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Effect of salts on the excited state of pyranine as determined by steady-state fluorescence

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ABSTRACT

Pyranine is a pH-sensitive fluorescent probe useful in the pH range of 4.5–8, and it has been extensively employed to determine pH inside cells, membranes and membrane models. The fluorescent properties of pyranine are a consequence of the excited states ROH* and RO^{-*}. The prototropic equilibrium of these excited species has a pK_a^* much lower than that of the ground state. In this paper we determined the pK_a^* (1.42 ± 0.06) and the relative quantum yield of pyranine in the pH range of 1–8 by analyzing the component peaks of the steady-state of the dye's emission spectrum. As pyranine is very sensitive to the medium we studied the influence of salts formed by mono-, di-, and trivalent ions on the apparent pK_a^*. In all cases, the presence of salts reduced the apparent pK_a^* to varying degrees depending on the valence of the cations. The strategy used to obtain this information was a dual emission ratiometric method at 441 and 511 nm after excitation at 350 nm. The results obtained demonstrate that pyranine is suitable to determine the pH of aqueous solutions in the range of 1–3.5.

1. Introduction

Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid, trisodium salt) (HPTS) is a water-soluble fluorescent molecule with three sulfonate groups and an 8-hydroxyl group. At a pH higher than 1, the three sulfonate groups are completely ionized (p$K_a \approx 0$–1), whereas in the ground state the 8-hydroxyl group is ionized at alkaline pH (pK_a = 7.2–7.3). Consequently, over a wide pH range and depending on the degree of ionization of the OH group, this molecule can exist as a trivalent or tetravalent anion.

For over 50 years, and due to its fluorescent properties, HPTS has been extensively used as a probe with a large number of scientific, medical and commercial applications. For instance, by means of steady-state measurements, HPTS has been employed to study the process of carbon monoxide binding to hemoglobin [1], as well as in sensors of pH [2–4], carbon dioxide [5] and ammonia [6]. Time-resolved emission has also been widely used with HPTS to study proton transfer reactions [7–9]. As a pH-sensitive fluorescence probe, HPTS has proved especially useful to determine the pH of liposome interiors and surfaces [10,11]. Due to its charge, HPTS shows membrane impermeability over a broad pH range. Consequently, this dye is a sensitive probe for monitoring the pH inside negatively charged vesicles and at the outer surface of positively charged vesicles.

These uses of HPTS derive from the absorption spectra of its protonated ROH and deprotonated RO$^-$ forms, and from the fluorescent properties of their corresponding excited states, ROH* and RO^{-*}. The absorption maximum of ROH is located at 403 nm, while that corresponding to RO$^-$ is at 454 nm. The fluorescence maximum of the RO^{-*} form, arising from the lowest excited state of the conjugated base, is at 510–515 nm, whereas the emission maximum of the lowest ROH* excited state is at 440–445 nm. The prototropic equilibrium of the electronically excited states has a pK_a^* much lower than that of the ground state and, consequently, the OH group of the excited HPTS is much more acidic than that of the ground state. Accordingly, the most common technique for pH determination is the dual wavelength excitation method [12]. This is based on the ratio of fluorescent intensity at a single emission wavelength, 510 nm, measured at two excitation wavelengths, usually 403 and 454 nm, and it is useful when the pH ranges, approximately, from 4.5 to 8.5.

HPTS is able to interact with different species and, as a consequence, its fluorescent properties may change. For example, the interaction of the dye with octadecylammonium, a positively charged molecule, has been described by Ray and Nakahara [13]. This process implies an important change in the emission spectrum of HPTS. In addition, Barrash-Shiftan et al. [14] have shown that not only the maximum of the ROH* fluorescence but also the UV–visible absorption spectra are solvent-dependent. Thus,

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the solvatochromic properties of pyranine can be used to measure the polarity of solvents and to probe microenvironments of organized host media. As indicated above, HPTS is very sensitive to the medium and, therefore, a non-realistic value of pH may be obtained as a result of changes in HPTS pK_a produced by any substance present in the medium. The main substances able to produce shifts in pK_a are salts, and in this regard Avnir and Barenholz [15] have systematically studied the effects of some electrolytes on the pK_a of HPTS ground state forms. Owing to its solubility and relative stability in acidic conditions it has been suggested that HPTS can also be used in acidic conditions. For example, Barrash-Shiftan et al. [14] have demonstrated the suitability of HPTS as a solvent probe of strong acidic solvent environments.

In this paper, we explored by means of steady-state fluorescence spectroscopy the possibility of using HPTS as a fluorescent pH probe in very acidic conditions (pH range 1–3.5). As salt composition and concentration affect the curve describing HPTS fluorescence as a function of pH range, we here tested the influence of several salts formed by mono-, di-, and trivalent ions on the apparent pK_a^*. As noted above the strategy used to obtain information on the fluorescent properties of HPTS at other pH intervals is based on the dual excitation ratiometric method. Here we used a different strategy: the sample was excited at a single wavelength, 350 nm, and the fluorescent intensities were recorded at two emission wavelengths: 441 nm, corresponding to the protonated form, and 511 nm, corresponding to the unprotonated form of excited HPTS. In addition, the pK_a^* and the relative quantum yield of HPTS were obtained by analyzing the component bands of the dye's steady-state spectrum.

2. Materials and methods

2.1. Materials

HPTS was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. Solutions were made in Ultrapure water (Milli Q® reverse osmosis system, 18.3 MΩ cm resistivity).

2.2. Methods

pH was measured at room temperature using a GLP22 pH-meter (Crison, Barcelona, EU) and adjusted by adding the appropriate volumes of HCl and NaOH solutions to the samples.

Fluorescence measurements were performed in a SLM Aminco Series 2 spectrofluorometer. After excitation at 350 nm, fluorescence emission intensities were recorded at 441, 487 and 511 nm using a bandpasss of 2 nm for excitation and emission. Emission spectra were acquired with a resolution of 1 nm. Changes in the fluorescence ratio at two wavelengths (511/441 nm or 487/411 nm) were adjusted to the measured bulk pH using the equation adapted from Avnir and Barenholz [15]:

$$\mathrm{pH} = \mathrm{p}K_a^{'} + \log\left(\frac{R - R_a}{R_b - R}\right) \quad (1)$$

where R is the 511/441 (or 487/441) fluorescence ratio, p$K_a^{'}$ is the apparent pK_a^* of HPTS, and R_a and R_b are the fluorescence intensity ratios of the protonated and unprotonated excited forms of the probe, respectively. The same equation was used in order to adjust the quantum efficiency to the bulk pH.

Component peaks of the fluorescent spectra were fitted to the original bands by iteration using Grams 3.2 (Galactic Inc.). The minimum number of component peaks necessary to obtain a good fit was estimated from the difference between the original and the fitted spectra. The curve fit was accepted when this difference was equal to the instrumental noise and the mathematical process converged to one solution.

3. Results and discussion

Inset of Fig. 1 shows fluorescence emission spectra of 6 μM HPTS in a pH range of 1–11 in 155 mM NaCl. After excitation at 350 nm, two major bands, both pH-dependent, appear at 441 ± 1 and 511 ± 1 nm corresponding, respectively, to the ROH^* and RO^{-*} forms of the dye. The first band increased when pH was reduced, whereas the band at 511 nm underwent a more complex effect: it increased from pH 1 to, approximately, pH 4.5, and was then reduced when pH became higher. No shift was observed in any band due to a pH effect. An isosbestic point appeared at 487 nm in a pH range from 1 to 4.5. At an acidic pH far from the pK_a the main molecule in the ground state is ROH. This fact and the excitation wavelength used (350 nm, where the absorption of RO^- is about 15% of the value for ROH [7]) assures that almost the only form that absorbs the excitation radiation is ROH. Consequently, the increase of the 441-band in an acidic medium and the concomitant decrease of the 511-band reflects the rise in ROH^* concentration. The decreased fluorescence when the bulk pH approaches the pK_a is due to the presence of the RO^- form, as it is not as efficiently excited as the ROH form. The changes in the spectra can be represented by means of the ratio between several fluorescence intensities. The fluorescence intensity ratio at 511 and 441 nm was higher than that observed with the ratio 487/441, although the slopes of both curves were similar.

All the fluorescent spectra obtained in water at different pH could be fitted to a minimum number of component peaks, which showed a non-pH dependent position for their maxima. The inset of Fig. 2 shows the result for a 6 μM HPTS solution at pH 1. The ROH^* band was efficiently fitted with two peaks at 433 ± 2 and 453 ± 4 nm and the RO^{-*} band also comprised at least two peaks centered at 506 ± 1 and 531 ± 1 nm. Although in our case the fitting

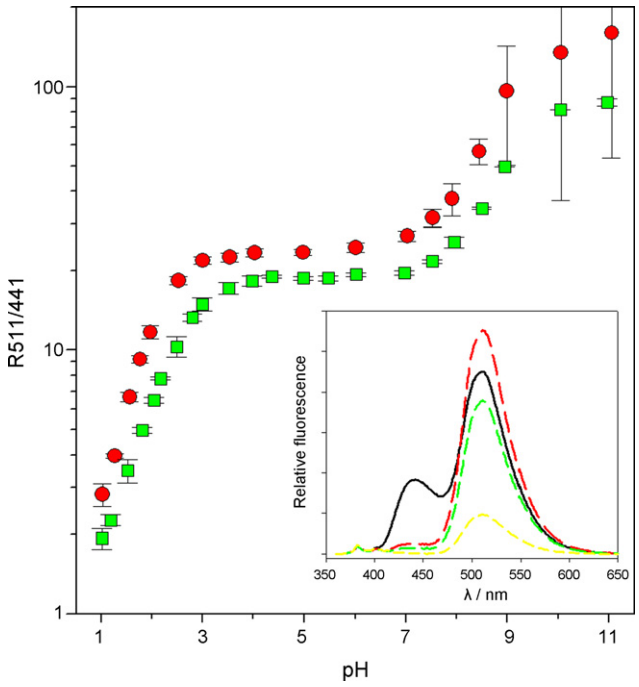

Fig. 1. Plot of the ratio of fluorescence intensities at 511 and 441 nm of HPTS 6 μM at two NaCl concentrations (squares: 0.018 mM; circles: 155 mM) as a function of pH (from 1 to 11). *Inset*: Fluorescence spectra of 6 μM HPTS in 155 mM NaCl at pH 1, 3, 7 and 11 (from continuous line to short dashed line).

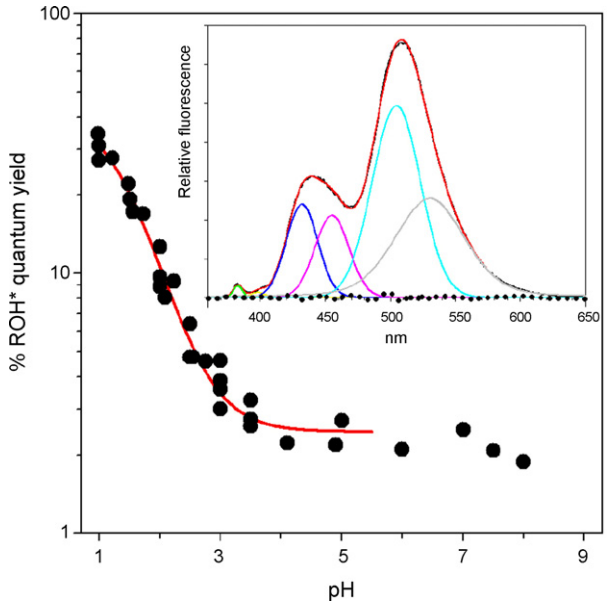

Fig. 2. Quantum yield of ROH^* band of HPTS at different pH calculated from the areas of the component peaks of the dye spectra. *Inset*: Component peaks of HPTS at pH 1. Black curve: original spectrum; red curve: adjusted spectrum; dotted line: residual; others: component peaks.

shows several Gaussians, the experimental spectra can be reproduced efficiently using other strategies. In a recent publication, Spry et al. [16] have shown that the fluorescent spectrum HPTS can be described by a Brownian oscillator model, in which the vibronic structure of HPTS is explained by the interaction of the solvent with the sulfonate groups.

In our results, the areas of the ROH^* and RO^{-*} component peaks showed a strong pH-dependence, in agreement with the changes in the fluorescent spectra. Using the steady-state fluorescence spectrum, Pines et al. [7] obtained the relative fluorescence yield of ROH^* at pH 5.5–6 from ratio between the area of ROH^* band and the total area. Quantifying the area under the two bands of the excited states they found that the ROH^* band occupied $4.5 \pm 1\%$ of the total area. When they employed transient experiments, the quantum yield calculated decreased to $3.5 \pm 0.7\%$. In our case the relative quantum yield of ROH^* was obtained from the relative area of the component peaks. Fig. 2 shows the results in a pH range from 1 to 8. As can be observed, the relative quantum yield has a constant value of 2.5 ± 0.4 in the pH range 3.5–7. This result is close to that calculated by means of transient experiments.

Fig. 1 plots the ratio of fluorescence intensities emitted at 511 and 441 nm as a function of the pH of an aqueous solution of 6 μM HPTS in the presence of two NaCl concentrations (0.018 and 155 mM). It should be borne in mind that an aqueous solution of HPTS corresponds to a 0.018 mM NaCl concentration, since the fluorescent probe is a trisodium salt. In this figure we can observe the high precision of the measurements carried out in the pH interval from 1 to 8, although other authors found that ROH^* fluorescence at pH 6.2 was practically unobservable [13]. When NaCl was present in the solution some changes in the fluorescence ratio were observed, thus indicating that the equilibrium between the excited fluorescent species has been modified.

It is known that the overlapping of bands in fluorescence titrations must, when produced, be corrected for the intensity component due to the other form. Usually, the so-called overlap ratios are calculated by obtaining the fluorescent spectra from solutions where only the protonated or deprotonated forms are present. Such samples are prepared by adding strong bases or acids to the dye solution. Once the correction has been made using the overlap ratios, and provided the fluorescent lifetimes of ROH^* and RO^{-*} are known (τ_o and τ_o', respectively), the pK_a^* can be calculated from the pH corresponding to the inflection point of the corrected titration curves according to the following equation [17]:

$$\mathrm{pH} = \mathrm{p}K_a^* - \log \frac{\tau_0}{\tau_o'} \quad (2)$$

Although the changes in HPTS fluorescence shown in Fig. 1 provide information about the protolytic behavior of the dye in the presence of the sodium cation, the broad bands of the spectra produce an inaccuracy in the pK_a^* if it is calculated from the inflection point of the corresponding titration curves. We performed the required correction by quantifying the component peaks of the HPTS spectra. This is shown in Fig. 2, where the relative quantum yield in a pH range from 1 to 5 was adjusted to Eq. (1).

It must be remarked that the lifetime of photoacids, specially the protonated state, is highly dependent on pH. It is standard practice using the same lifetime value for both states, $\tau_o \approx \tau_o' \approx 5.2$–6 ns [7,9,18]. Concerning to the possibility of quenching of both excited HPTS species by oxygen, it is known that is only a notorious quencher of fluorophores with decay times in excess of 20 ns [19]. Then, according to Eq. (2), the inflection point of the previous curve can be considered as the pK_a^*. The calculated value is 1.42 ± 0.06, in good agreement with the literature, where pK_a^* of 1.41 ± 0.07 and 1.38 ± 0.05 are reported [7,17]. Furthermore, the correction performed by analyzing the component peaks of the spectra circumvents the need to prepare the samples by adding strong alkalis or acids. In the case of Na^+, and as will be shown later for other substances, the presence of cations modifies the equilibrium between the excited forms of HPTS. Consequently, the overlap ratios obtained by this method may be affected by the presence of these ions.

Fig. 3 shows more accurately the effects of NaCl at low pH. At pH 1 these changes were minimal, but an increase in pH led to greater variation in the fluorescence ratio. From the values of emitted fluorescent intensities and by applying Eq. (1), it is possible to determine pK_a', R_a and R_b (Table 1). Thus, for HPTS in 0.018 mM NaCl,

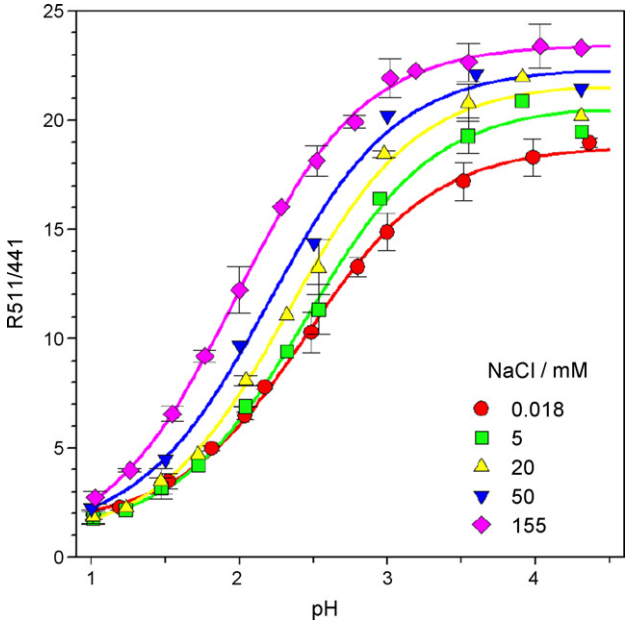

Fig. 3. Plot of the ratio of fluorescence intensities (511/441 nm) of HPTS 6 μM at several NaCl concentrations (0.018–155 mM) as a function of pH (from 1 to 4.3). The curved fitted to the experimental points follows Eq. (1).

Table 1
Values (average ± standard deviation, $n \geq 3$) of pK_a' (apparent pK_a^*) and fluorescent intensity ratio of protonated HPTS (R_a) and unprotonated HPTS (R_b) at different NaCl concentrations

NaCl (mM)	R_a	R_b	pK_a'	r^2
0.018	1.60 ± 0.28	19.0 ± 0.67	2.48 ± 0.03	0.9967
1	1.37 ± 0.26	20.1 ± 0.99	2.49 ± 0.03	0.9958
5	1.28 ± 0.29	20.9 ± 1.20	2.47 ± 0.04	0.9973
20	0.96 ± 0.33	21.9 ± 1.41	2.34 ± 0.06	0.9967
50	0.99 ± 0.08	22.4 ± 0.55	2.20 ± 0.08	0.9941
100	0.55 ± 0.33	23.7 ± 1.09	2.02 ± 0.05	0.9988
155	0.42 ± 0.43	23.5 ± 0.93	1.98 ± 0.04	0.9985
300	1.05 ± 0.30	23.1 ± 0.10	1.82 ± 0.02	0.9996

that is, in water, the following values were obtained (mean of five replicas): $pK_a' = 2.48 \pm 0.03$, $R_a = 1.60 \pm 0.28$ and $R_b = 19.0 \pm 0.67$. The presence of NaCl in the medium modified the values of pK_a', R_a and R_b. Values of pK_a' showed a significant reduction, making the solution pH as determined by HPTS more basic. The higher values of the fluorescence ratio obtained at any pH from 1 to 4.3 show that, at NaCl concentrations up to 300 mM, the relative amount of the RO^{-*} form is increased. From the pK_a' values the following mathematical equation, which relates the reduction of pK_a' with the increase in NaCl concentration, was obtained:

$$pK_a' = 1.91 + 0.555\, e^{-0.015[NaCl]} \quad (3)$$

In order to establish whether the effect of NaCl was dependent on probe concentration, the fluorescence ratio at pH 3 and in the presence of NaCl was determined at three HPTS concentrations (0.6, 6.0 and 12 μM). No variation independent of experimental error was found for these three concentrations (data not shown).

Some authors have studied the proton transfer from pyranine to water containing electrolyte concentrations in the molar range. It is accepted that at salt concentrations below 0.2 M the strong Coulomb attraction between RO^{-*} and the proton, generated by the dye's four negative charges, is partially screened [9], and that at concentrations higher than 1 M, the coulombic potential is almost completely screened. Leiderman et al. [9], working with NaCl and MgCl solutions, found that at salt concentrations higher that 0.5 M the intensity of the ROH^* band increases in line with an increasing salt concentration. This trend is opposite to that shown in Fig. 3 with NaCl concentrations between 0 and 0.3 M. The results of Fig. 4 were obtained in order to provide a more detailed description of the dependence of HPTS steady-state fluorescence on NaCl concentration. In a pH range from 1 to 7.5 the screening effect is detected at a salt concentration of about 50 mM. A subsequent increase of the salt concentration produces a progressive decrease of the attraction between RO^{-*} and H^+ and raises the relative amount and the intensity band of the deprotonated and excited HPTS. In contrast, the total electrostatic screening of the dye, detected as a breakdown in the trend of the RO^{-*} to ROH^* intensity ratio, depends upon the bulk pH. At pH 1 and 2.5, and for NaCl concentrations higher than 0.6 and 1.5 M, respectively, the relative intensity of the ROH band increases as the salt concentration increases. These results are similar to those described in the literature for high electrolyte concentrations [9,20], which are a consequence of the reduction of the number of water molecules solvating HPTS (for a review see Agmon [21]). In the case of pH 7.5 this point is reached at a NaCl concentration of 3 M.

Once the influence of NaCl on HPTS was established, the effect of other ions was studied according to their charge and valence. Monovalent cations with the same anion (Li^+, Na^+ and K^+, at 100 mM as chlorides) produced the same change in the fluorescent properties of HPTS, as can be deduced from Table 2. Moreover, the shape of the HPTS bands remained unaltered in all cations tested. Different anions with the same cation (NaCl, 100 mM; Na_2SO_4, 50 mM; Na_3PO_4, 33.3 mM) showed the same effect, giving also identical parameters of Eq. (1). It should be noted that during acidification, SO_4^{2-} and PO_4^{3-} undergo protonation. Thus, not only the chemical nature of the assayed anions, but also their different charges failed to have an effect on the fluorescence of HPTS. In contrast, Avnir and Barenholz [15] found that HPTS fluorescence is affected by the nature of the anions when it is measured by the dual excitation method at pH 7. However, important variations were observed when the valence of the cation changed. Fig. 5 shows the effect of a 100 mM cation concentration (such as 100 mM NaCl, 100 mM $CaCl_2$ or 100 mM $AlCl_3$) on the pK_a'. In the case of $AlCl_3$ solutions the maximum pH assayed was 3.5, since a higher value led to the precipitation of hydroxides of the cation. Fluorescent titration of HPTS with 100 mM $FeCl_3$ was also assayed but no results were obtained due to the high absorption of the sample and/or the quenching of the dye.

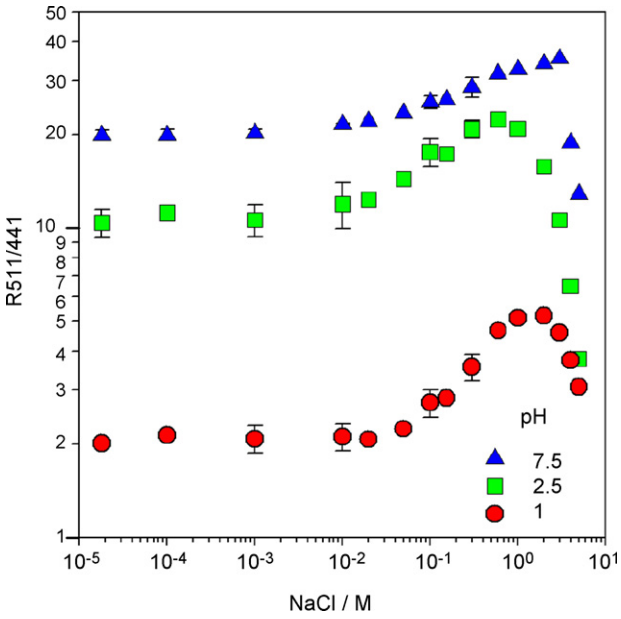

Fig. 4. Plot of the ratio of fluorescence intensities (511/441 nm) of HPTS 6 μM at three pH (1, 2.5 and 7.5) as a function of NaCl concentration (from 0.018 mM to 5 M).

In comparison with an aqueous HPTS solution, the presence of cations produced an increase of the unprotonated species, this increase being most evident at higher valence values. However, above a pH of 3 the titration curves corresponding to Ca^{2+} and Al^{3+} were coincident. As regards the pK_a', a reduction of this parameter was observed in line with increasing valence (Table 2). The values in this table clearly show that at this concentration, when valence was increased by one unit, the pK_a' was reduced by approximately

Table 2
Values (average ± standard deviation, $n \geq 3$) of pK_a' (apparent pK_a^*) and fluorescent intensity ratio of protonated HPTS (R_a) and unprotonated HPTS (R_b) at different cation and anion concentrations

Salt	R_a	R_b	pK_a'	r^2
LiCl 100 mM	0.69 ± 0.01	24.1 ± 0.1	2.04 ± 0.01	0.9997
NaCl 100 mM	0.55 ± 0.33	23.7 ± 1.1	2.02 ± 0.05	0.9988
KCl 100 mM	0.43 ± 0.19	25.5 ± 0.1	2.02 ± 0.02	0.9997
$MgCl_2$ 100 mM	0.28 ± 0.06	23.2 ± 0.1	1.78 ± 0.07	0.9995
$CaCl_2$ 100 mM	0.44 ± 0.42	25.1 ± 0.9	1.81 ± 0.04	0.9993
$FeCl_2$ 100 mM	0.0	19.1 ± 0.3	1.67 ± 0.01	0.9993
$AlCl_3$ 100 mM	0.0	24.8 ± 0.8	1.61 ± 0.10	0.9982
Na_2SO_4 50 mM	0.50 ± 0.29	25.2 ± 0.5	2.05 ± 0.04	0.9995
Na_3PO_4 33.3 mM	0.50 ± 0.25	25.9 ± 1.0	2.03 ± 0.01	0.9997

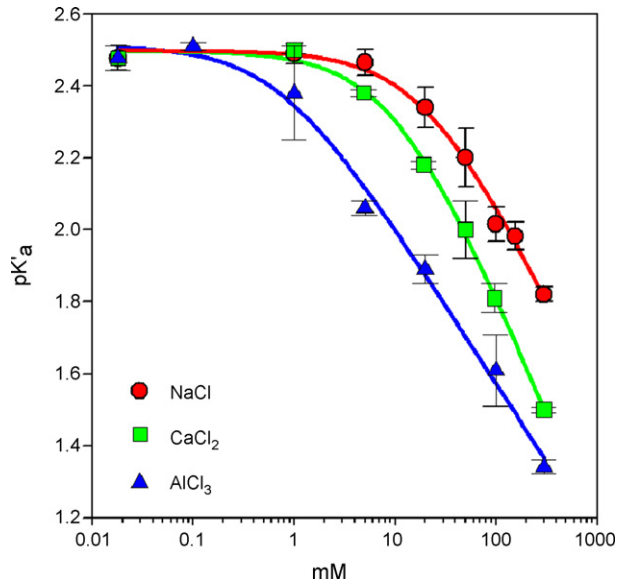

Fig. 5. Variation of $pK_a^{'}$ of a 6 μM HPTS solution as a function of NaCl, $CaCl_2$ and $AlCl_3$ concentration.

0.21 units. Table 3 shows the relative quantum yield of ROH^* in 100 mM solutions of NaCl, $CaCl_2$ and $AlCl_3$ at different pH. As can be observed, in a pH interval from 1 to 3, the reduction in ROH^* fluorescence depends on the cation charge. At a pH equal to or higher than 3.5 the decrease in yield caused by the three cations is the same. These results are partially coincident with that shown by Avnir and Barenholz [15] studying fluorescence changes in 460-nm excitation at pH 7. These authors suggest that pK_a changes of (ground state) HPTS depend on both the level cation hydration, given by Hofmeister series, and on their ability to form ion pairs with sulfonate groups. We found that the order in which the studied cations modify the $pK_a^{'}$ is $Al^{3+} > Fe^{2+} > Mg^{2+}$, $Ca^{2+} > Li^+$, Na^+, K^+. This order shows, first, a dependence of the $pK_a^{'}$ of the excited species on the charge of the cation, and, second, that there is no difference for cations of the same group despite their different hydration degree. Our results can be interpreted on the basis of the diffusion model of Agmon [21] for the dissociation and formation of a solvated proton in photoacids. The model includes the Debye–Hückel approximation for the screened potential caused by monovalent salts of strong electrolytes on the long-range potential of HPTS. In this model, and concerning to the cations, only their charge and concentration modulates the attraction between the proton and the dye and, consequently, the protonation of the excited HPTS. The observed effect of cations at low ionic strength is, indeed, produced by the screening, but this effect could be enhanced by the existence of a high negative molecular charge, which brings about an intense attraction between the pyranine and the counter ions present in the bulk. In the same conditions, other photoacids bearing less charge would show a fewer increase of deprotonation with cation concentration and would display, principally, the effect of water restriction when salt concentration is increased at molar level.

Table 3
Relative quantum yield of ROH^* band of HPTS in the presence of electrolytes (NaCl, $CaCl_2$ and $AlCl_3$) at a concentration of 100 mM and as a function of pH

pH	Relative quantum yield of ROH^* (%)		
	NaCl	$CaCl_2$	$AlCl_3$
1	21.3 ± 1.2	17.1 ± 0.9	11.1 ± 2.1
2	3.80 ± 0.01	2.97 ± 0.12	2.23 ± 0.26
3	1.83 ± 0.27	1.55 ± 0.18	1.43 ± 0.19
3.5	–	–	1.61 ± 0.10
4	1.47 ± 0.06	1.46 ± 0.13	–

Results: mean ± S.D., $n \geq 3$.

Regarding to the effect of Fe^{2+}, our results show that it is more intense than that of the other divalent cations studied. Some authors have proposed the existence of specific interactions between cations and sulfonated groups. Sulfonated lipids [22] and tensioactives [23] exhibit different tendency to form ion pairs with cations which does not depend only on their charge. On the other hand, it have been described the ability of the cation-exchange resin sodium polystyrene sulfonate to bind iron from ferrous sulfate solutions [24]. The facts indicated above, and the different behavior of Fe^{2+} in relation with the studied cations of the group IIA, mainly evidenced by and increased change of the $pK_a^{'}$, suggest a specific interaction between the sulfonate groups of HPTS and Fe^{2+}.

These results are concerned only for simple ions, and a different behavior could be expected in the case of larger ionic species, such as ionic surfactants, since it is known that micellization affects the spectral properties of fluorescent dyes [25], for instance, the anisotropy decay of HPTS to micelles is found to be much slower compared with that in bulk water [26].

4. Conclusions

Using the ratiometric determination between two emission wavelengths after excitation at a single wavelength, HPTS has been shown to be a suitable fluorescent probe to measure pH below 3.5. This ratio was enhanced in a concentration-dependent manner by salts from the medium. This effect produced a decrease in the HPTS $pK_a^{'}$ and thus led to the calculation of a more basic pH than the actual one. Hence, the exact measurement of pH must be carried out by using the appropriate calibration curves.

Due to the effect of salts on HPTS fluorescence, ratiometric determination can also be used to obtain the concentration of monovalent, divalent and trivalent cations.

For the same anion, monovalent cations have the same quantitative effect. However, the degree of change is different in divalent cations: their effect is higher than in monovalent ones, but if one compares the $pK_a^{'}$ values obtained with divalent cations, differences among cations of the IIA group and Fe^{2+} can be observed. The only trivalent ion tested, Al^{3+}, afforded the maximal reduction of $pK_a^{'}$. For the same cation, phosphate and sulfate groups have a similar effect to that obtained with chlorides of monovalent cations.

By analyzing the component peaks of the steady-state spectrum of HPTS it is possible to obtain the relative quantum yield of the excited species and the pK_a^* by fluorimetric titration. This methodology is an alternative to the classical methods based on the use of correction factors.

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