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Fluorescence News

Multiparametric Color-Changing Fluorescence Probes

KEY WORDS: Site-sensitive probes; steady-state fluorescence, multiparametric analysis; excited-state intramolecular proton transfer.

It is a dream of researchers to obtain a molecular tool that could probe a number of different types of intermolecular interactions simultaneously. This tool should allow separate determination of electronic and nuclear polarizability effects that are now commonly assembled under the term *polarity*. It should sense the magnitude and direction of local electric fields and recognize specific interactions, such as hydrogen bonds (H-bonding). Moreover, it should provide the local characteristics of H-bond donor ability (acidity) and H-bond acceptor ability (basicity). Such a tool is especially needed in the studies of interfaces and multicomponent and microheterogeneous systems (including all biologic applications), because different molecular probes, if applied for the studies of these properties separately, may exhibit different redistribution and binding properties leading to artifacts. We believe that the probes belonging to 3-hydroxychromone (3HC) and 3-hydroxyflavone (3HF) families, which is the subject of present communication, fulfill these criteria of multiparametric fluorescence probes. The existing methodology that originated about 50 years ago is still focused on single-parametric sensing. The probes are so designed or selected that the sensitivity to one parameter thought to sense the most important property is reinforced, whereas the sensitivity toward other properties is intentionally diminished (or even ignored). This is because in most cases the probe molecules exist as single excited-state and single ground-state species, and the electronic transition between them generates only one fluorescence emission band. To provide multiparametric response the probe molecules, depending on the conditions, should exist in different ground and excited states. Moreover, the transitions between these states can be represented by separate absorption/emission bands that can be differently sensitive to different types of perturbations. The 3HC and 3HF dyes (Fig. 1) show this property. In the parent 3HF molecule, the two bands in fluorescence emission are commonly observed because of the excited-state intramolecular proton transfer (ESIPT) reaction [1]. They reflect the presence of four states, the normal ground state (N), the normal excited state (N*), the tautomer excited state (T*), and the tautomer ground state (T) (see Scheme 1). The solvent-dependent dual emission can be observed in other dyes exhibiting ESIPT reaction, but 3HCs-3HFs are the unique case in which its appearance is not connected with conformational isomerizations and breakage of intramolecular hydrogen bonds.

In addition, in some cases the 3-OH group can dissociate yielding a ground-state anionic form (A) and an excited-state dissociated form (A*) that may be detected in absorption/excitation and emission spectra, respectively. The electronic transitions between correspondent forms are represented by highly intensive bands separated on the wavelength scale (Fig. 2).

It is remarkable that all these forms behave as distinct species that interact differently with the environment and are differently sensitive to a variety of interactions. Moreover, the interactions with the environment may produce a perturbation in the equilibrium between the ground-state and excited-state forms, which can dramatically influence the relative band intensities. A breakthrough in the probe design was the attachment at 4′ position of 3HF of the electron donor dialkylamino group, which made these dyes strongly solvatochromic. Further improvement of their spectroscopic properties (shift of excitation spectra to longer wavelengths, increase of fluorescence quantum yield, and optimization of two-band response) was provided by additional chemical modifications [2–4].

The new dyes show extreme sensitivity to solvent polarity [5] and thus may be used as sensors for polar/

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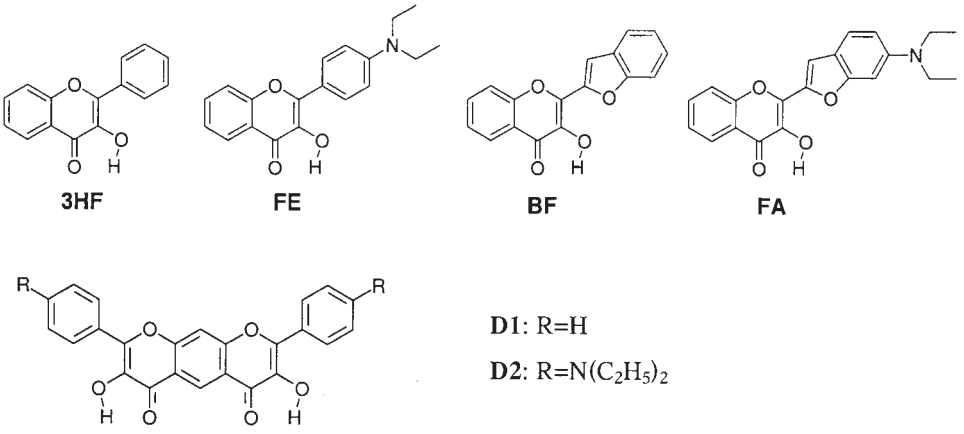
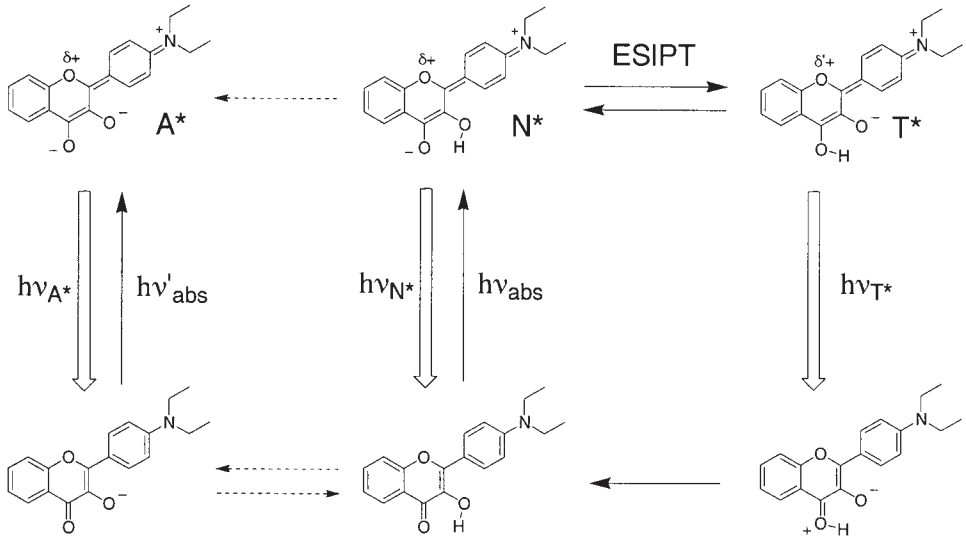


Fig. 1. The formulae of the probes, which are cited in the text, 3HF, FE, FA, F-BF, and diflavonols D1 and D2.



Scheme 1. Scheme of ground-state and excited-state transformations for probe FE. N*, T*, and A* are the normal (N), tautomer (T), and anionic (A) excited-state forms, correspondingly.

protic impurities in hydrophobic solvents with applications in probing of hydration in biomembranes [6,7]. They are also highly sensitive to the presence of water molecules in reverse micelles [8] and were found to be strongly electrochromic, that is, sensitive to the strength and direction of local electric fields [9]. The last property may find application for the determination of dipole potential in biological membranes.

Moreover, because the 3-OH and 4-carbonyl groups in 3HC and 3HF are connected by an intramolecular H-bond, which is the pathway for ESIPT reaction, the 3HC and 3HF molecules may constitute ideal H-bonding sensors [10]. The sensitivity to intermolecular H-bonding perturbation can be switched off by steric screening of the 4-carbonyl group [11].

Systematic analysis of excitation and fluorescence spectra of probe FE obtained in 21 representative solvents allows us to suggest a novel multiparametric approach for the simultaneous analysis of several physical-chemical properties of probe microenvironment: polarity (a function of dielectric constant), electronic polarizability (a function of refractive index), and H-bond donor ability [10]. The power of the proposed algorithm was demonstrated in the studies of solvent mixtures, reversed micelles, and binding sites

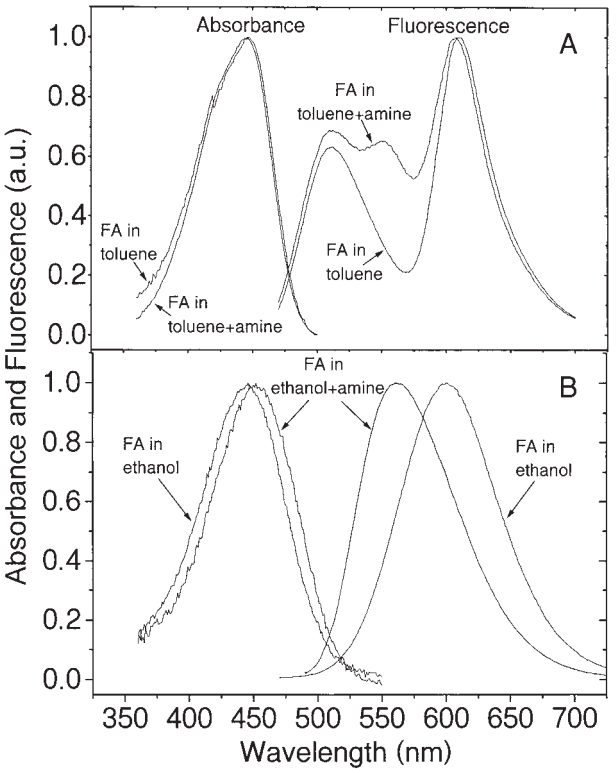


Fig. 2. Comparison of absorbance and fluorescence spectra of FA in toluene and on addition of 10% ethyldiisopropyl amine (A); in ethanol and on addition of 0.5% ethyldiisopropyl amine (B). The spectra are normalized at peak maxima.

of proteins [10]. The extension of this analysis to probe FA allows us to further characterize its highly specific binding site to bovine serum albumin [12].

Meanwhile, the attempts to find and characterize the A and A* forms of probe FA in common solvents were successful only in the presence of a strong proton acceptor such as amines (Fig. 2). If the environment is relatively polar, then the dissociated form can be formed in the ground state (Fig. 2B), but if the environment is hydrophobic, it can be observed only as a result of an excited-state process (Fig. 2A).

Previously Sytnik *et al.* [13] observed for parent 3HF, the presence of two binding sites in human serum albumin molecule. One of them binds the N form of the dye with the observation of fluorescence from the N* state, whereas the other binding site binds the A form with fluorescence emitting from the A* state. We made similar observations for the binding of BF to bovine albumin. We found three bands in emission and two bands in excitation and distinguished the excitation band of the A form and the emission band of the A* form (Fig. 3). The remaining two fluorescence bands (at shorter and longer wavelengths) belong to N* and T* emission and describe the binding of the probe to a low polar site. In these conditions, FA does not exhibit the presence of A or A* forms [12].

Another kind of fluorescent compounds, which are extremely sensitive to the nature of surroundings, are diflavonols [14]. These dyes contain two similar atomic

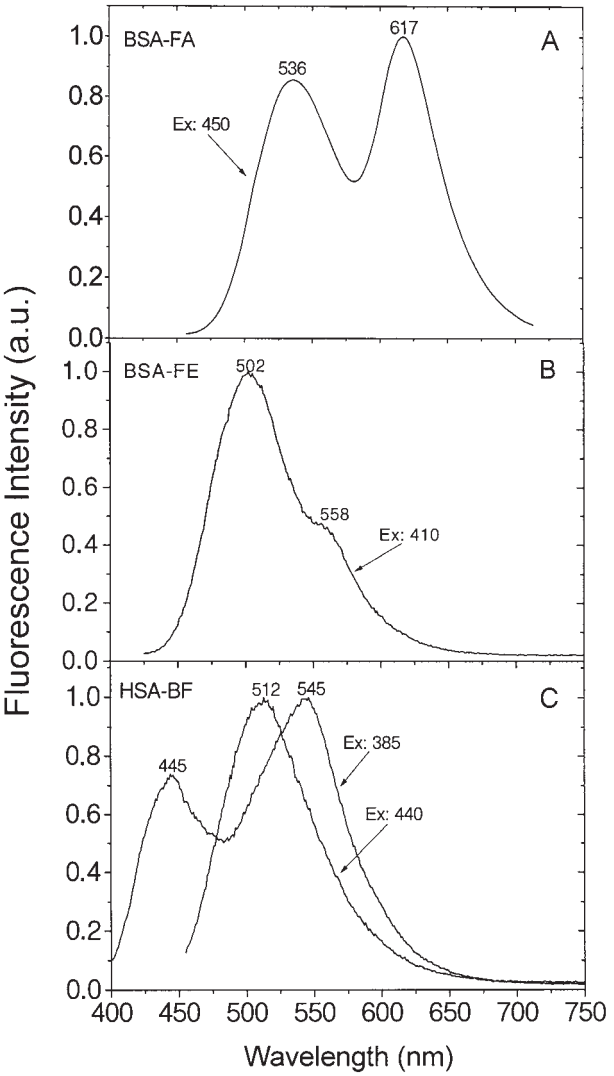


Fig. 3. Fluorescence emission spectra of the complexes of probes FA, FE, and BF with bovine (BSA) and BF with human (HSA) serum albumins. A, FA-BSA complex with excitation at the band maximum (450 nm). B, FE-BSA complex with excitation at the band maximum (410 nm). C, BF-BSA complex with excitations at two band maxima 385 and 440 nm. For the emission band at 445 nm we observe a single excitation peak at 385 nm, whereas for the two emission bands at 512 and 545 nm we observe two excitation peaks at 385 and 450 nm. Molar probe:protein ratio is 1:2. The spectra are normalized at peak maxima.

systems for ESIPT per molecule and are very promising in multiparametric probe design. In some environmental conditions they can undergo partial tautomerization in the excited state, and three bands may be observed in the fluorescence spectrum that belong to NN*, NT*, and TT* tautomeric forms. The experiment shows that nonsubstituted diflavonol (**D1**) can exist in NN* and NT* forms only, but in basic solvents such as ethanol it is capable to dissociate with the formation of anion. *Bis*-diethylamino-substituted compound (**D2**) can exhibit the formation of all three tautomers in aprotic solvents of intermediate polarity, such as ethylacetate, dichloromethane, or acetonitrile [14].

Thus we demonstrate the possibility of obtaining, from a single fluorescence probe, rich multiparametric information about the broad range of intermolecular interactions in the probe environment. This can be achieved if the probe can exist in several excited or ground states that interact differently with probe environment and when the interactions with the environment influence the energies and relative populations of these states. This information can be obtained by simple steady-state spectroscopic measurements. This information content can be further increased by application of time-resolved, quenching, emission anisotropy and other more sophisticated techniques. The prospective range of applications of these novel probes is very broad and includes surface chemistry, colloid chemistry, and polymer science. In life sciences the most prospective applications are in the studies of biomembranes, organelles, and the whole living cells, where the analysis of coupling between local electrostatics, hydration, and polarity becomes achievable.

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