sensitivity to solvent polarity and hydrogen bonding with protic solvents [7–13], 3-HF probes were used as sensors for polar/protic impurities in non-polar solvents [14,15], to detect the presence of water molecules in reverse micelles [16] or for biophysical studies in lipid vesicles and biomembranes [6,17–24]. Also, the electrochromic modulation

Abbreviations: FHE, 2-[4-di(2-hydroxyethyl)aminophenyl]-3-hydroxy-4*H*-4-chromenone; ATP, adenosine triphosphate disodium salt; 3-HF, 3-hydroxyflavone; ESIPT, excited-state intramolecular proton transfer; EYPC/PG, egg yolk phosphatidyl-choline/-glycerol; DMPC/DPPC, dimyristoyl/dipalmitoyl phosphatidylcholine; DMTAP, 3-(1,2-dimiristoyl-*sn*-propyl)-trimethylammonium bromide; 6-KC, 6-ketocholestanol; LUV, large unilamellar vesicles; FWHM, full width at the half-maximum

* Corresponding author. Tel.: +380 44 239 33 12; fax: +380 44 220 83 91.
E-mail addresses: Guy.duportail@pharma.u-strasbg.fr (G. Duportail), pvg_org@mail.ru, pvg@univ.kiev.ua (V.G. Pivovarenko).

1010-6030/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotochem.2006.03.037

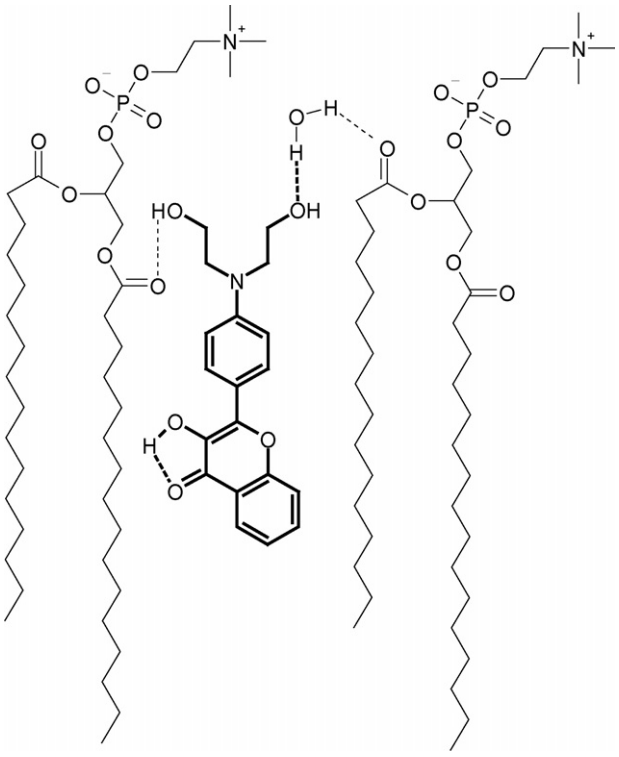


Scheme 1. Excited-state intramolecular proton transfer for the reference probe 4′-(dimethylamino)-3-hydroxyflavone and schematic representation of the energy levels.

of the ESIPT reaction has opened possibilities for convenient and sensitive observation of electrostatic field effects by recording the dramatic changes in the ratio of the two emission bands [25,26]. By using 3-HF derivatives possessing charged groups to anchor the probe to the membrane interface and hydrophobic substituents to orient it toward the low-polar part of the bilayer, this last property found an application for the determination of biomembrane potentials like surface [18,19] and dipole potential [21,24]. Moreover, due to the existence of the intermolecular H-bonded form, it is possible to simultaneously determine both polarity and hydration in phospholipid bilayers by using 3-HF probes [22,23].

As previously noticed, up to now all the 3-HF derivatives which were synthesized in order to get a fixed orientation in the lipid bilayer have positively charged group to ensure the anchoring of the probe at the membrane interface and accessorily to associate with negatively charged phospholipids. Due to the electrostatic interaction, this association with phospholipids can distort the local electric field of their phosphate group, and thus modify the distribution of electric fields existing in lipid membrane [27]. To clarify this problem, we need to conceive and to test 3-HF probes devoid of charged groups, but keeping their ability to be uniformly oriented in the lipid bilayer.

In the present work we describe the synthesis of the first flavonol probe of this series: 3-hydroxy-4′-[di-(2-hydroxyethyl) amino]flavone (FHE). We studied the fluorescence properties of this new probe dissolved in organic solvents as well as embedded in lipid vesicles considered as models for biomembranes. This probe has no charged groups, but possesses two hydroxyethyl groups which are able to form hydrogen-bonds with oxygen or nitrogen atoms of the phospholipid polar heads located at the membrane interface (Scheme 2). A third hydroxyl group is located at position 3 of the chromone ring, but should be much less prone to form intermolecular H-bonds due to the proximity of the carbonyl at position 4. Thus, the FHE molecule should incorporate into the lipid bilayer by keeping an



Scheme 2. Proposed preferential orientation and location of FHE probe molecule in a lipid membrane.

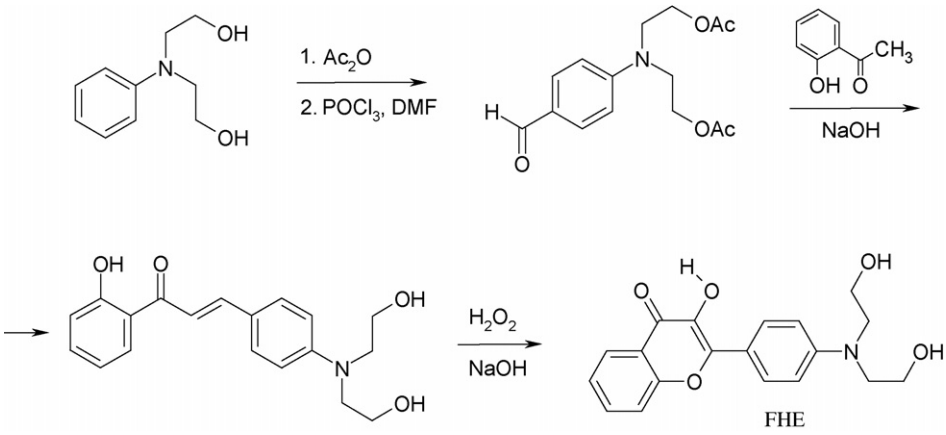
orientation parallel to the fatty acid chains, with the chromone ring in the down position.

2. Materials and methods

2.1. Synthesis

2-[4-Di(2-hydroxyethyl)aminophenyl]-3-hydroxy-4*H*-4-chromenone (FHE) was synthesized in four steps according to the next procedure (Scheme 3): *N*-phenyldiethanolamine (18.1 g, 0.1 mol) was boiled during 2 h in 50 ml of acetic anhydride, and the solvent was then evaporated in vacuo. The obtained oil (26.4 g) was dissolved in 50 ml of dimethylformamide, and phosphorus oxochloride (16.8 g, 0.11 mol) was added dropwise under stirring, at a temperature kept near 10–15 °C. The mixture was kept at 60 °C during 3 h, poured into ice and left for 10 h at room temperature. Then the formed oil was extracted by dichloromethane (3 ml × 50 ml), washed with water and dried with sodium sulfate. After, the solvent was evaporated in vacuo 20.5 g (70%) of crude aldehyde was obtained, containing 90–95% of pure substance, according to ^{1}H NMR data.

To the solution of aldehyde (5.39 g, 18 mmol) and 2-hydroxyacetophenone (2.5 g, 18 mmol) in ethanol (25 ml) a solution of sodium hydroxide (10 g in 15 ml of water) was added. After stirring during 2–3 h, the formed red–orange paste was left for 20 h at room temperature. Then the mixture was suspended in 150 ml of ethanol, cooled to 15 °C, and 7 ml of hydrogen peroxide (30%) were added under stirring. Since the temperature rose up to 60 °C, the mixture was stirred during 0.5 h, then neutralized by sulfuric acid and diluted by 150 ml of hot water. The precipi-



Scheme 3. Synthesis pathway to FHE probe.

tate formed was filtered off, recrystallised from ethanol and dried in vacuo. Yield 1.5 g (24%). The sample contained more than 99% of FHE probe according to ^{1}H NMR and HPLC data analysis. Yellow fibers with m.p. 179–180 °C. ^{1}H NMR spectrum: δ = 9.13 (s, 1H, OH), 8.08–8.06 (m, 3H, H-5, H-2′, H-6′), 7.75 (t, J = 8 Hz, 1H, H-7), 7.70 (d, J = 8 Hz, 1H, H-8), 7.42 (t, J = 8 Hz, 1H, H-6), 6.83 (d, J = 8 Hz, 2H, H-3′, H-5′), 4.84 (t, J = 5 Hz, 2H, OH), 3.58 (m, J = 6 Hz, 4H,–CH_2O–), 3.51 (t, J = 6 Hz, 4H,–CH_2N–). ^{13}C NMR spectrum, secondary carbons: 53.6; 58.6; tertiary carbons: 111.46; 117.75; 124.75; 125.07; 129.58; 133.49; quaternary carbons: 118.56; 121.91; 137.57; 147.4; 149.72; 154.71; 172.31. EI-MS spectrum, *m*/*z*: 341 (M^+), 157.

2.2. Chemicals

Solvents and chemicals used for synthesis were from Aldrich. 6-Ketocholestanol (6-KC), cholesterol, pluronic F-127, adenosine triphosphate disodium salt (ATP), egg yolk phosphatidylcholine (EYPC) and phosphatidylglycerol (EYPG), dimyristoyl (DMPC) and dipalmitoyl phosphatidylcholine (DPPC) were from Sigma. 3-(1,2-dimiristoyl-*sn*-propyl)-trimethylammonium bromide (DMTAP) was a gift from Dr. Heissler (Faculty of Chemistry, Strasbourg). The probe di-8-ANEPPS was from Molecular Probes. All these chemicals were used without further purification. Organic solvents were of spectroscopic grade for corresponding experiments.

2.3. Instrumentation

The following instrumentation was used. Melting points (uncorrected): PHMK apparatus ("VEB Analytik", Dresden). ^1H and ^{13}C NMR spectra: "Brucker-Spectrospin 500" spectrometer (solvent: DMSO-d_6, internal standard: tetramethylsilane). LC–MS analysis: "Agilent-1100" instrument with chemical ionization.

2.4. Vesicles

Large unilamellar vesicles (LUV) were obtained by the classical extrusion method [28] and were used in all experiments. Their final diameter was 0.11–0.12 μm and the final lipid concentration in all experiments was 200 μM in 15 mM phosphate–citrate buffer, pH 7.0. Probes were added to lipid vesicles, under stirring, at 1% ratio (mol/mol) from mM stock solutions in DMSO for 3-HF probes and in methanol for di-8-ANEPPS. 6-KC was incorporated into LUV from mM stock solutions in DMSO + 2.5% pluronic F-127 as previously described [29]. ATP was added from a 30 mM stock solution in 15 mM phosphate–citrate buffer, pH 7.0.

2.5. Spectroscopy

Fluorescence spectra were recorded on a Fluorolog (Jobin–Yvon Horiba) spectrofluorometer. The emission wavelengths for fluorescence excitation spectra were 645 nm for di-8-ANEPPS and 570 nm for FHE. For the latter probe, the excitation wavelength for the fluorescence emission spectra was 400 nm. All spectra were corrected for lamp intensity variations and signals from corresponding blank samples. All experiments were carried out at temperatures ensuring a liquid crystalline phase for the vesicles (20 °C for EYPC, 50 °C for DPPC and 30 °C or above for DMPC vesicles). With DPPC vesicles, experiments were also performed in the gel phase, namely at 20 °C. Emission anisotropy spectra were performed on a SLM-Aminco 8000 spectrofluopolarimeter (further computerized by Biologic, Chaix, France) in its L-configuration. The four emission polarized intensities curves versus wavelength (λ) needed for the determination of the emission anisotropy spectrum and the correction factor of the instrument were registered in a continuous way by scanning the emission monochromator, and simultaneously pair by pair, first $I_{VV}(\lambda)$ and $I_{VH}(\lambda)$, then $I_{HV}(\lambda)$ and $I_{HH}(\lambda)$.

2.6. Deconvolution procedure

Deconvolution of FHE fluorescence spectra into two bands, corresponding to normal (N^*) and tautomer (T^*) forms, was performed by using the "Siano" software kindly provided by its author (Dr. A.O. Doroshenko, Kharkov, Ukraine) [30]. The program is based on an iterative nonlinear least-square method

based on the Fletcher–Powell algorithm. The individual emission bands were approximated by a log-normal function [31] which accounts for three parameters: position, full width at the half-maximum (FWHM) and asymmetry (P). Band asymmetry is defined by dividing the FWHM (in cm^{-1}) of the band into blue and red parts according to the position of the band maximum and then calculating the ratio of the blue to the red part. In our case, the adopted fixed values were physically justified on the basis of previously obtained data in organic solvents and LUVs [11,22,23]. Thus, for the iteration process, the FWHMs of the short-wavelength band (N^*) were fixed only for the spectra in lower alcohols using the value obtained during the deconvolution of the spectra in 2-methyl-2-propanol. The other parameters, asymmetry of N^* and T^* bands, FWHM of the T^* band and relative intensities of the bands, were allowed to vary during the iteration process.

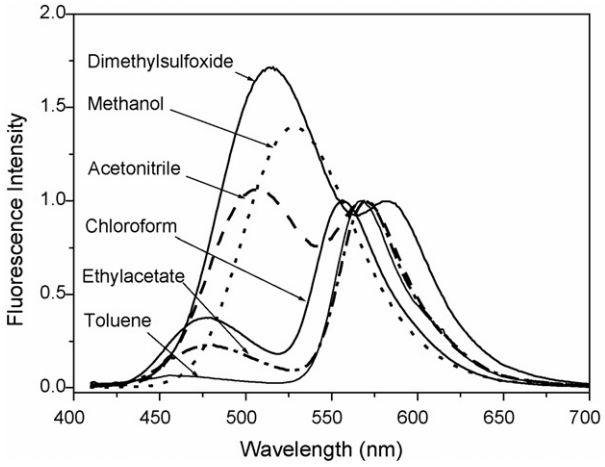


Fig. 1. FHE fluorescence spectra in organic solvents of different polarity. Excitation wavelength: 400 nm. Spectra are normalized at the T^* maxima (excepted for methanol).

3. Results and discussion

3.1. Fluorescence properties in organic solvents

We recorded the emission spectra in three categories of solvents: neutral (toluene, bromobenzene, dichloromethane, trichloroethylene and acetonitrile) basic (fluorobenzene, dioxan, tetrahydrofuran, ethyl acetate, acetone, *N*,*N*-dimethylformamide and dimethylsulfoxide) and protic ones (methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 2-methylpropanol-2 and chloroform). Some of the obtained spectra are given in Fig. 1 and the resulting spectroscopic parameters are summarized in Table 1.

Like most of the 3-HF derivatives previously studied [7–13], FHE probe shows a dual fluorescence in most of organic solvents, with the exception of the most polar ones such as water and lowest alcohols. Nevertheless, even in the last cases, although with a low precision, both emission bands can be extracted from the spectra by the deconvolution procedure.

Similarly to other 3-HF dyes, the emission spectra of FHE show a strong sensitivity to solvent properties. Indeed, an increase of the solvent polarity (a function of the dielectric constant ε) shifts the N^* band to the red and increases the I_{N^*}/I_{T^*} ratio. The position of both N^* and T^* bands shows a linear dependence with the solvent polarity (Fig. 2A). Moreover, the much larger slope observed for the N^* band as compared to the T^* band proceeds from the larger solvatochromism of the N^* state. This is in line with the classical solvatochromism of such dyes, due

Table 1
Absorption and emission parameters of FHE probe in organic solvents

Solvent	$f(\varepsilon)$	$f(n)$	$E_T(30)$	ν_{abs} (cm^{-1})	ν_{N^*} (cm^{-1})	ν_{T^*} (cm^{-1})	ΔS_{N^*}	ΔS_{T^*}	$\nu_{N^*} - \nu_{T^*}$	$\log(I_{N^*}/I_{T^*})$	ϕ (%)
Fluorobenzene	0.1926	0.1942	37.0	24910	20880	17670	4030	7240	3210	−0.45	19.0
1.4–Dioxan	0.2232	0.3358	36.0	25060	21510	17570	3550	7490	3940	−0.35	10.5
Toluene	0.2390	0.2261	33.9	24780	21930	17600	2850	7180	4330	−0.82	25.5
Diisopropyl ether	0.3067	0.1836	33	25030	22030	17670	3000	7360	4360	−0.68	11.0
Trichloroethylene	0.3087	0.2204	35.9	24850	21460	17790	3390	7060	3670	−0.69	28.0
Bromobenzene	0.3727	0.2442	36.6	24570	20880	17480	3690	7090	3400	−0.55	32.0
Ethyl acetate	0.3843	0.1853	38.1	25030	20920	17540	4110	7490	3380	−0.35	8.0
Tetrahydrofuran	0.4053	0.1968	37.4	24720	19790	17450	4930	7270	2340	0.34	10.5
Dichloromethane	0.4204	0.2033	40.7	24970	20620	17730	4350	7240	2890	−0.18	20.0
Acetone	0.4642	0.1803	42.2	25000	19890	17380	5110	7620	2510	0.28	7.0
Acetonitrile	0.4792	0.1748	45.6	25060	19700	17440	5360	7620	2260	0.47	9.0
N,*N*-Dimethylformamide	0.4801	0.2054	43.8	24600	19520	17100	5080	7500	2420	0.52	8.8
Dimethyl sulfoxide	0.4841	0.2009	45.1	24360	19410	16980	4950	7380	2430	0.66	13.8
Chloroform	0.3560	0.2074	39.1	25000	20960	17950	4040	7050	3010	−0.15	21.3
2-methyl-2-butanol	0.3805	0.1968	41.1	24420	20340	17850	4080	6570	2490	0.57	23.0
1-pentanol	0.4520	0.1986	49.1	24330	19350	17630	4980	6700	1720	1.02	54.0
1-butanol	0.4579	0.1948	50.2	24510	19260	17610	5250	6900	1650	1.23	53.0
1-propanol	0.4643	0.1898	50.7	24450	19150	17550	5300	6900	1600	1.47	55.5
Ethanol	0.4704	0.1812	51.9	24510	19070	17320	5440	7190	1750	1.41	57.0
Methanol	0.4773	0.1689	55.4	24720	18940	17850	5780	6870	1090	2.43	38.5

$f(\varepsilon) = (\varepsilon - 1)/(2\varepsilon + 1)$, dielectric permeability function [33,34]; $f(n) = (n^2 - 1)/(2n^2 + 1)$, refraction index function, reflecting high frequency polarizability component of a medium [33,34]; $E_T(30)$, empiric scale of solvent polarity, values taken from Ref. [36]; ν_{abs}, ν_{N^*}, ν_{T^*}, positions of absorption, N^* and T^* bands emission maxima, respectively; ΔS_{N^*} and ΔS_{T^*}, Stokes shifts; I_{N^*} and I_{T^*}, intensities of N^* and T^* bands, respectively. For calculation of $\log(I_{N^*}/I_{T^*})$, the integral intensities of each band obtained after deconvolution of the spectra were used.

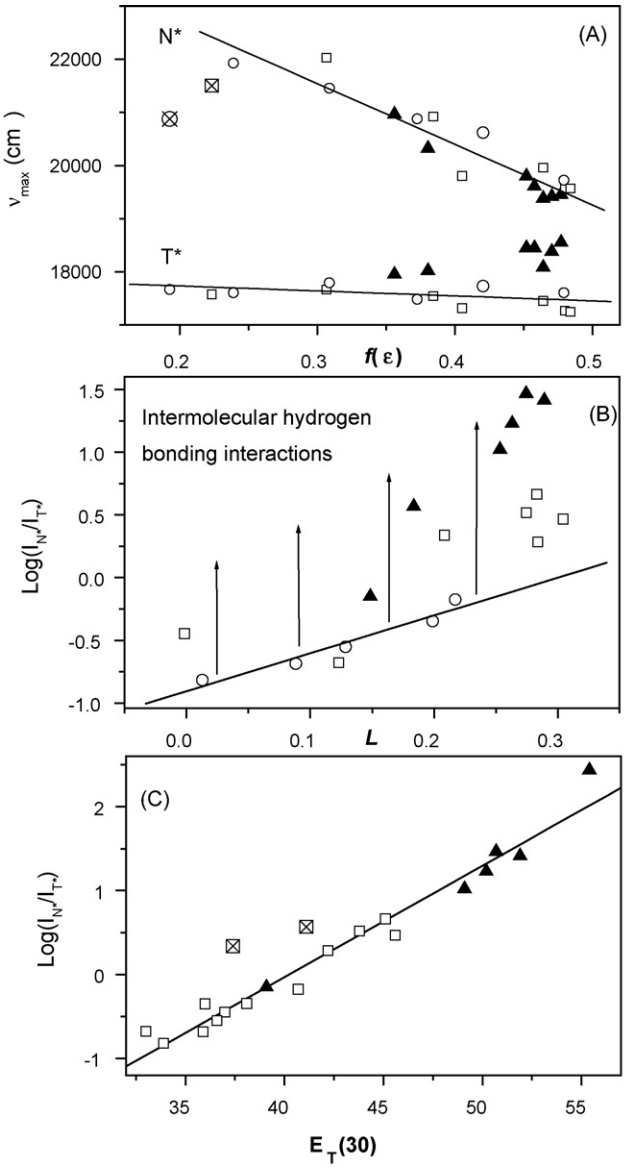


Fig. 2. (A) Positions of N^* and T^* emission bands maxima vs. polarity function $f(\varepsilon)$ for neutral (◯), basic (□) and protic (▲) solvents and corresponding linear regression fits. Are excluded from fitting: for N^* form the data from fluorobenzene and dioxan (crossed circle and square, respectively), for T^* form the data from protic solvents. (B) Dependence of $\log(I_{N^*}/I_{T^*})$ vs. Lippert polarity parameter L for neutral (◯), basic (□) and protic (▲) solvents and the corresponding linear regression fit. The linear regression is based on the data obtained from toluene, bromobenzene, dichloromethane, trichloroethylene and ethylacetate. The increase of the level of intermolecular hydrogen-bonding interactions is indicated by arrows. (C) Dependence of $\log(I_{N^*}/I_{T^*})$ vs. the empiric solvent polarity parameter $E_T(30)$ taken from Ref. [36] (□: neutral and basic solvents; ▲: protic ones) and the corresponding linear regression fit. The data from tetrahydrofuran and 2-methylpropanol (crossed squares) are excluded from the fitting.

to the difference in the dipole moments of the N^* and T^* excited states [12]. As the N^* state possesses a larger dipole moment than the T^* state, it is selectively stabilized by polar solvents and as a result the ESIPT equilibrium is shifted to the N^* state [13,32], thus increasing its relative fluorescence intensity. However, due to the presence of two additional hydroxyl groups, in close proximity to the positive pole of the fluorophore moiety, and to their ability to give H-bond interactions, it is not surprising to observe for FHE probe some unusual spectral behavior within the selected set of solvents. Thus, specific features can be observed with deviations from linearity of the plots of the bands positions versus the dielectric constant function (Fig. 2A). In the case of N^* band strong deviations are observed for the less polar of basic solvents as fluorobenzene and dioxan. In the case of T^* band protic solvents like alcohols exhibit systematic hypsochromic shifts, but this was previously observed with other 3-HF probes [11,32].

From these data, we can assert that the fluorescence properties of FHE do not considerably differ from those of other previously studied 3-HF probes with the same fluorophore moiety, except that some differences appears in non-polar basic solvents which may give additional interactions through hydrogen-bonding with the two terminal hydroxyl groups. This peculiar behavior of FHE in this class of solvents finds some confirmation by considering the parameter the most appropriate to the dual fluorescence of 3-HF probes, namely the intensity ratio I_{N^*}/I_{T^*} or $\log(I_{N^*}/I_{T^*})$. As shown in Fig. 2B, we expressed $\log(I_{N^*}/I_{T^*})$ versus the Lippert polarity function $L = (\varepsilon - 1)/(2\varepsilon + 1) - (n^2 - 1)/(2n^2 + 1)$, a function which is independent of polarisability effect [33–35]. In this representation, the data can be divided in two parts: either those concerning neutral and some polar aprotic (basic) solvents which lie on a straight-line regression, or those concerning non-polar basic and protic solvents, which lie much more higher from the linear regression. These excluded data concern fluorobenzene, dioxan, diisopropyl ether, chloroform, dimethylformamide and alcohols, all solvents which can give rise to strong hydrogen-bond interactions with FHE molecule. Thus, due to strong additional polarizing effect caused by hydrogen-bond formation, this dependence of $\log(I_{N^*}/I_{T^*})$ versus Lippert polarity function clearly separates solvents able to give strong hydrogen-bonding interactions with FHE probe from solvents where this interaction weak or does not exists. In such a representation, this probe could be considered as a good sensor for strong specific interactions in homogeneous solutions.

If on another hand, we choose to express $\log(I_{N^*}/I_{T^*})$ versus $E_T(30)$, which is a scale of solvent polarity developed by Reichardt [36], a fairly good linear dependence for all the tested solvents (with only a few exceptions) is observed as shown in Fig. 2C. In other words, the parameter $\log(I_{N^*}/I_{T^*})$ for FHE probe correlates rather well with this empiric scale of solvent polarity, $E_T(30)$, and can be itself considered as a new scale of solvent polarity.

3.2. Fluorescence properties in lipid membranes

3.2.1. General trends

The binding of FHE to large unilamellar vesicles results in a strong increase of the fluorescence intensity as compared to the weak fluorescence intensity observed in water, which proves that the dye incorporates easily into the lipid bilayer and is well isolated from the bulk water. In Fig. 3 are shown some of the emission spectra of FHE after incorporation into vesicles composed of lipids with differently charged polar heads: either vesicles composed of the anionic lipid EYPG, of the neutral lipid EYPC or of a 1:1 mixture of EYPC and the synthetic cationic

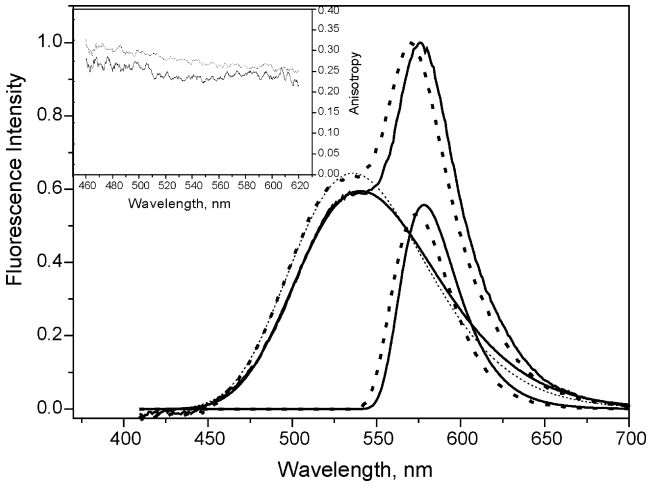


Fig. 3. Normalized fluorescence spectra of FHE embedded in EYPC-DMTAP 1:1 (solid) and EYPG (dot) lipid vesicles together with their separate N^* and T^* bands obtained by the deconvolution procedure (see Section 2.5). The spectrum corresponding to pure EYPC vesicles (not shown) is quite similar to EYPC-DMTAP one. Inset: corresponding fluorescence anisotropy spectra.

lipid DMTAP. In all cases, the dual emission characterizing ESIPT reaction is observed, with N^* emission band around 535 nm and T^* emission band centered at 575 nm. It appears from these spectra that the charge of the constituting lipids (−1, 0 and +0.5 for the three compositions, respectively) does not strongly modify the profile of fluorescence: to go from a positive (+0.5 for the EYPC/DMTAP mixture) to a negative charge (−1 for EYPG) provokes only a small increase of the N^* band intensity and a small blue shift of the T^* band. This poor sensitivity to surface potential was previously observed for charged 3-HF derivatives having a long spacer between the charged headgroup and the fluorophore moiety, and thus was explained by the deep location of the fluorophore in the lipid bilayer and, therefore, their significant screening from the membrane surface [37]. In sharp contrast, the 3-HF derivatives heaving shallower location of the fluorophore present a strong sensitivity to the surface potential [18,19,22]. In Fig. 3 are also shown the deconvoluted emission spectra and the steady state fluorescence anisotropy spectrum of FHE (insert). By combining these additional data, we obtain important informations:

First, we observe relatively stable fluorescence anisotropy through the whole spectrum which should point out a homogeneous distribution and orientation of the probe in the lipid bilayer, whatever the composition of the vesicles. Second, the deconvolution procedure leads to only two components, with an excellent accuracy ($r \geq 0.998$) corresponding to the N^* and T^* bands emission. The emission of N^* corresponds to a relatively narrow band with a halfwidth of about 3000 cm^{-1} close from the one obtained in pure solvents (2600–3300 cm^{-1}), whereas the T^* band presents the half-width (1250–1400 cm^{-1}) commonly observed for 3-HF dyes in organic solvents or in lipid membranes [12,22,23].

The existence of only two components is in sharp contrast with what was observed for other previously studied 3-HF derivatives, either neutral like 4′-dimethylamino-3-hydroxyflavone [22] or containing cationic ammonium groups with anchoring properties [23]. In previous studies, the deconvolution procedure was systematically leading to emission spectra composed of three different bands, corresponding to a H-bond-free form displaying a two-band emission due to the ESIPT reaction (N^* and T^* bands) and a H-bonded form (H–N^* form) displaying a single-band emission with no ESIPT. Obviously, in the case of FHE the H–N^* form emitting in a wavelength range between those of N^* and T^* forms, does not exist. The existence of only the H-bond-free form must correspond to a unique location of the probe molecule into the bilayer which explains the relatively stable fluorescence anisotropy through the emission wavelength range. Indeed, the probes presenting also the H–N^* form are characterized by fluorescence anisotropy values strongly decreasing at wavelengths corresponding to the H–N^* emission, which was mainly explained by a different location of this form [22,23].

The intensity ratio I_{N^*}/I_{T^*} we observed in lipid vesicles should correspond to a probe location close to the ester groups of phospholipids. However, the position of N^* form emission maximum is shifted considerably to the red by comparison with an ester-containing medium (478 nm in ethylacetate as compared to 535 nm in lipid vesicles and 540 nm in water) and should correspond to a much more polar environment. This discrepancy could be explained by the stabilizing influence of the electric fields existing in the membrane interior, and mainly the dipole potential Ψ_D which has the higher magnitude [27]. Depending on the probe orientation in the membrane, Ψ_D can either stabilize or destabilize the N^* excited state. Indeed, by increasing Ψ_D, we observed a red-shift of the emission of probes with a similar orientation as FHE and a blue-shift for probes with an opposite orientation [21,24,37]. These considerations were prompting us to study the influence of Ψ_D modulation on the fluorescence of FHE in lipid vesicles.

3.2.2. *Influence of membrane dipole potential modulation*

6-Ketocholestanol (6-KC) is a chemical effector known to increase the membrane dipole potential Ψ_D [38,39]. The styrylpyridinium probe di-8-ANEPPS was first used to measure, by fluorescence spectroscopy, the variation of Ψ_D in membrane systems, and became quite popular for this purpose. The method, making use of the electrochromic properties of this dye, is based on the fluorescence intensity ratio determination at two wavelengths on the edges of the excitation spectrum, namely I_{440}/I_{530} [29]. This probe was previously used for calibration of the effects caused by Ψ_D changes on the fluorescence parameters of two 3-HF probes which possess opposite orientations of the fluorescent moiety (in terms of direction of its dipole moment) in the lipid bilayer [21,24]. We demonstrated that after Ψ_D changes, due to the electrochromic properties of their common fluorescent moiety, the probe orienting in the bilayer similarly to di-8-ANEPPS shows similar shifts in the excitation spectra, whereas the probe with the opposite orientation shows the opposite shifts. Moreover, we established that this response of 3-HF probes to Ψ_D is also quantitatively correlated with dramatic changes of relative intensities of N^* and T^* emission bands [21,24]. We performed the same study with FHE probe incorporated into EYPC vesicles, by modulating Ψ_D by successive addition of

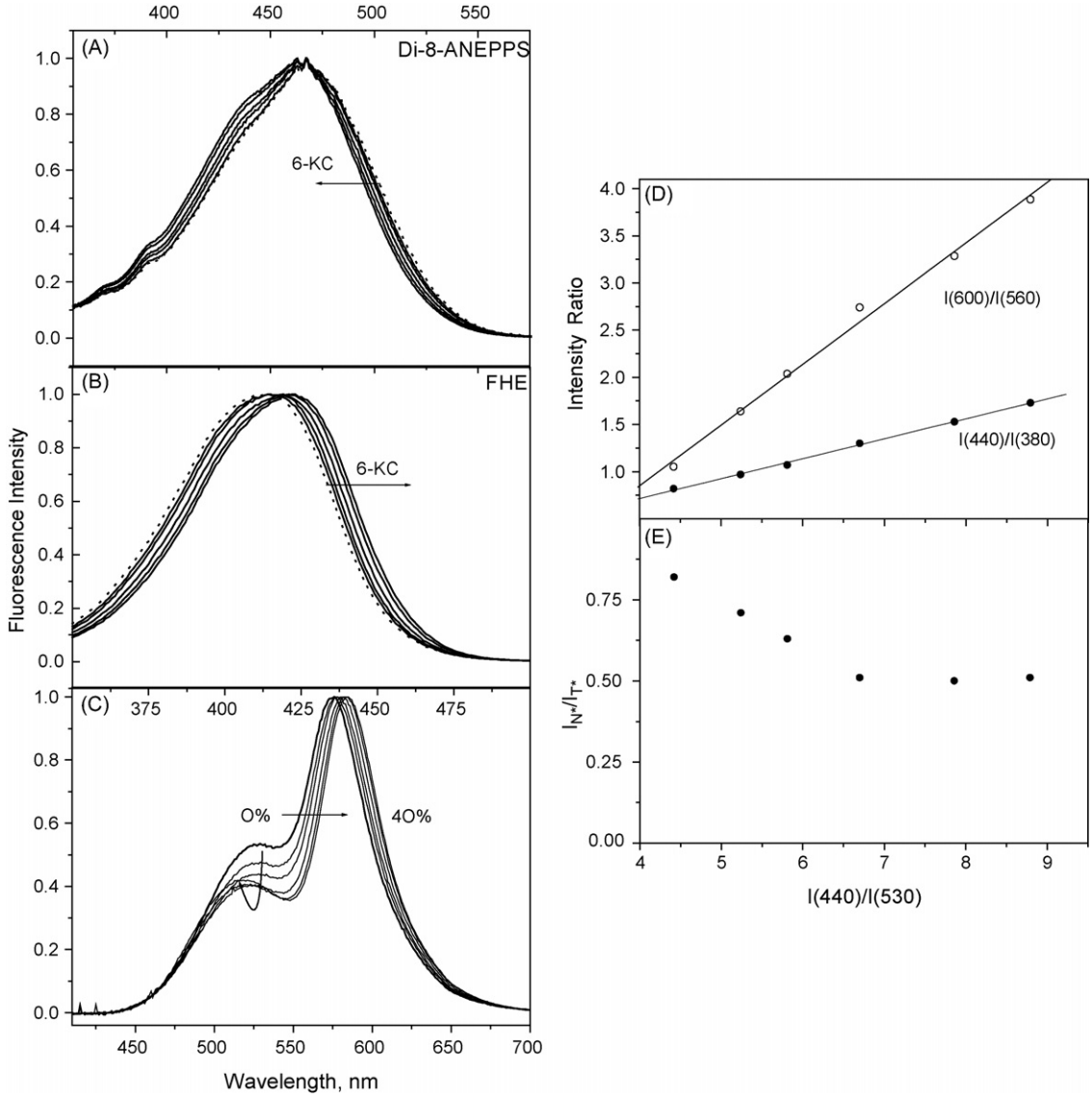


Fig. 4. Fluorescence results obtained on EYPC vesicles with varying amounts of 6-KC (0%, 5%, then 10–40% by step of 10%). (A and B) Fluorescence excitation spectra for vesicles labeled with di-8-ANEPPS and FHE respectively (dotted spectrum: 0%; the arrows indicate increasing 6-KC ratios); (C) Emission fluorescence spectra of FHE-labeled vesicles in the same conditions. (D) Intensity ratios I_{440}/I_{380} (●), measured on the excitation spectra, and I_{600}/I_{560} (◯), measured on the deconvoluted T^* band emission, for FHE-labeled vesicles vs. the corresponding ratio I_{440}/I_{530} for di-8ANEPPS-labeled vesicles; (E) Ratiometric response I_{N^*}/I_{T^*} of FHE-labeled vesicles vs. I_{440}/I_{530}. In panels D and E, 6-KC concentration is increasing from left to right.

6-KC. The different spectra (excitation and emission spectra) that we obtained are summarized in Fig. 4, together with the correlation curves obtained from the comparison between the ratiometric responses of FHE (either in excitation, I_{440}/I_{380}, or in emission, I_{N^*}/I_{T^*} and I_{600}/I_{560}) and the ratiometric response of di-8-ANEPPS (I_{440}/I_{530}). As shown in Fig. 4A and B, the sign and magnitude of the observed shifts in the excitation spectra with the addition of 6-KC are on the opposite direction for di-8-ANEPPS and FHE, which confirms that the respective fluorophores are oriented in opposite direction. This is a confirmation of the anchoring properties at the interface of the two terminal hydroxyl groups.

By considering now the emission spectra shown in Fig. 4C, it appears that the addition of 6-KC induces two different, although connected, events. First we observed the modification of the ratio I_{N^*}/I_{T^*}, the parameter we previously used to follow the modifications of membrane dipole potential in lipid vesicles and biological cells with other 3-HF derivatives [21,24,37]. Second, and this is an original consideration, we observed an appreciable red-shift of the T^* band, which indicates that in the present case, the tautomer excited state T^* is also presenting a rather strong electrochromism. We decided to quantify this shift by measuring the intensity ratio at two wavelengths on the edges of the deconvoluted T^* emission band, namely 560 and 600 nm. The correlation curves obtained by comparing the di-8-ANEPPS response I_{440}/I_{530} with FHE responses, both in excitation (I_{440}/I_{380}) and in T^* emission band (I_{600}/I_{560}) are shown in Fig. 4D. The correlation curves obtained in both cases are found linear on the whole range of 6-KC concentration. Thus, in some way the electrochromism of the T^* band

emission could be useful for measuring Ψ_D changes. Surprisingly, the correlation obtained with the ratio I_{N^*}/I_{T^*} (Fig. 4E) appears less conclusive since a saturation effect appears for the higher 6-KC concentrations. This discrepancy appearing at high 6-KC concentrations can be explained by considering the evolution of the emission spectra and by focusing on the N^* band (Fig. 4C). We observe that the increase of 6-KC concentration initially causes a decrease of the N^* form intensity, followed at the higher concentration by a clear blue-shift accompanied by a small increase of intensity. This is in contrast with the behavior of the T^* band which, as noticed before, undergoes a gradual red-shift with the increase of 6-KC concentration. Obviously, the two excited states, N^* and T^*, proceeding from the ESIPT reaction, are not responding in the same way to Ψ_D modulation. The N^* band emission appears to undergo like a saturation effect, which can be the indication of some interaction between FHE and highly concentrated 6-KC, maybe by the formation of intermolecular hydrogen-bonds facilitated by the increase of basicity of the N^* excited state due to intramolecular charge transfer effect [32] and leading to reorientation of a part of the FHE molecules. Thus, at these high 6-KC concentrations, the N^* form is in some way shielded from the influence of Ψ_D, with as a consequence the non-linearity of I_{N^*}/I_{T^*} ratio.

3.2.3. *Influence of cholesterol, temperature and ethanol*

Cholesterol, one of the main component of biomembranes, has a vertical position within the bilayer in such a way that its hydroxyl group is close to the ester carbonyl group of phospholipids and its opposite end is near the 14th carbon of an extended lipid chain [40]. It is well known to have a condensing effect on the packing of phospholipids in their liquid crystalline phase, in other words it decreases the membrane fluidity. We studied the influence of increasing concentrations of cholesterol on the fluorescence of FHE in EYPC vesicles. The first observation is the weak influence of cholesterol ratio on the fluorescence intensity (within 3%, not shown). This means that there is no direct interaction between cholesterol molecule and FHE. As shown in Fig. 5, the intensity ratio I_{N^*}/I_{T^*} decreases (from 0.65 to 0.42) when cholesterol ratio increases, and the positions of T^* band undergoes a small red-shift (~3–5 nm). Another way to modify membrane fluidity (to increase it) is to increase the temperature, what we did with DMPC vesicles in their liquid crystalline phases (from 50 to 30 °C). In this case, the increase of temperature induces a strong decrease of the overall fluorescence intensity (about 65%, not shown) due mainly to some increase of the non-radiative decay rates, but once normalized, the evolution of the emission spectra is very similar to the one observed by changing cholesterol ratio (see Fig. 5). This confirms that we are in presence of a "pure" fluidity effect. To increase the fluidity will allow the FHE molecule to slightly move towards a more polar environment, which means a less deep location, without any substantial changes of its orientation into the bilayer.

We also briefly studied the influence of ethanol. In the gel phase, for example in DPPC vesicles at 20 °C, ethanol is known to induce the formation of an interdigitated phase ($L_{\beta I}$ phase). This process was previously studied by fluorescence with probes like DPH [41], pyrene [42] or of 3-hydroxy-4′-aminoflavones [17]. In the present case the addition of ethanol (up to 100 mg/ml) to FHE-labeled DPPC vesicles at 20 °C does not modify at all the intensity and the profile of emission spectra (data not shown), which means that the interdigitation process does not influence, at least in terms of the environmental polarity, the deep location of FHE.

3.2.4. *Influence of ATP*

As a polyanion, adenosine triphosphate is a highly hydrophilic molecule with some amphiphilic character due to its planar and hydrophobic adenine moiety conferring it some ability to interact with lipid membrane. Meantime, we recently demonstrated that ATP can interact with 3-hydroxy-4′-(dimethylamino)flavone, this interaction being maintained even in the presence of lipid structures like mitochondria [43]. A work presently under progress in our laboratory, which will be published later on, shows that FHE is also prone to interact with ATP

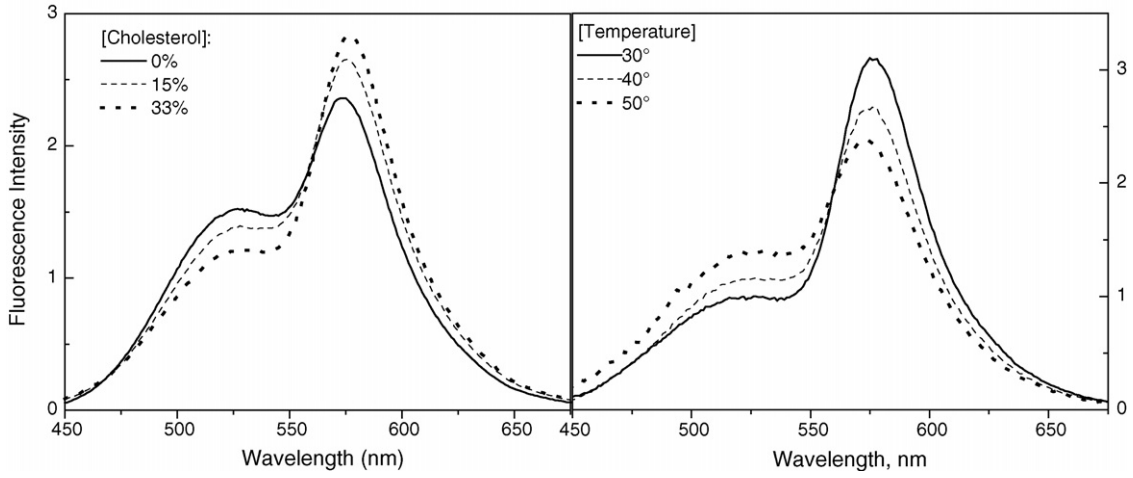


Fig. 5. Influence of cholesterol ratio and temperature on the emission spectra of FHE-labeled phosphatidylcholine vesicles. The cholesterol effect was determined on EYPC vesicles at 20 °C and the temperature effect on DMPC vesicles. The spectra obtained with increasing ratio of cholesterol are self-normalized at 562 nm. For comparison, the spectra obtained by varying the temperature are normalized at the same wavelength.

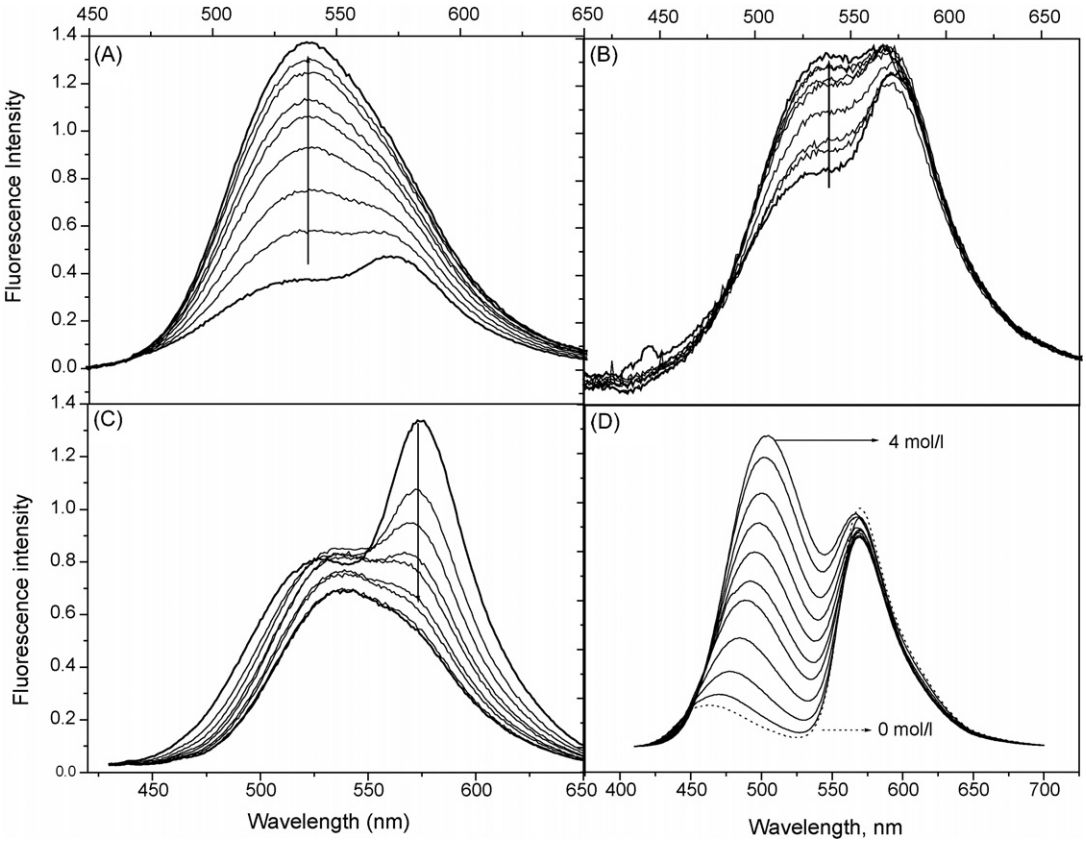


Fig. 6. Changes in the fluorescence spectra of FHE embedded in DPPC vesicles either at 20 °C (A, gel phase) or at 50 °C (B, liquid crystal phase), and in EYPC vesicles (C) at 20 °C; ATP concentration is increasing from 0 to 17 mM as shown by arrows. For comparison, panel D is showing the influence of increasing amount of water (from 0.25 to 4 mol/l) on the fluorescence spectrum of FHE in dioxan. Excitation wavelength: 400 nm.

in solution (data not shown). These considerations prompted us to study the influence of ATP on the fluorescence properties of FHE embedded in phospholipid vesicles. This was done by adding ATP, in a concentration range up to 17 mM, to FHE-labeled vesicles of EYPC and DPPC (in the latter case both in the gel phase at 20 °C and in the liquid crystal phase at 50 °C). In all cases, as shown in Fig. 6, ATP addition results in dramatic spectral changes which can be resumed as an increase in the relative intensity of the N^* band. This evolution is remarkable for DPPC vesicles in their gel phase, with a three fold intensity increase of the N^* band. In the liquid crystalline phase, this increase of intensity, although less pronounced, remains important. For EYPC vesicles which are spontaneously in the liquid crystal phase at 20 °C, the increase of intensity of the N^* band is still important, but is masked by the strong decrease of the T^* band intensity. Beyond the proof that ATP can interact with lipid vesicles at their polar interface, these results clearly indicate that, once bound to vesicles, ATP is capable to change the location of FHE from a deep site to a shallower one, since the obtained emission spectra is characteristic of a very polar environment. Indeed, the spectrum obtained at higher ATP concentrations, in the case of gel phase DPPC vesicles, is very similar to the spectrum obtained in methanol, and corresponding to a high quantum yield (see Table 1). The mechanism of this migration of FHE towards the interface is not yet clear and would justify supplementary experiments to study the influence of ATP binding on the structure of lipid vesicles. However, we can do the assumption that the new location of FHE allows the formation of H-bonds with protic molecules (probably water) leading to an increase in quantum yield and the locking of the ESIPT reaction in favor of some H–N^* state. This interpretation is confirmed by a titration of FHE in the low-polar and aprotic solvent dioxan with increasing concentration of water. As seen in Fig. 6D, we observe a dramatic effect of protic water molecules in the molar concentration range, as the N^* band position shifts to higher wavelengths and, most dramatically, the relative intensity of this band increases, as it does with ATP addition. Such a titration is in line with previous studies [12,44] and it was shown an intermolecular H-bonding is responsible for this effect rather than to a "preferential solvation" (an increase in the local concentration of the more polar water molecules in the fluorophore environment) [13].

3.3. Facing of the data in solvents and in lipid membranes

In order to avoid hasty conclusions which could be inferred by confronting the fluorescence data obtained from experiments performed in organic solvents and in lipid membranes, it appeared to us important to find a way to distinguish general polarity effects to more specific effects obtained in membranes, like the electrochromic influence of dipole potential as modulated by 6-KC, or the influence of surface-interacting molecules

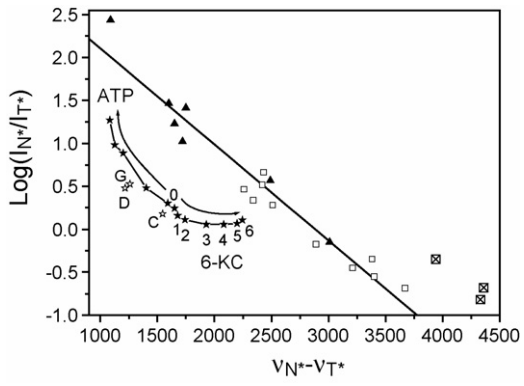


Fig. 7. Dependence of $\log(I_{N^*}/I_{T^*})$ vs. the difference of maximum positions of N^* and T^* bands ($\nu_{N^*} - \nu_{T^*}$). The linear regression is based on the data corresponding to the emission spectra obtained in neutral and basic solvents (□) or protic ones (▲) are excluded from the fit the points corresponding to dioxan, toluene and diisopropyl ether (crossed squares; see text). The data which correspond to the emission spectra obtained in lipid vesicles are represented by stars. Open stars: DMTAP-EYPC 1:1 (D); EYPG (G); EYPC with 33% cholesterol (C). Black stars: EYPC vesicles with varying amounts (as pointed by arrows) of 6-KC, from 0 to 40% (numbered 0–6), and ATP, from 0 to 17 mM.

like ATP. We found that the plots of $\log(I_{N^*}/I_{T^*})$ versus ($\nu_{N^*} - \nu_{T^*}$), the difference between the positions of both band maxima, represents an elegant and useful manner to proceed. It was previously shown that two 3-HF probes with the same fluorophore moiety as FHE, when tested in 21 different solvents [12] or in solvent mixtures [15], display a fair linear dependence of $\log(I_{N^*}/I_{T^*})$ versus ($\nu_{N^*} - \nu_{T^*}$). Meantime, it was also observed that the electric fields of reverse AOT micelles shifted the corresponding data far from the linear response observed with this set of solvents [16]. As shown in Fig. 7, FHE also presents, for the curve $\log(I_{N^*}/I_{T^*})$ versus ($\nu_{N^*} - \nu_{T^*}$), an excellent linear fit for the data in organic solvents. Only the data for low-polar solvents like toluene, dioxan and diisopropyl ether are far from the fit and were not considered since, in these solvents, some additional fluorophore stabilization can take place due to the close proximity between the polar hydroxyl groups and the positive pole of the molecule. If we add to this graph the data obtained with lipid vesicles, either without any additives or with increasing amounts of 6-KC or ATP, they clearly appear to be not correlated with the data obtained with solvents, as all the points are far from the linear regression curve. For example, the increase of membrane dipole potential induced by the addition of 6-KC causes a fall of the I_{N^*}/I_{T^*} ratio, but also an increase of ($\nu_{N^*} - \nu_{T^*}$), whereas the increase of polarity moves the data points in the reverse direction (increase of I_{N^*}/I_{T^*}, decrease of $\nu_{N^*} - \nu_{T^*}$) within the linear regression curve [12,15]. This is a key difference between the contribution to the spectral response of both the environment polarity and the membrane dipole potential, which stresses the differences between the respective influence of electric fields due to relatively small polar molecules of the probe solvation shell in an organic solvent environment and the electric fields appearing in a complex and highly ordered environment like the interior of a lipid bilayer. In some way, these considerations render illusive a common representation for a homogeneous environment like an organic solvent and a heterogeneous one like a lipid bilayer. The data obtained with varying concentrations of ATP confirm this point of view.

4. Conclusions

Within the general task of developing new 3-hydroxyflavone derivatives as ratiometric fluorescent probes for biomembranes, we presently synthesize 3-hydroxy-4′-[di-(2-hydroxyethyl)amino]flavone (FHE). This probe in organic solvents displays fluorescence properties close to that of other 3-HFs containing the same fluorophore, but the presence of the two additional hydroxyl groups induces an increased solvatochromism in non-polar basic solvents due to specific intermolecular H-bonding interactions in such solvents. Experiments performed in lipid vesicles showed that this probe is only weakly sensitive to surface potential, but exhibits a fairly good sensitivity to membrane dipole potential (as modulated by 6-ketocholestanol addition), as shown by the electrochromism of both the excitation spectra and the tautomer T^* band emission spectra. Interestingly, it is possible to make use of this T^* band electrochromism to determine the membrane dipole potential Ψ_D by measuring the ratio I_{600}/I_{560}. As for other 3-HF probes, the I_{N^*}/I_{T^*} ratio is also responding to Ψ_D, but the higher 6-KC concentrations are giving side effects. Being sensitive to changes of the membrane fluidity caused by varying the cholesterol ratio or the temperature, FHE also brightly senses the membrane binding of amphiphilic poly-anions like ATP. Finally, the differences appearing for FHE fluorescence parameters measured either in organic solvents or in lipid vesicles allowed us to underline the difficulty to find a common representation for environments so different as an "isotropic" solvent and a highly anisotropic lipid bilayer.

As a more prospective conclusion, we have to underline two main characteristics of FHE incorporated into a lipid bilayer: firstly, the two hydroxyethyl groups linked to the nitrogen atom of 3-HF moiety are able to form hydrogen bonds with the phospholipid ester groups and thus confer to the molecule anchoring properties and a precise vectorial orientation in the bilayer without the need of any charged head-group in its structure. Secondly, as compared to previously developed 3-HF probes, this new probe does not exhibit any H-bonded emitting form due to H-bond interactions between its 3-hydroxy group and protic molecules in the environment. This last point, together with the weak sensitivity to the surface potential, renders FHE able to measure membrane dipole potential with a minimum of side corrections. Joined together, the information gleaned in the present study will be useful in the further development of new 3-HF derivatives as fluorescent probes for membrane studies.

Acknowledgments

This work was supported by CNRS, Université Louis Pasteur and by the French *Ministère des Affaires Etrangères* through the ECO-NET Program between France, Ukraine and Russia. GM is a fellow from *Agence Universitaire de la Francophonie*.

ENAMINE Co. (Kyiv) is acknowleged for providing chemicals and NMR and LC–MS support.

References

[1] P.K. Sengupta, M. Kasha, Excited state proton transfer spectroscopy of 3-hydrohyflavone and quercetin, Chem. Phys. Lett. 68 (1979) 382–385.

[2] M. Itoh, Y. Fujiwara, M. Sumitani, K. Yoshihara, Mechanism of intramolecular excited-state proton transfer and relaxation process in the ground and excited states of 3-hydroxyflavone and related compounds, J. Phys. Chem. 90 (1986) 5672–5678.

[3] P.-T. Chou, M.L. Martinez, J.-H. Clements, Reversal of excitation behavior of proton-transfer vs. charge-transfer by dielectric perturbation of electronic manifolds, J. Phys. Chem. 97 (1993) 2618–2622.

[4] T.C. Swinney, D.F. Kelley, Proton transfer dynamics in substituted 3-hydroxyflavones: solvent polarization effects, J. Chem. Phys. 99 (1993) 211–221.

[5] O.S. Wolfbeis, A. Knierziger, R. Schipfer, pH-Dependent fluorescence spectroscopy XVII: first excited singlet state dissociation constants, phototautomerism and dual fluorescence of flavonol, J. Photochem. 21 (1983) 67–79.

[6] V.V. Shynkar, A.S. Klymchenko, Y. Mély, G. Duportail, V.G. Pivovarenko, Anion formation of 4′-(dimethylamino)-3-hydroxyflavone in phosphatidyl-glycerol vesicles induced by HEPES buffer: a steady-state and time-resolved fluorescence investigation, J. Phys. Chem. B 108 (2004) 18750–18755.

[7] A.S. Klymchenko, T. Oztürk, V.G. Pivovarenko, A.P. Demchenko, A 3-hydroxychromone with dramatically improved fluorescence properties, Tetrahedron Lett. 42 (2001) 7967–7970.

[8] A.S. Klymchenko, T. Oztürk, A.P. Demchenko, Synthesis of furanochromones: a new step in improvement of fluorescence properties, Tetrahedron Lett. 43 (2002) 7079–7082.

[9] S. Ercelen, A.S. Klymchenko, A.P. Demchenko, An ultrasensitive fluorescent probe for hydrophobic range of solvent polarities, Anal. Chim. Acta 464 (2002) 273–287.

[10] A.S. Klymchenko, V.G. Pivovarenko, T. Oztürk, A.P. Demchenko, Modulation of the solvent-dependent dual emission in 3-hydroxychromones by substituents, New J. Chem. 27 (2003) 1336–1343.

[11] A.S. Klymchenko, V.G. Pivovarenko, A.P. Demchenko, Elimination of hydrogen bonding effect on the solvatochromism of 3-hydroxyflavones, J. Phys. Chem. A 107 (2003) 4211–4216.

[12] A.S. Klymchenko, A.P. Demchenko, Multiparametric probing of intermolecular interactions with fluorescent dye exhibiting excited state intramolecular proton transfer, Phys. Chem. Chem. Phys. 5 (2003) 461–468.

[13] V.V. Shynkar, A.S. Klymchenko, E. Piémont, A.P. Demchenko, Y. Mély, Dynamics of intermolecular hydrogen bonds in the excited states of 4′-dialkylamino-3-hydroxyflavones. on the pathway to an ideal fluorescent hydrogen bonding sensor., Phys. Chem. A 108 (2004) 8151–8159.

[14] W. Liu, Y. Wang, W. Jin, G. Shen, R. Yu, Solvatochromogenic flavone dyes for the detection of water in acetone, Anal. Chim. Acta 383 (1999) 299–307.

[15] V.G. Pivovarenko, A. Wróblewska, J. Błażejowski, 2-[4-(Dimethylamino)phenyl]-3-hydroxy-4*H*-chromene-4-one: a H-bond-sensitive fluorescent probe for investigating binary mixtures of organic solvents, Anal. Chim. Acta 545 (2005) 74–78.

[16] A.S. Klymchenko, A.P. Demchenko, Probing AOT reverse micelles with two-color fluorescence dyes based on 3-hydroxychromone, Langmuir 18 (2002) 5637–5639.

[17] O.P. Bondar, V.G. Pivovarenko, E.S. Rowe, Flavonols—new fluorescent membrane probes for studying the interdigitation of lipid bilayers, Biochim. Biophys. Acta 1369 (1998) 119–130.

[18] G. Duportail, A.S. Klymchenko, Y. Mély, A.P. Demchenko, Neutral fluorescence probe with strong ratiometric response to surface charge of phospholipid membranes, FEBS Lett. 508 (2001) 196–200.

[19] G. Duportail, A. Klymchenko, Y. Mély, A.P. Demchenko, On the coupling between surface charge and hydration in biomembranes: experiments with 3-hydroxyflavone probes, J. Fluoresc. 12 (2002) 181–185.

[20] A.S. Klymchenko, G. Duportail, T. Oztürk, V.G. Pivovarenko, Y. Mély, A.P. Demchenko, Novel two-band ratiometric fluorescence probes with different location and orientation in phospholipid membranes, Chem. Biol. 9 (2002) 1199–1208.

[21] A.S. Klymchenko, G. Duportail, Y. Mély, A.P. Demchenko, Ultrasensitive two-color fluorescence probes for dipole potential in phospholipid membranes, Proc. Natl. Acad. Sci. USA 100 (2003) 11219–11224.

[22] A.S. Klymchenko, G. Duportail, A.P. Demchenko, Y. Mély, Bimodal distribution and fluorescence response of environment-sensitive probes in lipid bilayers, Biophys. J. 86 (2004) 2929–2941.

[23] A.S. Klymchenko, Y. Mély, A.P. Demchenko, G. Duportail, Simultaneous probing of hydration and polarity of lipid bilayers with 3-hydroxyflavone fluorescent dyes, Biochim. Biophys. Acta 1665 (2004) 6–19.

[24] G. M'Baye, V.V. Shynkar, A.S. Klymchenko, Y. Mély, G. Duportail, Membrane dipole potential as measured by ratiometric 3-hydroxyflavone fluorescence probes: accounting for hydration effects, J. Fluoresc. 16 (2006) 35–42.

[25] A.S. Klymchenko, A.P. Demchenko, J. Am. Chem. Soc. 124 (2002) 12372–12379.

[26] N.A. Nemkovich, W. Baumann, V.G. Pivovarenko, Dipole moments of 4'-aminoflavonols determined using electro-optical absorption measurements or molecular Stark-effect spectroscopy, J. Photochem. Photobiol. A: Chem. 140 (2002) 19–24.

[27] P. O'Shea, Intermolecular interactions with/within cell membranes and the trinity of membrane potentials: kinetics and imaging, Biochem. Soc. Trans. 31 (2003) 990–996.

[28] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812 (1985) 55–65.

[29] E. Gross, R. Bedlack Jr., L.M. Loew, Dual-wavelength ratiometric fluorescence measurement of the membrane dipole potential, Biophys. J. 67 (1994) 208–216.

[30] A.O. Doroshenko, L.B. Sychevskaya, A.V. Grigorovich, V.G. Pivovarenko, Fluorescence probing of cell membranes with azacrown substituted ketocyanine dyes, J. Fluoresc. 12 (2002) 455–464.

[31] D.B. Siano, D.E. Metzler, Band shapes of the electronic spectra of complex molecules, J. Chem. Phys. 51 (1969) 1856–1861.

[32] V.G. Pivovarenko, A. Wróblewska, J. Błazejowski, The effect of hydrogen bonding interactions between 2-[4-(dimethylamino)phenyl]-3-hydroxy-4*H*-chromene-4-one in the ground and excited states and dimethylsulfoxide or methanol on electronic absorption and emission transitions, J. Mol. Struct. 708 (2004) 175–181.

[33] E. Lippert, in: J.B. Birks (Ed.), Organic Molecular Photophysics, vol. 2, Wiley & Sons, New York, 1975, pp. 1–31.

[34] W. Liptay, in: E.C. Lim (Ed.), Excited States, vol. 1, Academic Press, New York, 1974, pp. 129–229.

[35] N. Mataga, T. Kubota, Molecular Interactions and Electronic Spectra, Marcel Dekker, New York, 1970.

[36] C. Reichardt, Solvatochromic dyes as solvent polarity indicators, Chem. Rev. 94 (1994) 2319–2358.

[37] V.V. Shynkar, A.S. Klymchenko, G. Duportail, A.P. Demchenko, Y. Mély, Two-color fluorescent probes for imaging the dipole potential of cell plasma membranes, Biochim. Biophys. Acta 1712 (2005) 128–136.

[38] J.C. Franklin, D.S. Cafiso, Internal electrostatic potentials in bilayers measuring and controlling dipole potentials in lipid vesicles, Biophys. J. 65 (1993) 289–299.

[39] S.A. Simon, T.J. McIntosh, A.D. Magid, D. Needham, Modulation of the interbilayer hydration pressure by the addition of dipoles at the hydrocarbon/water interface, Biophys. J. 61 (1992) 199–786.

[40] C. Bernsdorff, R. Winter, Differential properties of the sterols cholesterol, ergosterol, β-sitosterol, *trans*-7-dihydrocholesterol, stigmasterol and lanosterol on DPPC bilayer order, J. Phys. Chem. A 107 (2003) 10658–10664.

[41] P. Nambi, E.S. Rowe, T.J. McIntosh, Studies of the ethanol-induced interdigitated gel phase in phosphatidylcholines using the fluorophore &,6-diphenyl-1,3,5-hexatriene, Biochemistry 27 (1988) 9175–9182.

[42] H. Komatsu, E.S. Rowe, Effect of cholesterol on the ethanol-induced interdigitated gel phase in phosphatidylcholine: use of fluorophore pyrene-labeled phosphatidylcholine, Biochemistry 30 (1991) 2463–2470.

[43] V.G. Pivovarenko, O.B. Vadzyuk, S.O. Kosterin, Fluorometric detection of adenosine triphosphate with 3-hydroxy-4′-(dimethylamino)flavone in aqueous solutions, J. Fluoresc. 16 (2006) 9–15.

[44] Z. Turkmen, A.S. Klymchenko, S. Oncul, G. Duportail, G. Topcu, A.P. Demchenko, A triterpene oleanolic acid conjugate with "-hydroxyflavone derivative as a new membrane probe with two-color ratiometric response", J. Biochem. Biophys. Methods 64 (2005) 1–18.