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A wide-range fluorescent pH-indicator based on 3-hydroxyflavone structure

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Abstract

With the aim to develop wide-range pH-indicators, we synthesized a new water-soluble compound, 3,4′-dihydroxy-3′,5′-bis-(dimethylaminomethyl)flavone (FAM345), which displays a spectral sensitivity to pH in the range from 2 to 12 in absorption spectra as well as in fluorescence excitation or emission spectra. In all three cases, the estimation of pH is possible, either from the position of the maxima or from the absorbance or fluorescence intensity at one or several separated wavelengths, or also from absorbance or fluorescence intensity ratios. This last feature allows determining pH value of the medium independently from the probe concentration, from its possible photobleaching, as well as from any variation of light source intensity (at least in fluorescence methods).

Keywords: Absorbance; Fluorescence; pH-Indicator; Flavonol; 3-Hydroxyflavone; Ratiometric probe

1. Introduction

Flavonols (3-hydroxyflavones, 3HF) are presenting a considerable interest not only in drug design [1], but also in the design of new fluorescence probes with broad applications in the study of molecular interactions in solutions and biological systems. The attractive features of the flavonols are related to their high sensitivity to physico-chemical parameters of the environment. Flavonols usually exhibit two bands in their fluorescence spectrum, due to an excited state intramolecular proton transfer (ESIPT) reaction [2], leading to two excited forms, the normal N* and the tautomer T* ones, and thus resulting in two strongly separated bands in the fluorescence spectrum. Their positions and relative intensities depend on several parameters of the medium. Due to this unique phenomenon many flavonol derivatives were shown to be very effective probes in the analysis of the structure of micelles [3–6] and phospholipid vesicles [6–13], as well as in the fluorescence recognition of cations of different radii [14–16].

The presence of a single hydroxyl group in the position 3 of the molecule renders the flavonols able to sense pH in the range from 8 to 12 [17]. Such a sensor molecule can be further derivatized by other chemical groups also submitted to some acid–base equilibrium, but at quite distant pHs. Provided all the corresponding species are fluorescent, this sensor should work properly in a relatively large pH range.

Several examples of dyes with two pH transitions are described in the literature. There are many different molecules like phenols or nitrogen-containing substances such as aminonaphtols [18], 2-(pyridyl)benzimidazoles [19,20], polyamines [21], hydroxyphenylbenzoxazoles [22] or acridones [23]. However, for most of them, their practical use is quite uneasy because of low solubility, low fluorescence quantum yield, low fluorescence sensitivity to pH or of acid–base transitions too far away on the pH-scale.

With the aim to develop wide-range pH-indicators, we propose in the present work a new fluorescence pH sensor based on flavonol structure that works properly in a

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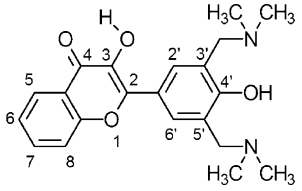


FAM345

Scheme 1.

pH range from 2 to 12. This probe, 3,4′-dihydroxy-3′,5′-bis-(dimethylaminomethyl)flavone (FAM345), contains two hydroxyl groups, both of them being conjugated to a carbonyl group pertaining directly to the chromophore system of the molecule (Scheme 1). Due to this feature, it is possible to drive the position of excitation and/or emission spectrum simply by the deprotonation of one of the hydroxyl groups. Due to their phenolic nature, the acidity constants pK_a of the hydroxyls are in a pH range from 8 to 10. Since the molecule also contains two dimethylaminomethyl groups localized in close proximity to the positive head of the molecular dipole, we can expect that not only deprotonation of hydroxyl groups, but also protonation of dimethylaminomethyl groups should have an influence on intensities and positions of both excitation and emission bands of FAM345. Additional spectral effects could be caused by intramolecular acid–base interactions between hydroxyl and amino groups of FAM345. For example, such an interaction can cause the deprotonation of 4′-OH group at a lower pH [24], rendering possible pH measurements in a pH range near neutrality.

2. Experimental

FAM345 was prepared from 3,4′-dihydroxyflavone and *N*,*N*,*N*′,*N*′-tetramethyldiaminomethane by a well known procedure [25]. A solution of 0.254 g (1 mmol) of 3,4′-dixydroxyflavone (INDOFINE, U.S.A.), and 0.404 g or 0.54 ml (4 mmol) of *N*,*N*,*N*′,*N*′-tetramethyldiaminomethane (from Aldrich) in 5 ml of dry dioxane was boiled 4–5 h, until a yellow–orange precipitate was formed. It was filtered off and washed with dioxane. The obtained reaction yield was 41% (0.151 g). The product was homogeneous according to ^{1}H NMR spectroscopy data and TLC (eluent–chloroform-methanol–triethylamine mixtures, 95:4:1 or 85:14:1, v/v). Melting point was 154–155 °C (uncorrected). It was determined on a PHMK apparatus (“VEB Analytik” Dresden). ^{1}H NMR spectrum was obtained in DMSO-d_6 with TMS as internal standard on Varian Mercury-400 spectrometer. Below chemical shifts (δ, ppm), form of a signal (s, singlet; d, doublet; t, triplet), coupling constants (J, Hz) and signal intensity are given: 8.11d (J = 8) 1H; 7.94s 2H; 7.71t (J = 8) 1H; 7.65d (J = 8) 1H; 7.38t (J = 8) 1H; 3.57s 4H; 2.30s 12H. Mass spectrum (obtained on Thermabeam Mass Detector, Waters Integrity System, U.S.A.) showed a molecular ion peak at 368 corresponding to the calculated molecular mass.

For absorption and fluorescence spectroscopy experiments, FAM345 was first dissolved at 5 μM concentration in phosphate–citrate–borate buffer, 15 mM (5 mM of each component, ionic strength μ = 0.05 M, pH 7.2), and the pH were then adjusted to the desired values by addition of microquantities either of 5 M NaOH or 5 M HCl, never exceeding 3% of the initial volume. The absorption spectra were recorded on a Cary 4 spectrophotometer, and the fluorescence spectra on a Jobin–Yvon Fluoromax spectrofluorimeter. pH were determined directly into the quartz cuvette (1 cm × 1 cm) on a 713 pH-meter from Metrohm. Temperature was kept at 20 °C for all the experiments.

The effective pK_a values (presented in Table 1) were calculated by the Fletcher–Pawl algorithm using a nonlinear least squares method, as developed in Doroshenko’s program [26,27], which minimizes the sum of squared deviations of the experimental and calculated absorbance (A) or fluorescence data in the approximation of one-step protonation process for each transition. The program utilizes the common formula [28]:

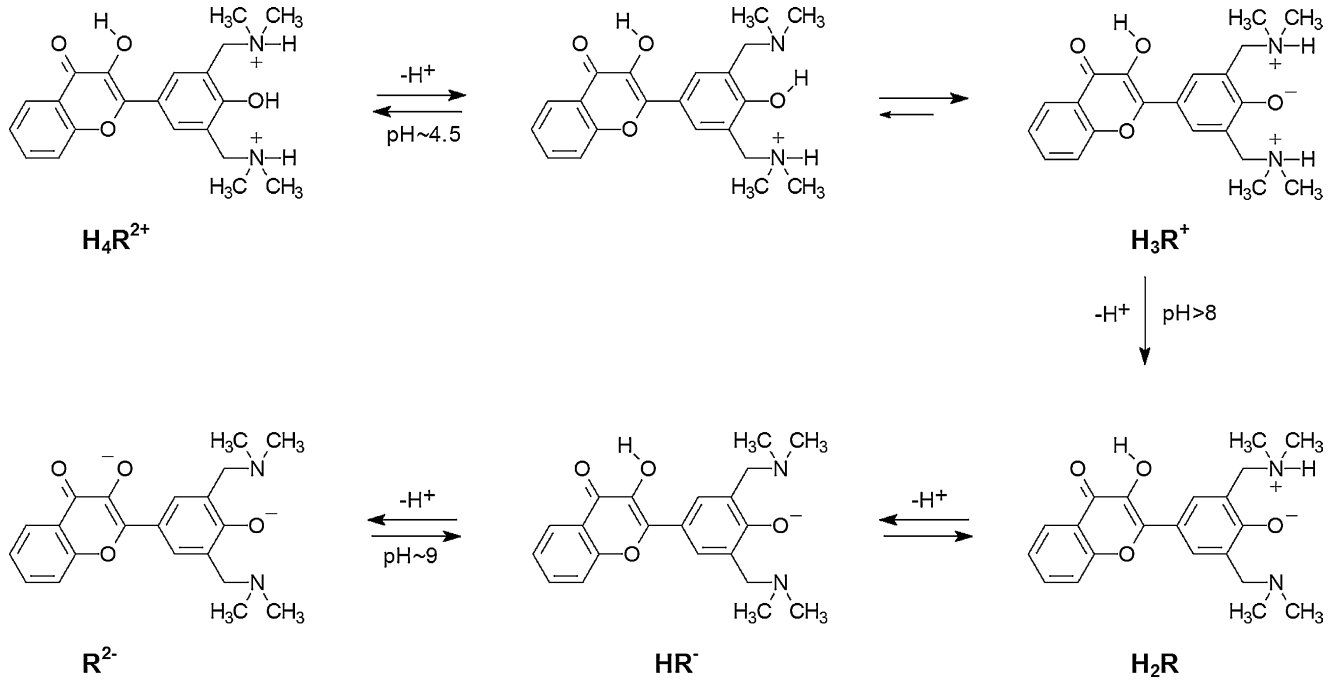
$$A = \frac{A_{HA}10^{-pH} + A_A 10^{-pK_a}}{10^{-pH} + 10^{-pK_a}},$$

where A_{HA} is the absorbance or fluorescence intensity of each protonated species (H_4R^{2+} and H_3R^+ for the first and second transitions, respectively, see Scheme 2), A_A is the absorbance or fluorescence intensity of each deprotonated species (H_3R^+ and R^{2-} for first and second transitions, respectively). In such an approximation, we obtained for the second transition (pK_2

Table 1
Effective pK_a values of FAM345 determined by the absorption, fluorescence excitation and emision parameters

Method	Constant	By maximum position	Registration wavelength			Averaged value
			λ = 350 nm	λ = 385 nm	λ = 430 nm	
Absorption	pK_1	4.43 ± 0.10	4.29 ± 0.08	4.17 ± 0.04	4.32 ± 0.08	4.30 ± 0.08
Excitation		4.38 ± 0.10	4.51 ± 0.15	4.29 ± 0.08	4.47 ± 0.16	4.41 ± 0.12
Emission		[b]	4.33 ± 0.08[a]	[b]	4.34 ± 0.09	4.34 ± 0.09
Absorption	pK_2	9.28 ± 0.06	8.97 ± 0.05	9.03 ± 0.04	8.93 ± 0.05	9.05 ± 0.05
Excitation		8.33 ± 0.06	9.00 ± 0.13	8.90 ± 0.08	8.88 ± 0.05	8.78 ± 0.08
Emission		8.84 ± 0.12	8.67 ± 0.17[a]	[b]	8.55 ± 0.10	8.69 ± 0.13

[a] Calculated from the intensity ratio I_{440}/I_{530}.
[b] There is no possibility to determine.



Scheme 2.

in Table 1) an averaged pK_a resulting from three protonation processes with close pK_a values: $R^{2-} \rightarrow HR^{-}$, $HR^{-} \rightarrow H_2R$ and $H_2R \rightarrow H_3R^{+}$.

3. Results and discussion

3.1. Absorption spectra

An increase of pH from 2 to 6 induces a red shift of the absorption maximum from 350 to 385 nm (Fig. 1a and b). A further pH increase from 6 to 12 shifts the spectra more to the red, up to 430 nm for the maximum. Two isosbestic points, at 359 and about 410 nm, are present in the absorption spectra. They confirm the existence of two acid–base equilibria within the tested pH range. The second isosbestic point (410 nm) is eroded in some degree, which may be due to the formation of more than one FAM345 species in the higher pH range.

To present the spectral changes in a more simple way, we plotted the absorbance versus pH at the three wavelengths corresponding to the maxima observed in the absorption spectra network (Fig. 1c). These curves, as well as the curve showing the variation of the maximum wavelength of the absorption spectra versus pH (Fig. 1b), distinctly show the formation of the different species corresponding to the two acid–base equilibria existing in the studied pH range. The values of effective acidity constants (pK_a) are calculated from these data (Table 1). These different curves are leading to very close values of pK_a, pointing out the possibility to perform pH determination at one separate wavelength or even by the position of absorption maximum. However, all these curves are presenting only a very small variation in the pH region from 6 to 8, that renders pH measurements unprecise in this range. Also it should be pointed out that evaluation of pK_a from the maximum position can give only an approximate value.

Concentration independent pH determination is also possible by measuring the absorbance ratio at two different wavelengths. As shown in Fig. 1d, the ratio A_{430}/A_{350} is changed by more than two orders of magnitude, thus allowing a simple estimation of pH.

It is obvious from the changes observed in the absorption spectra that some chemical processes are occuring for the dye already in acidic conditions, below pH 5. By considering the structure of FAM345 and the changes in the spectra, it is quite evident that the pH increase from 2 to 6 causes the deprotonation of one of the hydroxyl groups, namely the one in 4′ position (see Scheme 2, conversion $H_4R^{2+} \rightarrow H_3R^{+}$). It follows that such an unusually low pK value for an hydroxyl pertaining to a phenolic compound is possible only because this phenolic group is coupled and interacts with the adjacent dimethylamino groups in this part of the molecule [24].

Other important changes in the absorption spectra occurs around pH 9 and can be associated with the dissociation of other proton-containing groups that are present in the molecule. The observed erosion in the absorption spectra of the isosbestic point at 410 nm confirms that several chemical processes are taking place in this pH range. Taking into account the chemical structure of FAM345, it is likely that a stepwise dissociation of the 3-OH-group (phenol-like) and the two tertiary ammonium groups occurs. According to their known properties they dissociate approx-

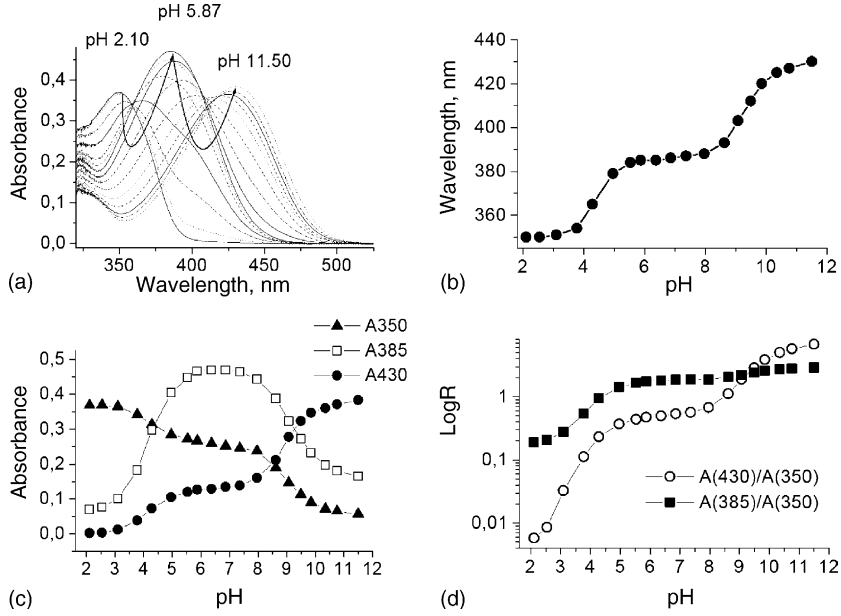


Fig. 1. (a) Absorption spectra of FAM345 in the range of pH from 2.5 to 11.8. (b) Position of the absorption maximum vs. pH. (c) Absorbance at 350, 385 and 430 nm vs. pH. (d) Absorbance ratios in the base 10 logarithm ($\log R$) at two pairs of wavelengths: A_{430}/A_{350} and A_{385}/A_{350} vs. pH.

imately in the same pH range, from 8 to 10. Thus, there are three dissociation steps taking place in this pH range. On our opinion, the dissociation of the 3-OH-group should mainly contribute to the absorption and emission spectra of the dye, while the dissociation of ammonium groups, which are uncoupled with the chromophore moiety, should contribute to a lesser extent. Thus, the whole prototropic equilibria for FAM345 can be represented by Scheme 2 (conversions $H_3R^+ \rightarrow R^{2-}$).

3.2. Fluorescence excitation spectra

The same trends as in absorption spectra can be identified in the fluorescence excitation spectra of FAM345 performed within the pH range from 2 to 12 (Fig. 2). The first acid–base reaction occurs at pH between 3 and 6, inducing a red-shift of the spectra, with band maxima going from 360 to 400 nm, and an isoemissive point at 370 nm. The second reaction occurs at pH between 8 and 12 and presents a more complex

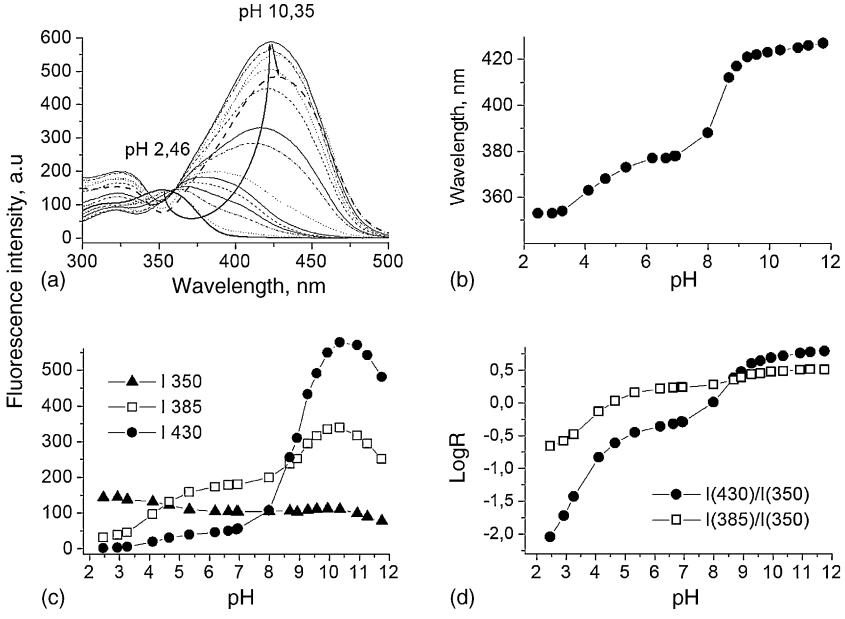


Fig. 2. (a) Excitation spectra of FAM35 in phosphate–citrate–borate buffer in the range of pH from 2.46 to 11.75. (b) Position of the excitation maximum vs. pH. (c) Fluorescence excitation intensity vs. pH at 350, 385 and 430 nm. (d) Fluorescence intensity ratios in the base 10 logarithm ($\log R$) at two pairs of excitation wavelengths: I_{430}/I_{350} and I_{385}/I_{350} vs. pH. Emission wavelength was 515 nm in all cases.

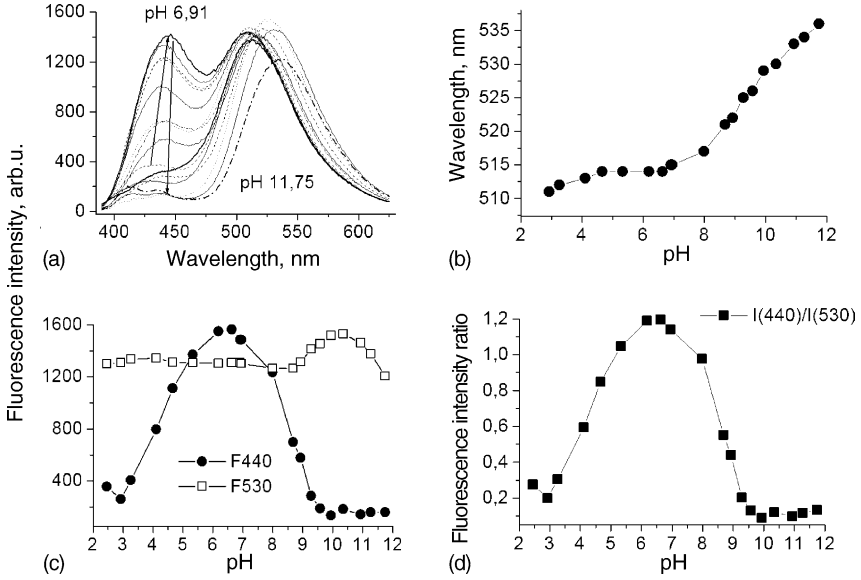


Fig. 3. (a) Emission spectra of FAM35 in the range of pH from 2.46 to 11.75. (b) Position of the long-wavelength maximum vs. pH. (c) Fluorescence emission intensities at 440 and 530 nm vs. pH. (d) Fluorescence intensity ratio I_{440}/I_{530} vs. pH. Excitation wavelength was 360 nm in all cases.

character, displaying a strong increase of fluorescence intensity together with a red-shift of the maximum. All observations are in agreement with the scheme of chemical conversions proposed above.

As for absorption spectra, the variation with pH of the intensity at different wavelengths of the excitation spectra allows to calculate the pK_a values of the dye and also to determine the pH value of the solution in the pH range from 3 to 10 (Fig. 2c, Table 1). Concentration-independent pH determination is also possible by considering the position of the maximum (Fig. 2b) or by measuring the fluorescence intensity ratio at two different wavelengths. As shown in Fig. 2d, the ratio I_{430}/I_{350} is changed by more than two orders of magnitude allowing a simple estimation of pH. In contrast with absorption data, the pH dependency obtained from the excitation spectra (Fig. 2d) allows a relatively precise pH determination even in the range near pH 7.

3.3. Fluorescence emission spectra

Due to the appearance of different emitting species when the pH changes, FAM345 shows a dual fluorescence in the whole range of tested pH (Fig. 3a). However, it is hard to estimate whether intramolecular or intermolecular proton transfer is involved in the observed dual fluorescence because of the rather complex structure of this molecule. Two pairs of basic and acidic groups could be considered for the occurrence of intramolecular proton transfer. Also the simultaneous existence of several differently protonated forms of the molecule could be considered in this case. Thus, a blue-shifted band that is emitting with a maximum at 440 nm and presents a continuous change in its intensity (see Fig. 3c for details) could be a result either of intramolecular or intermolecular reactions. However, since the intensity changes continuously when pH increases, there are no doubts that an intermolecular deprotonation of one or several groups occurs.

The intensity of the red-shifted emission band does not change substantially in the pH range from 2 to 9. This emission band cannot be assigned to one of the possible prototropic forms of FAM345 as it does not present any stable position of its maximum. The position of this maximum shifts from 509 to 527 nm when pH increases up to 9. At higher pH, this position of the emission maximum is further shifted to the red, up to 535 nm (Fig. 3b), clearly showing a further deprotonation of the molecule. Taking into account this phenomenon it can be concluded that pH measurements could be performed by considering the position of maximum in the pH range from 7 to 12.

It is also possible to measure pH just by recording fluorescence intensities of FAM345. Indeed, the present data show that a ratiometric measurement, by using the intensity ratio I_{440}/I_{530} (Fig. 3d), is probably the better way to measure the pH in the range between 3 and 9. However, in contrast with the data obtained from the absorption and fluorescence excitation spectra, the curves obtained from the emission spectra are symmetrical relatively to pH 7. This imposes to perform the pH measurements separately depending on whether we are in acidic or in basic conditions.

4. Conclusions

The synthesized fluorescent dye FAM345 displays spectral sensitivity to pH in the range from 2 to 12 in absorption spectra as well as in fluorescence excitation or emission spectra. In all three cases, the estimation of pH is possible, either from the position of the maxima or from the absorbance or fluorescence intensity at one or several separated wavelengths,

or also from absorbance or fluorescence intensity ratios. This last feature allows determining pH value of the medium independently from the probe concentration, from its possible photobleaching, as well as from any variation of light source intensity (at least in fluorescence methods).

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