Novel Two-Band Ratiometric Fluorescence Probes with Different Location and Orientation in Phospholipid Membranes

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Summary

3-hydroxyflavone (3-HF) derivatives are very attractive fluorescence sensors due to their ability to respond to small changes in their microenvironment via a dramatic alteration of the relative intensities of their two well-separated emission bands. We developed fluorescence probes with locations at different depths and orientations of 3-HF moiety in the phospholipid bilayer, which determine their fluorescence behavior. While the spectral shifts of the probes correlate with their binding site polarity, the intensity ratio is a complex parameter that is also sensitive to the local hydration. We demonstrate that even the deeply located probes sense this hydration effect, which can be modulated by the charge of the lipid heads and is anisotropic with respect to the bilayer plane. Thus the two-band ratiometric fluorescence probes can provide multiparametric information on the properties of lipid membranes at different depths.

Introduction

Fluorescence microscopy of the living cell is a rapidly developing field of research with countless potentialities [1–3], and the success in visualization of cellular substructures, membranes, and macromolecules provided a strong impulse for further development of this method. One of the very promising prospects that is still poorly explored at the biological membrane level is the possibility of providing the color-changing response to different structural perturbations in cell membranes that can be offered by two-color ratiometric probes [4]. The strong demand for these probes cannot be satisfied without introduction of new fluorophores and their chemical modifications in order to endow them with the desirable properties. Requirements imposed on these properties are much more stringent than that for the commonly applied probes for fluorescent labeling. In addition to high chemical and photochemical stability and high fluorescence quantum yield, they should provide strong change of color in response to different membrane perturbations. In this sense the common polarity-sensitive [5] and electrochromic [6] dyes have very limited capabilities, as these probes commonly provide the response by shifting one broad band that is present in emission, and the magnitude of the shift is usually smaller than the bandwidth. In order to be sensitive to the two-band ratiometric probe, the dye is required to exhibit an excited-state reaction: isomerization, electronic charge transfer, or proton transfer [7]. In addition, both initial and product forms of the reaction should be present in emission and provide high-intensity and well-separated fluorescence bands. Then, the ratio of intensities of these bands can become a signal of membrane perturbations, which is very attractive for precise measurement and analysis.

There is one family of fluorophores, 3-hydroxyflavones (3-HF), which potentially satisfies these requirements. They exhibit the excited-state intramolecular proton transfer (ESIPT) reaction, which results in two emission bands belonging to normal excited state (N*) and to the photo-tautomer (T*) reaction product [8–12]. The latter is shifted dramatically to longer wavelengths so that the two forms can be easily seen in emission as resolved separate bands. The positions of the two bands and, what is most essential, the ratios of their intensities are very sensitive to different perturbations. Therefore, 3-HF derivatives have found important applications in the studies of reverse micelles [13, 14], phospholipid [15–17] and natural [18] membranes. New strategies have recently been found for improvement of spectroscopic properties of the parent 3-HF chromophore [19, 20]. They allow shifting its absorption and fluorescence spectra to longer wavelengths, increasing the quantum yield and modulating the two-wavelength sensitivity within the desired ranges.

The other unique sensor property of 3-HF chromophore is the ability to report on different properties of environment simultaneously. Its heterocyclic π-electronic system, which provides the strong increase in asymmetry of charge distribution in the excited state, allows the shifts of fluorescence spectra similarly to common solvatochromic and electrochromic dyes; but since the two bands in emission originate from two separate excited states, N* and T*, with different magnitudes and orientations of their dipole moments [11, 12], the sensitivity of these states to polarity and electric field perturbation of their microenvironment is different. The ESIPT reaction site is strictly localized between 3-hydroxyl and 4-carbonyl groups, which form a hydrogen bond that closes a low-stable five-membered ring [8]. Therefore,
this reaction shows extreme sensitivity to intermolecular hydrogen bond perturbations [9] that should have a certain directionality in space. Moreover, because of the asymmetric nature of the chromophore and unidirectional nature of ESIP reaction, the fluorescence spectra are expected to be sensitive to anisotropic properties of the environment and in particular to electrostatic fields. This is extremely attractive in the studies of different structurally anisotropic systems such as micelles, monolayers, and biological membranes.

Application of these properties cannot be efficient without providing the chromophore the ability of occupying definite location and orientation in the membrane. The most efficient method for the emplacement of the chromophore at the desirable site in the bilayer is the attachment of a positively charged group. This allows its localization at the bilayer interface due to interaction of this group with the negative charge of phosphate groups [21, 22]. Particularly, this can be important for those low-polar chromophores that are intended to protrude deeply into the bilayer [6]. Additional stabilization and orientation of the probe can be achieved by introduction at proper places of aliphatic hydrocarbon chains of different lengths, which make the probe properties close to that of lipids.

In this study, we synthesized novel flavone derivatives containing quaternary ammonium group as an attached positive charge, and hydrocarbon chains at different positions. These modifications are made with strong concern toward maintaining, unchanged, the property of the 3-hydroxyflavones to undergo ESIP reaction, allowing very sensitive and convenient two-band ratiometric detection in fluorescence.

Results and Discussion

Synthesis

In order to introduce a charged group to a 3-hydroxyflavone chromophore from the chromone side, the starting material, 5-chloromethyl-2-hydroxyacetophenone, was prepared (Figure 1). Chloromethylation of 2-hydroxyacetophenone with paraformaldehyde in concentrated HCl at 40°C occurs at the 5 position. The product, which is pure enough for the next step, was obtained in high yield. Applying a common procedure [23], the latter was converted into corresponding chalcone with subsequent oxidative heterocyclization with hydrogen peroxide. Resultant 6-ethoxymethylflavones were transformed into bromomethyl derivatives by heating at 100°C in 62% HBr. The reactions proceed quickly and in good yields.

6-Bromomethyl-3-HF derivatives are very reactive fluorescence labels in which bromine can be easily substituted with amino or mercapto groups. To synthesize the target compounds F2N8 and F4N1, the substitution of bromine was carried out with different tertiary amines. In order to introduce a positive charge from the opposite side of the flavone chromophore (the phenyl ring), the starting material, 4-[4-(4-pyridyl)piperazino]benzaldehyde, was prepared in two steps from 4-chloropyridine and 1-phenylpiperazine, followed by introduction of the formyl group into the intermediate, 1-phenyl-4-(4-pyridyl)piperazine (Figure 1). Both steps proceeded with relatively high yields. Then, the aldehyde was condensed with 2'-hydroxyacetophenone, and the resultant chalcone was transformed into corresponding flavone. Finally, the obtained flavone was transformed into corresponding zwitterionic compound PPZ by treating with 1,3-propanesultone. It should be noted that PPZ, unlike all the other products, is less soluble in common organic solvents except DMSO.

Absorption and Fluorescence Properties in Neat Solvents

All the new probes display two bands in emission spectra. Thus, they retain the property of being able to undergo ESIP, which is manifested by the presence of two N* and T* emission bands, but the relative intensities of these bands exhibit broad variations (Figure 2). Flavone probes F2N8 and F4N1, which possess the positively charged group attached to chromone unit, demonstrate almost identical absorption and fluorescence spectra in various neat solvents (Table 1). However, when compared with the parent flavone F, they show both absorption and fluorescence spectra shifted to longer wavelengths together with significant increase of relative intensity of the N* band. In contrast, flavone PPZ shows considerable blue shifts in the spectra along with a decrease of the relative intensity of N* band (Figure 2; Table 1). These differences can be attributed to an effect of proximity to the chromophore of positive charge, which, interacting electrostatically with 3-HF moiety, either stabilizes (F2N8, F4N1) or destabilizes (PPZ) its N*-excited state. This phenomenon was recently studied in detail for a series of charged and uncharged 3-HF derivatives in the solvents of different dielectric properties [24]. It was found that the intensity ratio of N* and T* bands can exhibit dramatic electrochromic modulation (internal Stark effect), and the dielectric screening of the proximal charge by the dipolar molecules or ions decreases the effect.

Similarly to the parent flavone F, all the novel flavones demonstrate strong sensitivity to the polarity of their environment. Increase of solvent polarity for all the probes results in gradual red shift of the N* band, which is accompanied by dramatic increase of fluorescence intensity ratio between N* and T* bands, I_N*/I_T* (Figure 2). This is typical for 3-HF derivatives possessing 4′-dialkylamino substituent in the phenyl ring. The presence of the donor group induces a large dipole moment in the excited N* state, making it strongly solvatochromic[10–12].

Binding of the Probes to Phospholipid Membranes

Binding to lipid vesicles was monitored by the change of fluorescence intensity in the presence of increasing lipid concentrations. A strong, up to 100-fold, increase of intensity upon binding to large unilamellar vesicles composed of dioleoyl phosphatidylcholine (DOPC) is observed for all the probes considered (Figure 3). With the probe concentration of 1 μM, the binding appears to be quite efficient in the cases of F, F2N8, and F4N1, since the plateau is reached at a lipid concentration of 200 μM. PPZ seems to be less prone to binding to vesicles since the plateau is approached only at 400–600
Figure 1. Chemical Structures of the Studied Probes and Pathways of Synthesis for F2N8, F4N1, and PPZ

R = C,H₄ or n-C₄H₉ (a), CH₂O, HCl, 35°C; (b), 4-diethylaminobenzaldehyde, OH⁻, EtOH; (c), H₂O, OH⁻, EtOH, 0°C; (d), N,N-dimethyloctylamine or trimethylamine, EtOH, t; (a), diisopropylethylamine, nitrobenzene, 200°C; (b), dimethylformamide, POCl₃, 60°C; (c), 2-hydroxyacetophenone, OH⁻, EtOH; (d), H₂O, OH⁻, EtOH, 0°C; (e), 1,3-propanesultone, DMF, 100°C.

The probe and DOPC concentrations for subsequent quenching experiments were selected according to these data.

Location of the Probes in Phospholipid Bilayer

In order to provide information on the probe location in the bilayer, fluorescence quenching experiments of these probes by spin-labeled lipids were performed by using the parallax method [25, 26]. Their fluorescence was quenched with shallow (TempoPC), medium (5-SLPC), or deep (12-SLPC) nitroxide-labeled phosphatidycholines in DOPC vesicles prepared by either ethanol or octylglucoside dilution. The relative amount of fluorescence quenching obtained by the introduction of different quenchers was employed to calculate fluorophore depth using the parallax equation (see Experimental Procedures). The location of the dyes was expressed as Z₀, which corresponds to the distance of the fluorophore from the center of the lipid bilayer (more precisely, the distance from the border between the two leaflets to the center of the fluorophore). The quenching experiments with the vesicles obtained by ethanol dilution method were repeated three times. As these vesicles appeared to have a dimension corresponding to large unilamellar vesicles, namely 0.12 μm, this series of experiments was emphasized. For comparison one set of quenching experiments was performed with vesicles obtained by octylglucoside dilution. According to their dimension, these vesicles are probably multilamellar, and despite more scattered quenching data, the results remained coherent with those obtained with unilamellar vesicles (Table 2).

According to these data, the probes F and F2N8 locate at 16 ± 1 and 15 ± 1 Å, respectively, from the center of the bilayer. This location is at the level of the ester groups and glycerol residues of phospholipids [27] and is somewhat deeper than the location of the most common dyes with charged groups, like rhodamine B, fluorescein, and ANS (16.5–18.5 Å) [28]. Meanwhile, chromophores of probes F4N1 and PPZ locate much deeper in the region of hydrocarbon chains at 10 ± 1 and 7 ± 1.5 Å from the center, respectively. These quite contrasting results were expected considering the chemical structures of the probes. Probe F is an uncharged and relatively hydrophobic molecule; therefore, it can locate at any depth inside the low-polar bilayer interface. However, the presence of two polar groups, 3-hydroxyl and 4-carboxyl, which tend to form intermolecular hydrogen bonds with water and polar groups of lipids, can be sufficient for its location preferentially near the interface. In an analogous case of another hydrophobic aromatic molecule, 9-methylnaphthalene, which shows deep location in the bilayer, an introduction of hydroxyl group results in translocation of the chromophore next to the interface [29]. F2N8 is a charged molecule with a quaternary ammonium acting as an anchor at the interface [30]. The presence of strongly hydrophobic octyl hydrocarbon chain, probably aligned along the fatty acid chains of the phospholipids, imposes on the fluorophore an oblique orientation exposing 3-hydroxyl and 4-carboxyl groups to the membrane interface. This results in apparent similarity in the location of probes F and F2N8. However, F2N8 seems to be located more precisely than F, as can be seen from the quenching data (Table 2). Indeed, the medium nitroxide-PC quenches F and F2N8 with the same efficiency, while shallow and deep quenchers affect much stronger probe F, which is probably due
While in the case of F and F2N8, it locates on the level of sn₃ carbonyls of phospholipids, for F4N1 and PPZ it is close to the deeper sn₁ carbonyls (Figure 4).

**Fluorescence Behavior of Probes in Phospholipid Vesicles**

**Effect of Incorporation into Vesicles**

Incorporation of the probes into vesicles results in a very strong increase of fluorescence intensity (Figure 3), which is in line with their much higher quantum yields in vesicles with respect to water (Table 1). Only for PPZ is this difference in quantum yields lower. The quenching of 3-HF fluorescence in water is well known [9]. The screening from bulk water together with restriction in mobility of the environment are probably the two major factors responsible for the increase in probability of radiative transition from both N* and T* states. Importantly, for all the studied probes, the quantum yield in vesicles is much higher than in different organic solvents, which suggests that the restriction of mobility is an important factor.

For all the studied probes, the excitation spectra in phospholipid vesicles match closely the absorption spectra and do not differ considerably when recorded at N* or T* emission band maxima. This result, which is similar to that observed in neat solvents, allows us to rule out the presence of the anion form in emission and consider the two bands as originating from the same ground-state species.

Fluorescence spectra of probe F in the studied PC vesicles (Figure 5) are similar to those obtained in aprotic solvents and correspond to the solvent polarity range between ethyl acetate and acetonitrile (Figure 2). This is in correspondence with the data on preferable location of the dye at the level of the glycerol moiety observed by the parallax method. Surprisingly, the differences in fluorescence spectra between the probe F and the charged probe F2N8 in DOPC and EYPC (egg yolk phosphatidylcholine) vesicles are less pronounced than those observed in neat organic solvents. While in these solvents (see Figure 2) the N* band of probe F2N8 is substantially red shifted (by 26 nm in ethyl acetate), in PC vesicles its position is almost the same as that of probe F (Figure 5). A similar observation is made for fluorescence intensity ratio, I₅/I₇ (Table 1). For flavone F2N8, this ratio is higher by approximately three times in ethyl acetate as compared to F, while in DOPC vesicles the difference is by 1.3 times only. The same tendency is observed for probes F4N1 and PPZ. The absorption spectra for probes F, F2N8, F4N1, and PPZ in the PC vesicles are very similar to those obtained in neat solvents (Table 1); therefore, the origin of these variations should be in the excited-state electrostatic influence of positively charged substituent, which becomes screened on incorporation into the bilayer. Most probably, it is the screening of positively charged groups of probes F2N8, F4N1, and PPZ by the negatively charged phospholipid phosphate headgroups.

It should also be noted that the fluorescence spectra of the studied probes do not differ in the range of the lipid concentration 25–800 µM (data not shown). The only exception is probe PPZ since its fluorescence spec-

![Figure 2. Fluorescence Spectra of the Studied Probes in Different Solvents: Excitation Wavelength 410 nm](image_url)
Table 1. Spectroscopic Properties of Flavone Probes in Solvents and Phospholipid Vesicles

<table>
<thead>
<tr>
<th>Probe</th>
<th>Solvent/Vesicles</th>
<th>$\lambda_{\text{max}}^{\text{abs}}$</th>
<th>$\lambda_{\text{max}}^{N^*}$</th>
<th>$\lambda_{\text{max}}^{T^*}$</th>
<th>$I_{N^<em>}/I_{T^</em>}$</th>
<th>$\phi$</th>
</tr>
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<tbody>
<tr>
<td>F</td>
<td>Ethyl acetate</td>
<td>394</td>
<td>475</td>
<td>567</td>
<td>0.229</td>
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<td></td>
<td>Acetonitrile</td>
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<td>509</td>
<td>569</td>
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<td>0.09</td>
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<tr>
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<td>410</td>
<td>554</td>
<td>–</td>
<td>–</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>DOPC vesicles</td>
<td>406</td>
<td>518</td>
<td>569</td>
<td>0.862</td>
<td>0.42</td>
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<tr>
<td></td>
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<td>515</td>
<td>569</td>
<td>1.089</td>
<td>0.42</td>
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<tr>
<td></td>
<td>EYPC vesicles</td>
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<td>511</td>
<td>569</td>
<td>0.698</td>
<td>–</td>
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<tr>
<td></td>
<td>EYPG vesicles</td>
<td>404$^c$</td>
<td>510</td>
<td>565</td>
<td>0.866</td>
<td>–</td>
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<td></td>
<td>Water</td>
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<td>560</td>
<td>–</td>
<td>–</td>
<td>$\leq$0.002</td>
</tr>
<tr>
<td></td>
<td>DOPC vesicles</td>
<td>421</td>
<td>515</td>
<td>573</td>
<td>1.111</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>DOPG vesicles</td>
<td>424</td>
<td>510</td>
<td>572</td>
<td>1.351</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>EYPC vesicles</td>
<td>421$^c$</td>
<td>511</td>
<td>574</td>
<td>1.125</td>
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<td></td>
<td>EYPG vesicles</td>
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<td>510</td>
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<td>F4N1</td>
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<tr>
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<td>565</td>
<td>0.669</td>
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<td>538</td>
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<td>–</td>
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<tr>
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<td>EYPC vesicles</td>
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<td>520$^c$</td>
<td>572</td>
<td>0.480</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>EYPG vesicles</td>
<td>396$^c$</td>
<td>519$^c$</td>
<td>568</td>
<td>0.490</td>
<td>–</td>
</tr>
</tbody>
</table>

$\lambda_{\text{max}}^{\text{abs}}$, position of absorption maxima; $\lambda_{\text{max}}^{N^*}$ and $\lambda_{\text{max}}^{T^*}$, position of fluorescence maxima of N* and T* forms. $\phi$ is the fluorescence quantum yield.

*a* Reference [12].

*b* The band appears as a shoulder.

*c* The values correspond to excitation maxima recorded at the T* band peak.

Insensitivity to Variation in Fatty Acid Residues

The parent uncharged probe F shows lower relative intensity of N* band ($I_{N^*}/I_{T^*}$) in EYPC as compared to DOPC vesicles, demonstrating the decreased polarity of its surrounding (Figure 5; Table 1). This can be explained by redistribution of the probe to greater depths in the EYPC bilayer, the hydrophobic region of which displays broader structural heterogeneity due to the larger variety of fatty acid species. The same is observed with probe PPZ, which can exhibit broader distribution of locations due to long distance between the chromophore and the charged group and lower hydrophobicity of 3-HF moiety. Meanwhile, this effect is absent for F2N8 and F4N1 (Figure 5; Table 1). This can be a result of their much more precise location in a layer 10–15 Å from the bilayer center, the properties of which do not depend significantly on the structure of phospholipid hydrocarbon chains. The same regularity is observed in negatively charged egg yolk phosphatidylglycerol (EYPG) with respect to dioleoyl phosphatidylglycerol (DOPG) vesicles: F2N8 and F4N1 compared to F and PPZ show much smaller decrease in $I_{N^*}/I_{T^*}$ ratio (Figure 5; Table 1). This allows for probes F2N8 and F4N1, avoiding the effect of the fatty acid composition and focusing the analysis on the effects of polarity, electrostatics, and hydrogen bonding.

**Figure 3.** Dependence of Fluorescence Intensity of Flavone Probes at Concentration 1 µM in LUV Vesicles on DOPC Concentration

The excitation wavelength 400 nm: the integral fluorescence emission intensity was obtained from the area under the spectra (in wavelength scale). The results were normalized with the account of quantum yields of the probes in 800 µM DOPC vesicles.
Table 2. Quenching of 3-Hydroxyflavone Probes by Nitroxide-Labeled Lipids

| Probe | Experiment | F_E1/F_0 | F_E2/F_0 | F_E3/F_0 | Zcf, Å | Zcf>H1Å
|-------|------------|----------|----------|----------|--------|----------
| F     | E1         | 0.49     | 0.495    | 0.55     | 15.9   | 15.9     |
|       | E2         | 0.47     | 0.485    | 0.53     | 16.1   | 16.0     |
|       | E3         | 0.50     | 0.50     | 0.57     | 15.8   | 15.8     |
|       | OG         | 0.49     | 0.49     | 0.52     | 15.8   |          |
| F2N8  | E1         | 0.515    | 0.49     | 0.56     | 15.3   |          |
|       | E2         | 0.53     | 0.49     | 0.59     | 15.0   | 15.0     |
|       | E3         | 0.53     | 0.475    | 0.61     | 14.7   |          |
|       | OG         | 0.55     | 0.54     | 0.59     | 15.6   |          |
| F4N1  | E1         | 0.535    | 0.45     | 0.49     | 9.9    |          |
|       | E2         | 0.50     | 0.41     | 0.45     | 10.1   | 10.0     |
|       | E3         | 0.56     | 0.43     | 0.46     | 9.8    |          |
|       | OG         | 0.67     | 0.51     | 0.58     | 10.5   |          |
| PPZ   | E1         | 0.64     | 0.54     | 0.49     | 7.8    |          |
|       | E2         | 0.58     | 0.53     | 0.46     | 7.3    | 7.0      |
|       | E3         | 0.60     | 0.49     | 0.39     | 6.3    |          |
|       | OG         | 0.87?    | 0.47     | 0.40     | 7.1    |          |

Zcf is the distance between the middle of the bilayer and the chromophore center for each individual type of preparation (OG refers to the vesicles obtained by octylglucoside dilution); <Zcf> is the average of the three Zcf values obtained with unilamellar vesicles. The error is probably higher than the one that could be estimated from the deviations from the average and is estimated around ±1 Å, even more in the case of PPZ (±1.5 Å). Ftc/F_0, F_5/F_0, and F_12/F_0 are the values of fluorescence quenching ratios in vesicles containing 15 mol% TempoPC, 5-SLPC, or 12-SLPC, respectively, to DOPC vesicles lacking nitroxide-labeled lipid.

absorption spectra (Table 1). The shift to the blue of the N* band for 3-HF derivatives always indicates the decrease of polarity of the chromophore surrounding [10, 11], and this is in line with results of the parallax measurements suggesting the deeper location of F4N1 in the bilayer. What then is the origin of substantial increase of the I_N*/I_T*- ratio, which is the opposite of the expected polarity effect?

A reasonable explanation could be that the N* band position and the I_N*/I_T*- ratio of the 3-HF probe characterize different properties of the microenvironment. The N* band position is sensitive to the polarity of the probe surrounding, with resolution comparable to the size of the N*-excited state dipole, ca. 10 Å. Meanwhile, the I_N*/I_T*- ratio is directly connected with the energetic and kinetic variables of ESIPT reaction between the proximal 3-hydroxyl and 4-carbonyl groups and, therefore, can report on perturbation of this reaction at the distance of a single hydrogen bond [8]. Since the ESIPT reaction center for probe F4N1 is estimated to locate on the level of sn carbonyl (Figure 4), the water molecules bound at this level [30] may provide this perturbation by intermolecular hydrogen bonding, decreasing the proton transfer efficiency. This result demonstrates that the 3-HF probes can provide an extremely sensitive response to the changes in properties of their binding sites in membrane, and this response is multiparametric. Sensitivity to the Surface Charge

Comparative studies were performed on LUV composed of anionic DOPG and EYPG with respect to neutral
actions at the ESIPT reaction site of the probes. As it was previously proposed for probe F [17], they are connected with the increased hydration of this site in EYPG as compared to EYPC vesicles. For probes F and F2N8, this probably occurs on the level of sn_2 carbonyl and phosphate groups, while for the deeper located probes F4N1 and PPZ, the hydration site may be located at the level of sn_1 carboxyls (Figure 4). The deep penetration of water molecules inside the bilayer is a known fact that was established in experiment [30, 31] and confirmed in molecular dynamics simulations [32–34]. The observed opposite effect for PPZ (in comparison with other probes) suggests that all the probes are able to sense the differences in anisotropic properties of the bilayer, which may be connected with oriented dipoles of trapped water molecules.

Significance

Introduction and development of two-band ratiometric probes is an important breakthrough that should expand enormously the possibilities of fluorescence spectroscopy and microscopy, including the image recording in real color. The new 3-hydroxyflavone derivatives offer unique possibilities in this respect by providing observation of two emission bands, blue-green (at about 500 nm) and yellow-red (at about 600 nm), the spectral positions and relative intensities of which are strongly sensitive to the properties of their microenvironment. Because variations of these properties occur at atomic distances, this sensitivity cannot be applied in full without providing their definite location and orientation in the bilayer. Solution of this problem is presented in the present work. A family of advanced probes has been synthesized which retain all major properties of 3-HF, such as two bands in emission spectra and the dramatic solvent-dependent variations of their intensity ratio. On incorporation into phospholipid vesicles, their well-resolved two emission bands are maintained, and moreover, the fluorescence quantum yields are greatly increased. Dependent on the probe structure, their 3-HF chromophores can locate at different depths and in different orientations.

We show that the two-band fluorescent 3-HF probes inserted into the membrane bilayer allow for obtaining several parameters that characterize the properties of their location sites simultaneously. Similarly to common polarity-sensitive dyes, the shifts of their two emission bands are correlated with polarity of their environment. The intensity ratio of these bands in addition to polarity effects is sensitive to specific interactions that affect ESIPT reaction, and in particular, to intermolecular hydrogen bonding. This provides the possibility of characterizing the hydration of probe binding sites in membranes. Because of unidirectional character of ESIPT reaction, anisotropic properties of probe location sites can also be revealed. We are on the beginning steps of exploration of these unique possibilities.

Experimental Procedures

General Methods
Melting points of the synthesized compounds were determined on a Büchi 512 melting point apparatus and presented as uncorrected
values. Microanalyses were performed with a Carlo Erba 1106 Ele-
mental Analyzer. Proton NMR spectra were recorded at 200 MHz
on a JEOL PMX 270 MHz spectrometer. Tetramethylsilane (TMS)
was used as an internal standard in all NMR spectra run in CDCl3
or DMSO-d6. Mass spectra were recorded on a Kratos MS-25 mass
spectrometer using EI or FAB methods. All column chromatogra-
phy was performed on silica gel (Merck, Kieselgel 60H, Art 7736).
Absorption spectra were recorded on Cary 3 Bio (Varian) spectro-
photometer. Fluorescence spectra in solvents were recorded on Quanta
Master (PTI) and those in vesicles on SLM 48000 (SLM-Aminco)
spectrofluorometers.

Chemicals
All the solvents used for absorption and fluorescence measure-
ments were of spectroscopic grade purchased form Aldrich and Fluka
Chemical Co. Acetonitrile and ethyl acetate were additionally dried
over phosphorus pentoxide and sodium sulfate, respectively, with
subsequent distillation. Diethyle and egg yolk phospholipids and
octylglucoside were purchased from Sigma Chemical Co. Tem-
poPC, 5-SLPC, and 12-SLPC were purchased from Avanti Polar
Lipids. The concentration of phospholipid stock solutions in chloro-
form was determined by dry weight. The nitrooxide content of ni-
trooxide-labeled lipids was calculated using the electron spin reso-
nance (ESR) integrations of corresponding diluted stock solutions
in chloroform by comparing with a temokoholine reference solution
in the same solvent.

Synthesis of Probes
4'-Dimethylamino-3-Hydroxyflavone (F)
4'-dimethylamino-3-hydroxyflavone (F) was synthesized and puri-
fied as described elsewhere [12].

5-Chloromethyl-2-Hydroxyacetophenone
A suspension of 1 ml of 2-hydroxyacetophenone (Aldrich) and 0.27
g of paraformaldehyde in 5 ml of concentrated hydrochloric acid was
stirred at 35°C for 4–5 hr until the formation of yellow precipitate,
which was filtered and washed thoroughly with water. The precipi-
tate was dried and washed with minimum amount of methanol. Yield
60%; mp 78°C–81°C; 1H NMR (200 MHz, CDCl3) 2.64 (3H, s), 4.56
(2H, s), 6.97 (1H, d, J 8 Hz), 7.49 (1H, dd, J 8.4 Hz, 2.1 Hz), 7.72 (1H,
J 8.2), 12.315 (1H, s); Anal. Calcd. for C9H9ClO2 C 58.55%, H 4.91%;
found C 58.74%, H 4.99%.

6-Ethoxymethyl-4'-N,N-Diethylamino-3-Hydroxyflavone
1 g of 5-chloromethyl-2-hydroxyacetophenone was dissolved in 10 ml of
ethanol. The solution was treated with 0.87 g of sodium hydrox-
ide while stirring. The reaction was controlled by toluene; yield, 89.5%; mp, 119
°C.

8:2). Yield, To a solution of 0.12 ml of 2-hydroxyacetophenone and 0.25 g of
4-dibutylaminobenzaldehyde (prepared from aniline and n-butyliodid and further formylation).

-Dimethylamino-3-Hydroxyflavone (F)
A solution of 0.1 g of 6-bromomethyl-4'-N,N-dietyl-
aminoflavone in two steps starting from 5-chloromethyl-
2-hydroxyacetophenone and 4-dibutylaminobenzaldehyde (prepared in two steps from aniline and n-butyliolid and further fotmylation).

3.56 (8H, m), 6.68 (2H, d, J 6.0 Hz), 6.92 (2H, d, J 8.8 Hz), 7.79 (2H,
d, J 8.2 Hz), 7.81 (2H, t, J 7.2 Hz), 8.16 (2H, d, J 9.1 Hz), 8.21 (1H,
J 8.2), 8.22 (1H, s), 8.35 (1H, d, J 8.4); MS (FAB) m/z 479.2 (M+
1)^-, 322.1, 239.6, 154.0.

N-[4'-N,N-Diethylamin]-3-Hydroxy-6-Flavonyl[4-Methyl-
N,N-DimethyloctylAmmoniumBromide(F2N8)]
A mixture of 30 mg of 6-bromomethyl-4'-N,N-diyethy-
limino-3-hydroxyflavone and 20 mg of N,N-dimethyloctylamine (Aldrich) was
solubilized in 3 ml of dry tetrahydrofururan. After stirring for 4 hr, the
formed precipitate was filtered and washed with cold tetrahydrofuran.
yield, 86%; mp, 150°C–154°C; UV max in acetone 417 nm, ε =
34,000 l mol−1 cm⁻1; 1H NMR (200 MHz, CDCl3) 0.85 (3H,
t, J 6.80), 1.2–1.4 (18H, multiplet), 3.33 (6H, s), 3.4–3.6 (6H, multiplet),
5.28 (2H, s), 6.7–7.0 (3H, multiplet), 7.59 (1H, d, J 8.4), 8.15 (2H, d,
J 8.17), 8.22 (1H, s), 8.35 (1H, d, J 8.4); MS (FAB) m/z 479.2 (M+
1)^-, 322.1, 239.6, 154.0.

4-Bromomethyl-4'-N,N-Dibutylamino-3-Hydroxyflavone
This compound was prepared as 6-bromomethyl-4'-N,N-diety-
lamino-3-hydroxyflavone in two steps starting from 5-chloromethyl-
2-hydroxyacetophenone and 4-dibutylaminobenzaldehyde (prepared in two steps from aniline and n-butyliolid and further fotmylation).

1-Phenyl-4-(4-Pyridyl)Piperazine
A mixture of 4 g of 4-chloropryidine hydrochloride (Aldrich), 4 ml of
1-phenylpiperazinone (Aldrich), and 14 ml of ethylolidesopropylamine
(Fluka) in 20 ml of nitrobenzene (Aldrich) was heated with stirring
for 4 hr at 200°C. The resultant solution was concentrated on a
rotary evaporator and then water was added. The precipitate was
filtered and washed with water and hexane. Crystallized from tolu-
en/hexane = 1/1, yield 60%; mp, 163°C. This compound was used in
the next step without any further characterization.

4-(4-Pyridyl)PiperazinoneBenzyaldehyde
To 8 ml of dry dimethylformamide (DMF), 2.4 ml of POCl3 was added
dropwise with stirring and cooling on ice bath. To the solution, 2 g
of 1-phenyl-4-(4-pyridyl)piperazinone was added, and the mixture
was heated at 60°C for 1 hr, then cooled and poured into ice bath.
The precipitate formed after neutralization with sodium carbonate
was filtered and washed with water and hexane. It was crystallized from
toluene/hexane = 1/1; yield 60%; mp, 119°C–120°C; 1H NMR (200 MHz, CDCl3)
3.56 (8H, m); 6.88 (2H, d, J 6.0 Hz), 6.92 (2H, d, J 8.8 Hz), 7.79 (2H,
d, J 8.2 Hz), 8.31 (2H, d, J 6.0 Hz), 9.80 (1H, s).

3-Hydroxy-4-(4-Pyridyl)PiperazinoneFlavone
A solution of 0.12 ml of 2-hydroxyacetophenone and 0.25 g of
4-(4-pyridyl)piperazinonebenzyaldehyde in ethanol, 0.36 g of 70%
KOH aq was added, and the mixture was treated with 0.7 ml of 30% hydrogen peroxide. After 4 hr, it
was diluted with water, and the suspension was neutralized with diluted HCl. The precipitate was filtered and washed with water.

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yield 90%; mp 270°C–275°C (decomp.); UV max in acetone-triite 380
nm, ε = 30,000 l mol⁻¹ cm⁻¹; 'H NMR (200 MHz, CDCl₃) 2.11
(2H, m), 2.37 (2H, t, J 7.0 Hz), 3.57 (4H, t, J 5.6 Hz), 3.89 (4H, t, J 5.6
Hz), 4.32 (2H, t, J 6.7 Hz), 7.09 (2H, d, J 9.0 Hz), 7.26 (2H, d, J 7.4
Hz), 7.44 (1H, ddd, J 8.2, 6.1, 1.8 Hz), 7.61 (1H, d, J 8.4 Hz), 7.76
(1H, m), 8.09 (1H, d, J 8.2 Hz), 8.16 (2H, d, J 9.0), 8.35 (2H, d, J 7.4
Hz); MS (FAB) m/z 522.0, 400.0, 232.

Preparation of Vesicles Samples and Fluorescence Measurements
For binding studies, large unilamellar vesicles (LUV) of DOPC (0.12
μm in diameter) were used. They were obtained by the classical
extrusion method as described previously [35]. LUVs were labeled
by adding an aliquot (generally 1 μl) of probe stock solution (2 mM)
in methanol (DMSO in the case of PPZ) to 2 ml solutions of vesicles
in increasing concentrations (from 0 to 800 μM in lipids). Preparation
and labeling of EYPC and EYPG vesicles were performed applying
the same procedures. The fluorescence intensities, corresponding
to the integrated areas under the emission spectra, were determined
immediately after the labeling for F₂ZN, and F₄N₁. In the case of
PPZ, the intensity was recorded after 10 min, the time required to
gain a stable intensity. The fluorescence quantum yields of the probes
embedded in a lipid environment were determined as previously
described [36], the reference being 4'-diethylamino-3-hydroxyflavo-
none with a quantum yield of 52% in ethanol [10].

For quenching experiments by the parallax method, lipid vesicles
were prepared according to Kachel et al. [28] with small modific-
tions. DOPC (85%) and nitroxide-labeled PCs (15%) were mixed in
chloroform in order to obtain a final concentration of 400 μM. The
mixtures were dried under N₂ and kept under vacuum for 30 min,
and then resuspended in ethanol or in octylglycoside solution at 50
mM (120 μl) by continuous rotation using a rotary evaporator for 30
min. Finally, 6 ml of buffer (15 mM HEPES, pH 7.4) was added and
vortexed briefly. The sizes of vesicles were determined by light
scattering method using a NISAD Coultronics Nanosizer. The vesicles
obtained by the ethanol dilution method were of the same size
of LUV (0.12 μm), while those obtained with octylglycoside were
somewhat larger (0.4 μm). The final concentration of the probes and
the labeling procedure were the same as for the binding experi-
ments.

Quenching Experiments and the Parallax Method
Fluorescence intensity of labeled vesicles, either DOPC or DOPC
mixed with 15% nitrooxide lipids, was measured in a 1 cm semimicro
quartz cuvette as described in the subsection on binding studies. The
measured fluorescence intensities, corresponding to the inte-
grated areas under the emission spectra, were corrected for the
background signal of the corresponding unlabeled vesicles. This
background intensity was less than 4% of the measured intensity,
except for PPZ (about 10%). The excitation wavelength was 400
nm and the excitation and emission slits were set at 4 nm (8 nm in
the case of PPZ)

Using the corrected F/F₀ values, the distance of the fluorophores
from the center of the bilayer was calculated using the parallax
equation as developed by London and collaborators [25, 26, 28,
37]; Zₚ = L₀ + [1 − ln(F/F₀)/C = Lₚ/2L₀], where Zₚ is the distance
of the fluorophore from the center of the bilayer, F₀ and F are the
fluorescence intensities in the presence of the shallow quencher
(quencher 1) or the deeper quencher (quencher 2), respectively, L₀ is
the distance of the shallow quencher from the center of the bilayer,
Lₚ is the distance between the shallow and deep quenchers, and C
the concentration of quencher in molecules/Å² (equals mole frac-
tion of nitroxide-labeled phospholipid/area per phospholipid;
presently C = 0.15/70 Å² [32]). For a given chromophore, the quenching
by the two most efficient quenchers (TempoPC/5-SLPC or 5-SLPC/19.
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