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Membrane Dipole Potential as Measured by Ratiometric 3-Hydroxyflavone Fluorescence Probes: Accounting for Hydration Effects

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We previously applied the electrochromic modulation of excited-state intramolecular proton-transfer (ESIPT) reaction for the design of novel 3-hydroxyflavone (3-HF) derivatives as fluorescent probes for measuring the dipole potential, Ψ_D, in lipid bilayers (Klymchenko *et al*., *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 11219). In the present work, this method was revisited to take into account the influence of the bilayer hydration on the emission ratiometric response of 3-HF probes. For this reason, it was necessary to deconvolute the whole fluorescence spectra into three bands corresponding to the non H-bonded forms, normal N^* and tautomer T^* forms, both participating to the ESIPT reaction, and to the H-bonded H–N^* form, excluded from this reaction. This allowed us to determine the pure N^*/T^* intensity ratio, without any contribution from the H–N^* form emission depending essentially on the bilayer hydration. This new approach allowed us to confirm the correlation we obtained between the response of 3-HF probes on dipole potential modifications and the corresponding response of the reference fluorescent probe di-8-ANEPPS, thus further confirming the potency of 3-HF probes as excellent emission ratiometric probes to measure dipole potential in lipid membranes.

KEYWORDS: 3-Hydroxyflavones; dipole potential; ratiometric fluorescence; lipid bilayers; hydration .

INTRODUCTION

By considering the lipid bilayer structure, it is obvious that charged groups of different nature (e.g., charged or zwitterionic lipid head-groups, water dipoles and dipolar lipid residues) are not uniformly distributed through the membrane. Therefore, the electrical potential, Ψ, does not change linearly across a lipid or cell membrane. In fact, Ψ follows a complex profile with the contribution of three different electrical potentials defined as the "trinity" of membrane potentials [1]: (1) $\Delta\Psi$, the transmembrane potential related to the difference in ion concentrations between the two bulk aqueous phases separated by the membrane; (2) Ψ_S, the surface potential which arises from charged residues at the membrane-solution interfaces, and (3) Ψ_D, the dipole potential which results from the alignment of dipolar residues of the lipids and from associated water molecules within the membrane. The transmembrane potential, $\Delta\Psi$, is known to regulate the function of a number of membrane proteins, especially cation channels [2]. By applying a defined voltage across the membrane via electrodes in the aqueous phases on both sides of the membrane, the effect of $\Delta\Psi$ on the closing–opening processes of these channels can be studied in detail. However, the molecular site of action of $\Delta\Psi$ is not in the aqueous phases, but rather within the membrane or at its interface, where the electric field created not only by $\Delta\Psi$ but also by Ψ_S and Ψ_D can influence the conformation of membrane proteins and the distribution of their charged substrates.

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The transmembrane potential, $\Delta\Psi$, can be accurately measured by electrodes allowing the control of its influence on membrane processes in great detail. The effect of surface potential, Ψ_S, has also given rise to a number of studies, as it is relatively easy to determine the zeta potential of cell and membrane suspensions by electrophoretic methods. In contrast, the dipole potential, Ψ_D, is much more difficult to control and measure. The estimates of the absolute Ψ_D values for phosphatidylcholine bilayers vary from −280 mV, as evaluated from different penetration rates of hydrophobic ions [3], to −500 mV, as computed from molecular dynamic simulation data [4]. Direct measurements of this potential, which are possible only on phospholipid air–water or water–mercury monolayers [5,6] support Ψ_D existence but disagree quantitatively with the values obtained in bilayers [7,8]. Consequently, the dipole potential in lipid bilayers can only be theoretically calculated or inferred from indirect experimental methods.

One of these indirect methods is the use of fluorescent voltage-sensitive probes. Styrylpyridinium probes such as RH421 or di-8-ANEPPS were the first popular dyes for the determination of Ψ_D [9,10]. Both probes bind to lipid membranes with their chromophore in the lipid headgroups region, where they are sensitive to the local electric field originating from the dipole potential. As a consequence of this binding, two effects could be used to quantify the dipole potential, the modification of the pK_a and the shift of the fluorescence excitation spectra of both membrane-bound probes.

Gross *et al*. [9] were the first to use the shift in the fluorescence excitation spectrum of membrane-bound di-8-ANEPPS to quantify Ψ_D. They found that the binding to the membrane of the dipolar compound phloretin, which decreases Ψ_D [3,11], induces a red shift of the fluorescence excitation spectra while the binding of 6-ketocholestanol (6-KC), which increases Ψ_D [3,12] induces a blue shift. In order to quantify these shifts, Gross *et al*. used a ratiometric method by determining the ratio, *R*, of the fluorescence intensities measured at a single emission wavelength (620 nm) after excitation at two wavelengths on the edges of the excitation spectrum (440 and 530 nm). A decrease in *R* thus corresponds to a Ψ_D decrease and inversely, an increase corresponds to a Ψ_D increase. By comparing, at different concentrations of phloretin and 6-KC, the *R* values with Ψ_D values provided from kinetic measurements of hydrophobic ion transport across lipid bilayers [3], they found that the determined *R* values were linearly correlated to the Ψ_D values [9]. Indeed, a change of *R* of 0.8 corresponds to a change in Ψ_D of about 100 mV.

Thus, styrylpyridinium dyes like di-8-ANEPPS allow ratiometric recording of Ψ_D, provided that the sample could be excited with two different wavelengths. With lipid vesicles or biomembrane suspensions, this possibility is easily provided by common spectrofluorimeters. In contrast, for studies with cells, this possibility could not be easily achieved with most fluorescence microscopes. Thus, it would be much more convenient to dispose of dyes with a ratiometric response in emission that would be adapted for multi-color imaging microscopes. Moreover, ratiometric measurements in emission eliminate distortions of data caused by photobleaching and variations of probe loading as well as by instrumental factors such as light source stability [13,14]. In this context, we designed new Ψ_D-sensitive fluorescent probes by using 3-hydroxyflavone (3-HF) derivatives which exhibit two emission bands well separated on the wavelength scale due to the presence of two forms in the excited state (normal N^* and tautomer T^* forms) resulting from an excited state intramolecular proton transfer (ESIPT) reaction. In a number of systems, it was shown that electrochromic shifts of these two bands are connected with strong changes of their relative intensities [15,16]. In our previous and pioneering work [17], two 3-HF derivatives, BPPZ and F4N1, were synthesized and used to measure Ψ_D in phospholipids vesicles. The dipole moment of BPPZ is oriented toward the centre of the bilayer, while the dipole moment of F4N1 is oriented in the opposite direction. It results that the excitation and emission bands shift in accordance with the probe orientation in the bilayers and with variations of Ψ_D induced by incorporation of the Ψ_D-modifiers phloretin and 6-KC.

However, even if the correlation of the emission ratiometric responses of both probes F4N1 and BPPZ with the excitation ratiometric response of di-8-ANEPPS was found good enough to validate 3-HF derivatives as membrane probes for the determination of membrane dipole potentials, it recently appears that our experimental approach should be confirmed. Indeed, we recently showed [18,19] that the fluorescence spectra of 3-HF derivatives incorporated in phospholipid vesicles cannot be explained by considering only the normal N^* and tautomer T^* emission bands. In fact, these probes are simultaneously present in H-bond-free forms displaying the two-band emission (N^* and T^*) due to the ESIPT reaction and in an H-bonded form (H–N^*) displaying a single-band emission with no ESIPT. The individual emission profiles of these forms were obtained by deconvolution of the fluorescence spectra. The maximum of the H–N^* emission band appears roughly positioned at a wavelength between those of the N^* and T^* forms. This additional emission, which was not considered in our previous paper, could

exert a non negligible influence on the "true" N^*/T^* intensities ratio as compared to the ratio we measured on the rough fluorescence spectrum. Moreover, such an influence has to be considered since the two dipole potential modifiers we used, phloretin and 6-KC, themselves locally influence the lipid bilayer hydration [19,20]. The same consideration is also valid if we consider the substitution of the ester phospholipids by their corresponding ether analogues which induces a decrease of dipole potential concomitantly with a significant decrease of the bilayer hydration [19,21]. Thus, it appears that our previous study which validated the use of 3-HF derivatives as fluorescent probes for measuring Ψ_D must be revisited to confirm the correlation between the excitation ratiometric response of di-8-ANEPPS and the N^*/T^* emission ratio for 3-HF measured on the N^* and T^* emission bands obtained after deconvolution of the overall emission spectra. This is the aim of the present work.

MATERIALS AND METHODS

Chemicals

Phloretin, 6-ketocholestanol (6-KC), pluronic F-127, and egg yolk phosphatidylcholine (EYPC) were purchased from Sigma. Dimyristoyl phosphatidylcholine (DMPC) and 1,2-di-*o*-tetradecyl-*sn*-glycero-3-phosphocholine (DTPC) were from Avanti Polar Lipids. All these chemicals were used without further purification.

The probe di-8-ANEPPS was purchased from Molecular Probes. The synthesis of *N*-[4′-dimethylamino) -3-hydroxy-6-flavonyl]methyl-*N*,*N*-trimethyl ammonium bromide (F4N1) and 3-[2-(4-(dioctylamino)-3-hydroxyflavonylmethyl) (dimethyl)ammonio]-1-propane sulfonate (F8N1S) have been described in detail [22,23].

Vesicles

Large unilamellar vesicles (LUV) were obtained by the classical extrusion method [24]. 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. Phloretin and 6-KC were incorporated into LUV from mM stock solutions in DMSO + 2.5% pluronic F-127 as previously described [9].

Spectroscopy

Fluorescence spectra were recorded on a Fluorolog (Jobin-Yvon Horiba) spectrofluorometer. The emission wavelengths for fluorescence excitation spectra were 620 nm for probe di-8-ANEPPS and 570 nm for probes F4N1 and F8N1S. For the latter probes, 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, namely 20°C with EYPC vesicles and 38°C with DMPC and DTPC vesicles.

Deconvolution Method

Deconvolution of F4N1 and F8N1S fluorescence spectra into three bands, corresponding to normal (N^*), normal H-bonded ($H–N^*$) and tautomer (T^*) forms, was performed by using the "Siano" software kindly provided by its author (Dr A.O. Doroshenko, Kharkov, Ukraine) [25]. 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 [26] 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 [16,18,19]. Thus, for the iteration process, the FWHMs of the two short-wavelength band (N^* and $H–N^*$) were fixed at 3000 cm^{-1}. For the $H–N^*$ band, the asymmetry and the band position were fixed at 0.9 and 18,400 cm^{-1}, respectively. The other parameters, asymmetry of N^* and T^* bands, FWHM of the T^* band and relatives intensities of the bands, were allowed to vary during the iteration process.

RESULTS

Two relatively similar 3-HF derivatives were used in the present study. The first one is the previously reported F4N1 probe, whose location and orientation with respect to the lipid bilayer was determined by the parallax quenching method [19,22]. The probe F4N1 contains a small positively charged trimethylammonium group that interacts electrostatically with the lipid phosphate groups,

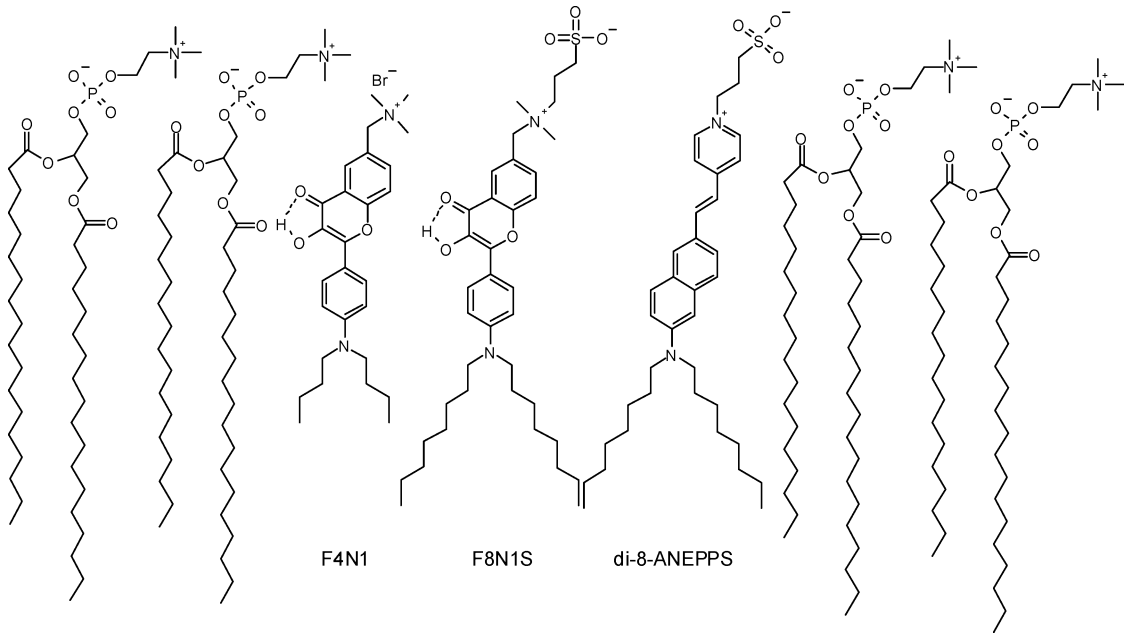

Fig. 1. Structure and estimated locations of F4N1, F8N1S and di-8-ANEPPS in a PC layer.

while the low-polar flavone chromophore is extending into the hydrophobic part of the bilayer as the large hydrophobic 4′-dibutylamino substituent favors its vertical orientation. The second probe is a novel one, F8N1S, which was shown to present higher affinity and selectivity toward the cellular plasma membrane, and thus is particularly designed for cellular microscopy experiments [23]. Probe F8N1S, with respect to its parent analog F4N1, possesses a zwitterionic group and longer hydrocarbon chains which should significantly diminish the penetration rate of the probe through the bilayer, thus explaining its plasma membrane retention, but without modifying the vertical insertion. The expected locations of these two probes, as well as the location of the styryl probe di-8-ANEPPS, are shown in Fig. 1. The probe BBPZ, presenting the reverse orientation of the flavone chromophore [17], was no more used in the present study as the reverse electrochromic effect due to its reverse orientation was well established, and also because of the relatively low intensity of the first emission band which renders the deconvolution of the emission spectrum into three bands relatively unprecise.

The dipole potential in EYPC vesicles can be modulated either by the addition of phloretin and 6-KC which decreases and increases its value, respectively, [27–30]. Another way to decrease the dipole potential of the bilayer is to substitute ester phospholipids by ether phospholipids [21], what we did by comparing DMPC with DTPC vesicles. We observe that with the addition of 40% of 6-KC, the excitation spectra of probes F4N1 and F8N1S are shifted to shorter wavelengths. As expected, the excitation spectra are shifted on the opposite direction by addition of phloretin. As shown in Fig. 2A, the sign and magnitude of the observed shifts in the excitation spectra with the addition of phloretin and 6-KC are similar to those observed with di-8-ANEPPS. Let us remind that before the introduction of 3-HF derivatives, the dipole potential changes were inferred from the shifts of the excitation spectrum of this latter probe by measuring the intensity ratio at the edges of the spectrum, I_{440}/I_{530} [9]. The shifts of the excitation bands of our probes can be evaluated in the same way, by measuring the corresponding ratio I_{390}/I_{445}. As seen in Fig. 2B the correlations obtained with di-8-ANEPPS data are satisfactory for both F4N1 and F8N1S except for the points corresponding to the higher phloretin concentration (20%), probably because of side effects of phloretin. Indeed, the response to phloretin is not only due to its vectorial electrochromic effect, but also contains a contribution due to its direct interaction with incorporated probes as revealed by its fluorescence quenching effects [31]. Despite this last point, we clearly confirmed that these 3-HF derivatives are responding to the same electrochromic mechanism as di-8-ANEPPS and are thus putative candidates for quantitative measurements of Ψ_D changes.

In our previous work [17], we demonstrated also a good correlation between the di-8-ANEPPS

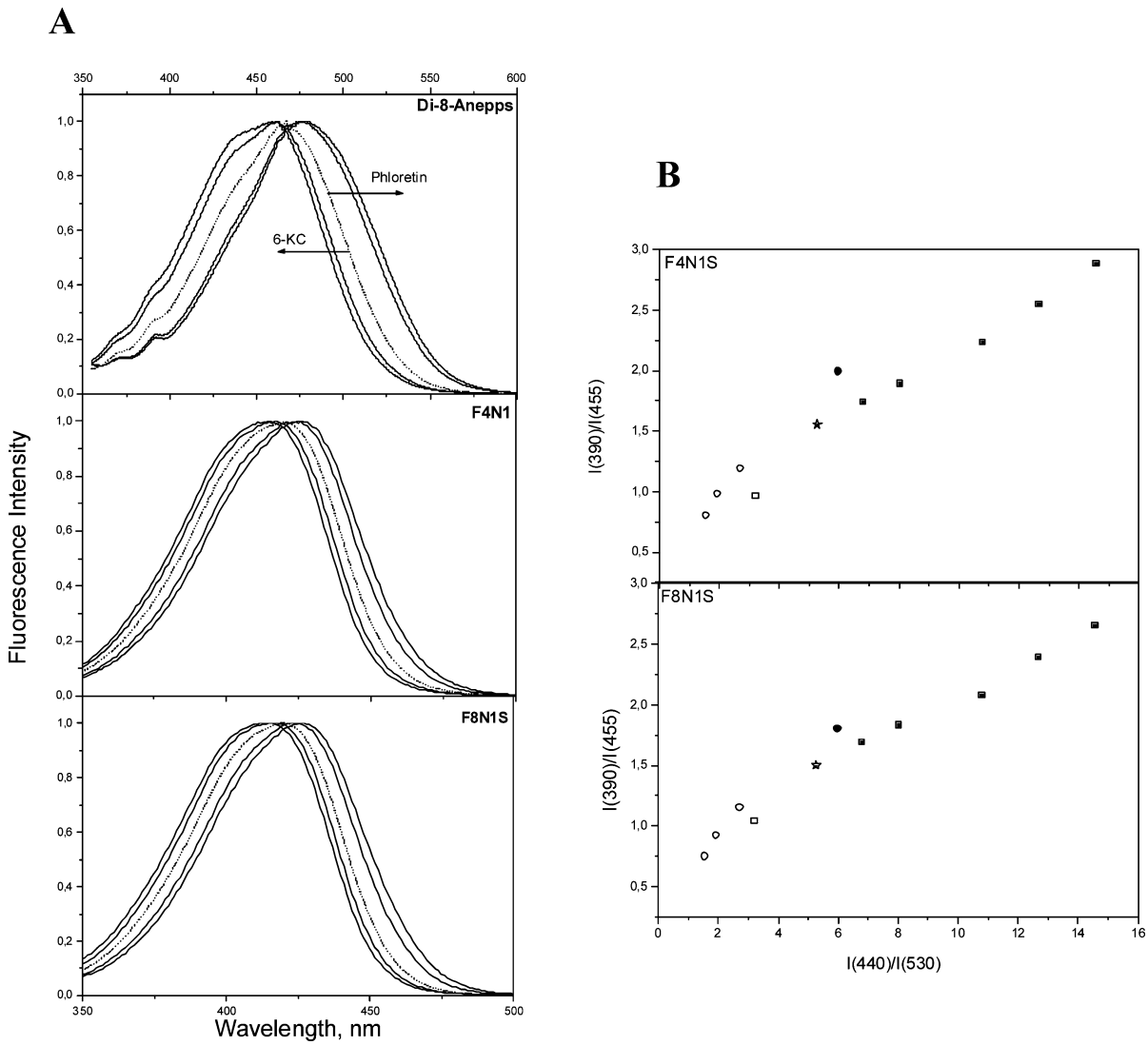

Fig. 2. (A) Response in the excitation spectra of di-8-ANEPPS, F4N1, and F8N1S to variations of the dipole potential in EYPC LUV induced by the addition of 6-KC and phloretin. The spectra were recorded at the following emission wavelengths: 645 nm for di-8-ANEPPS and 570 nm for F4N1 and F8N1S. The spectra were normalized at the band maximum. Compared to the spectra obtained without any additive (dotted spectra), the direction of the spectral shifts are shown by arrows and are the results of increasing percentages of 6-KC (20 and 40%) and phloretin (10 and 20%). (B) Correlation of the excitation ratiometric response of probes F4N1 and F8N1S to the dipole potential with that of di-8-ANEPPS. Intensity ratios were measured at the edges of the spectra (390 and 455 nm for 3-HF probes, 440 and 530 nm for di-8-ANEPPS). Symbols: EYPC (star); EYPC + 6-KC (5 to 40%) (■); EYPC + phloretin (5, 10, and 20%) (○); DMPC (●) and DTPC (□) vesicles at 38°C.

excitation response and the ratiometric response I_{T^*}/I_{N^*} at the two band maxima of the rough emission spectrum, which were supposed to correspond to the emission of the N^* and T^* forms. In fact, we recently demonstrated [18,19] that the emission spectra of 3-HF derivatives incorporated into lipid vesicles result not only from the classical normal (N^*) and tautomer (T^*) excited forms due to the ESIPT reaction, but also contain a third emission band, with its maximum at ca. 543 nm. This additional emission band corresponds to the normal H-bonded species (H–N^*) of the probes. With respect to the N^* state, the H–N^* state is stabilized by an intermolecular H-bonding with a water molecule which prevents its own ESIPT reaction. We showed that the relative intensities of the N^* and T^* forms are strongly correlated with the surrounding polarity, while the relative intensities between the

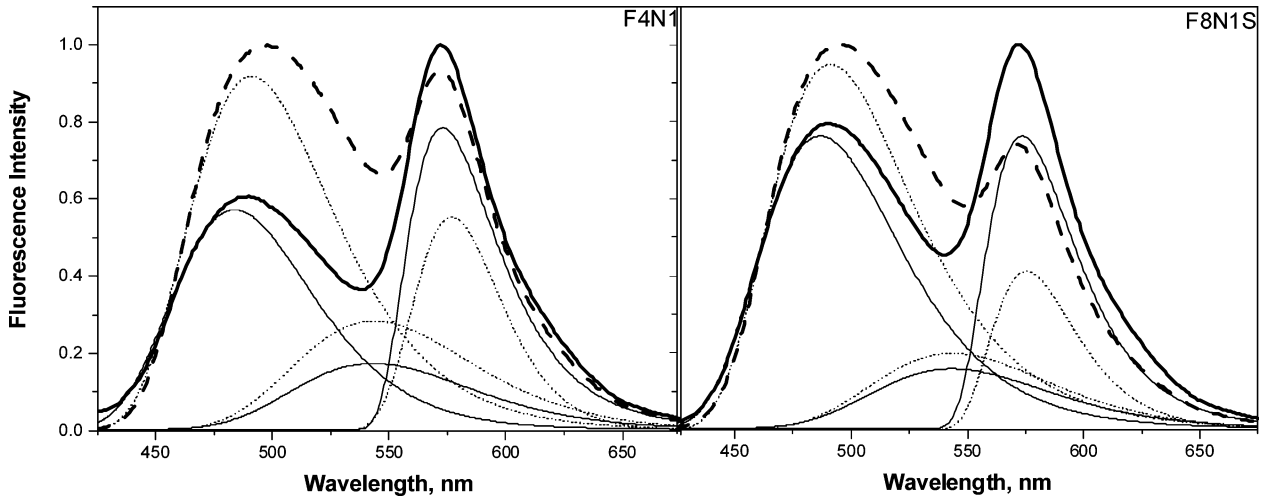

Fig. 3. Emission fluorescence spectra of probes F4N1 and F8N1S in EYPC vesicles and their deconvolution into N*, H–N* and T* bands, from left to right, respectively. Symbols: Broken lines, EYPC vesicles (dashes: whole spectrum; dots: deconvoluted spectra); Continuous lines, EYPC vesicles + 40% 6-KC (heavy line: whole spectrum; thin lines: deconvoluted spectra). Excitation wavelength set at 400 nm. The whole spectra are normalized at their intensity maxima.

H-bonded form (N–H*) and H-bond free forms (N* + T*) are more correlated to the hydration level at the probe location site. Thus, such a fluorescence approach allows evaluating simultaneously polarity and hydration of lipid bilayers [18,19]. These considerations prompted us to reconsider the scaling of Ψ_D by considering no more the two-bands intensities of the rough dual fluorescence spectrum, but the intensities of the true N* and T* forms.

To characterize the three emissive bands, we deconvoluted the emission fluorescence spectra of probes F4N1 and F8N1S by using log-normal functions after having selected the most optimal parameters (band maximum, full width at half maximum, and asymmetry) for each of the log-normal components, as we previously described [18,19]. We performed this analysis for probes F4N1 and F8N1S in EYPC vesicles differing in their content in 6-KC (up to 40%) and in phloretin (up to 20%). As an example, the deconvoluted spectra obtained either without any additives or with 40% 6-KC are presented in Fig. 3. The changes of the spectroscopic characteristics of the N* and

Table I. Spectroscopic Characteristics of Probes F4N1, F8N1S, and Di-8-ANEPPS in Large Unilamellar Vesicles[a]

Sample	F4N1		F8N1S				Di-8-ANEPPS
	λ_{N^*} (nm)	λ_{T^*} (nm)	I_{T^*}/I_{N^*}	λ_{N^*}(nm)	λ_{T^*} (nm)	I_{T^*}/I_{N^*}	I_{440}/I_{530}
EYPC + 40% 6-KC	490	572	1.64	491	572	1.25	14.6
EYPC + 30% 6-KC	489	572	1.48	492	572	1.01	12.7
EYPC + 10% 6-KC	491	574	1.1	494	572	0.91	8.0
EYPC	495	572	0.94	497	572	0.74	5.25
EYPC + 10% Phloretin	498	572	0.57	499	570	0.47	1.95
EYPC + 20% Phloretin	498	572	0.48	503	570	0.41	1.55
DMPC (38°)	497	572	1.19	499	570	0.94	6.0
DTPC (38°)	490	572	0.65	495	570	0.53	3.2

[a] λ_{N^*}, λ_{T^*} and I_{T^*}/I_{N^*}—positions of fluorescence maxima of the N* and T* bands and their intensity ratios. All the values were obtained from the deconvoluted N* and T* bands.

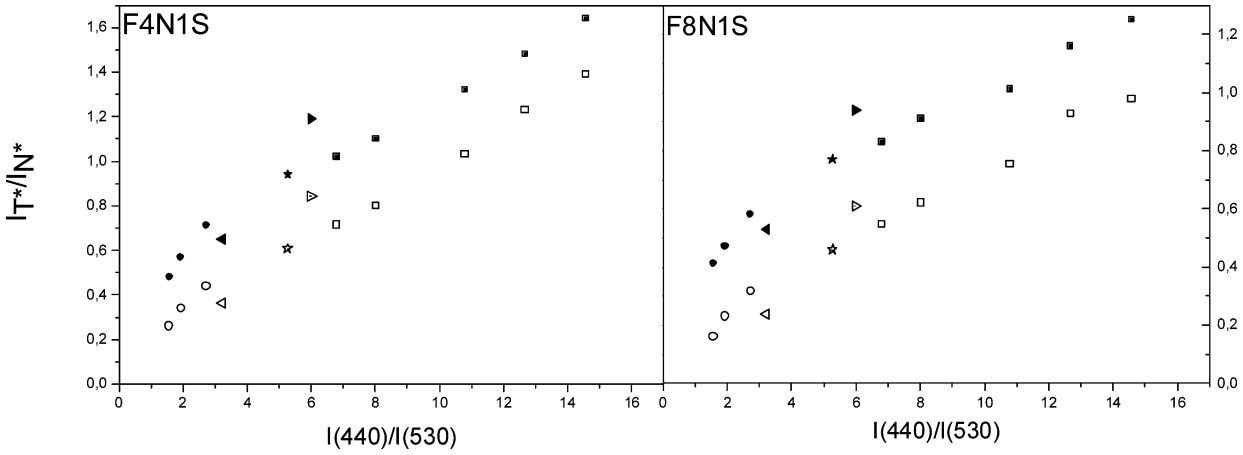

Fig. 4. Correlation of the emission ratiometric response (I_{T^*}/I_{N^*}) of probes F4N1 and F8N1S with the excitation ratiometric response of di-8-ANEPPS. Symbols: EYPC vesicles (stars), with addition of 5, 10, 20, 30, and 40% of 6-KC (squares) or of 5, 10, and 20% of phloretin (circles), DMPC (right triangles) and DTPC (left triangles) vesicles at 38°C. For comparison, two correlation curves are presented: one corresponding toI_{T^*}/I_{N^*} ratio obtained from the deconvoluted N* and T* bands (see text; white symbols), the other to the same ratio obtained from the apparent N* and T* maxima of the whole spectra (black symbols).

T* emission bands (position of the band maxima, λ_{N^*} and λ_{T^*}, and intensity ratio, I_{T^*}/I_{N^*}) with addition either of 6-KC or phloretin are summarized in Table I together with the corresponding excitation intensity ratio obtained with di-8-ANEPPS for the same samples. The data obtained from the fluorescence spectra (excitation spectra of di-8-ANEPPS, deconvoluted emission spectra of F4N1 and F8N1S; not shown) measured for vesicles made from ester phospholipids (DMPC) and from the corresponding ether phospholipids are added to Table I.

The correlations of the emission ratiometric responses of both probes to the dipole potential with the excitation ratiometric response of di-8-ANEPPS are presented in Fig. 4. In fact, for comparison reasons, we present here not only the correlation curves obtained with I_{T^*}/I_{N^*} ratio calculated after the deconvolution procedure, but also the ones obtained with I_{T^*}/I_{N^*} calculated from the apparent N* and T* bands of the whole emission spectra, as previously determined [17]. This comparison obviously leads to correlation curves of similar quality and as satisfactory as the correlation curves obtained with the ratio I_{390}/I_{345} from the corresponding excitation spectra (*vide supra*, Fig. 2B). Thus, the probe hydration effects in the present case do not influence considerably the response of the probes to the dipole potential. These curves bring us the ultimate proof that such 3-HF derivatives are excellent fluorescent probes for the quantitative measurement of membrane dipole potential Ψ_D, through the recording of the relative intensities of the two well-resolved emission bands proceeding from the non H-bonded forms (normal N* and tautomer T*) which result from the ESIPT reaction. However, as seen from the comparison shown in Fig. 4, the present restatement does not preclude the use of the rough I_{T^*}/I_{N^*} ratio obtained without deconvolution of the emission spectra. This last point is of particular importance in case of the deconvolution procedure would be quite impossible to proceed, like for example in ratiometric confocal fluorescence imaging of living cells [23].

CONCLUSION

The application of the highly electrochromic ESIPT reaction of 3-hydroxyflavone derivatives allowed us to consider their strong two-color fluorescence ratiometric response as a new method for measuring the dipole potential in lipid bilayers and biomembranes. We presently confirmed that the response of probes F4N1 and F8N1S, both in excitation and in emission, show quantitative correlations with the response in excitation of the reference probe di-8-ANEPPS. The first correlation, obtained by considering the response in the excitation spectra, provides the proof that these 3-HF derivatives can be considered as good alternatives to di-8-ANEPPS, as they are responding identically to the dipole potential variations. The second correlation was obtained by considering the ratiometric response in intensities of the two well-resolved emission N* and T* bands resulting from the ESIPT reaction. In this latter case, we took care to deconvolute the overall fluorescence spectra in order to determine (and eliminate) the probe hydration effects on this ratiometric response. This analysis, which was not previously taken into ac-

count, confirms that 3-hydroxyflavone probes are prototypes of emission ratiometric probes for electric fields in lipid membranes, much more convenient than excitation ratiometric probes which need to determine a ratiometric effect on the edges of a single-band excitation spectrum.

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