pH Sensors

An Efficient Fluorescent Probe for Ratiometric pH Measurements in Aqueous Solutions

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The development of new powerful microscopy approaches,[1–4] as well as the availability of various synthetic and natural fluorescent molecules,[5–7] has made fluorescence microscopy an essential tool for cellular biology. Fluorescent labels that specifically target molecules or organelles are currently used to investigate the structures and functions of cells. In particular, fluorescent probes have been developed to locally measure the concentrations of important metabolites, such as ions that are involved in signaling and energy transduction. Despite their significance in many essential biological processes, protons have not been studied as much as other ions (e.g. Ca^{2+}) by fluorescence microscopy;[8] a possible explanation for this is the absence of entirely satisfactory fluorescent indicators to measure pH in the relevant range (4.5–7.4).[9] In fact, pH does not significantly alter the emission spectra of most current fluorescent indicators, for instance, fluorescein

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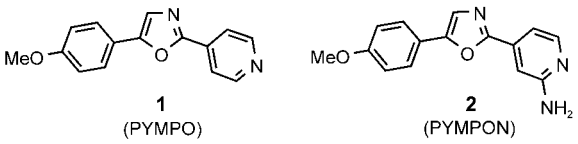
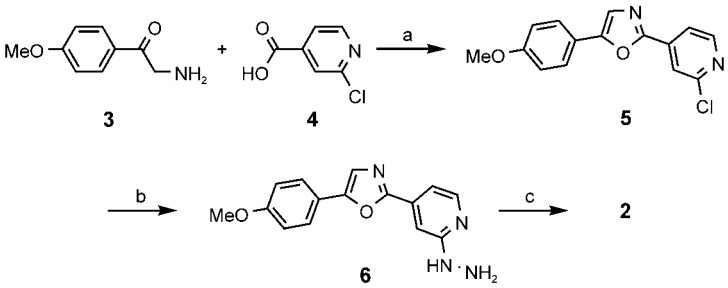
and pyranin, other than to modify the intensity of their fluorescence emission. To measure pH by fluorescence spectroscopic techniques is a delicate process and requires preliminary calibrations. Herein, we report a new fluorescent indicator designed to measure pH in the range of 5–7 in aqueous solution by means of a ratiometric method that relies on fluorescence emission after one- and two-photon excitation.

To measure pH by a ratiometric fluorometric method, both the acidic and basic states of the indicator should ideally be fluorescent and exhibit shifted absorption and emission spectra. In principle, this is the case when either the electron-donor or the -acceptor moiety of a donor–acceptor fluorophore exhibits acid or base properties. However, in practice, numerous pathways compete with fluorescence emission for the relaxation of the excited state in donor–acceptor chromophores; for example, basic donor amino groups can act as fluorescence quenchers.[6,7] An important constraint is thus to have similar quantum yields of fluorescence for the acidic and basic states of the pH indicator. Furthermore, the pK_a values of the ground and excited states are prone to be significantly different in many donor–acceptor molecules owing to the redistribution of the electron density about the molecule upon light absorption. This feature may complicate the analysis.[10–12]

A 2-(4-pyridyl)-5-aryloxazole backbone was chosen to engineer the donor–acceptor fluorescent pH probe. A series of oxazole derivatives was originally investigated to develop laser dyes[13] owing to the favorable photophysical features exhibited after one-photon excitation: $\varepsilon \approx 3\times10^4\,\text{M}^{-1}\text{cm}^{-1}$ at $\lambda \approx 350$ nm and strong fluorescence emission ($\Phi_F \approx 1$) at $\lambda \approx 500$ nm. 2-(4-pyridyl)-5-(4-methoxyphenyl)oxazole (PYMPO, **1**) has already been reported to be an attractive fluorescent probe to measure pH,[8,14] but its use is restricted to the most acidic cellular compartments as it has a low pK_a value (p$K_a \approx 4$). A related boronic acid probe with a larger pK_a value (≈ 8)[15] was sensitive to interference from solutes other than protons.[16] Our design relied on the introduction of substituents on the pyridine ring of **1** to induce a shift in its pK_a value without altering too strongly its favorable photophysical features or its solubility in water. The 2-aminopyridyl derivative **2** (PYMPON) was hence prepared—the pK_a value of 2-aminopyridine is 6.9 at 20 °C whereas that of pyridine is 5.3 at the same temperature.[17]



PYMPON **2** was obtained in three steps (Scheme 1): The oxazole **5** was prepared in 88% yield by the condensation of 4-methoxyphenacylamine (**3**) with 2-chloroisonicotinic acid (**4**) and was subsequently converted into the hydrazine **6** in 41% yield. Reduction of the hydrazine moiety in the latter compound yielded the aniline derivative **2** (PYMPON) in 51% yield. Figure 1 displays the absorption and emission



Scheme 1. Pathway for synthesis of the target compound PYMPON **2**. a) $POCl_3$, Δ, 8 h; b) NH_2NH_2, H_2O, Δ, 16 h; c) Raney Ni, EtOH, H_2 (1 bar), room temperature.

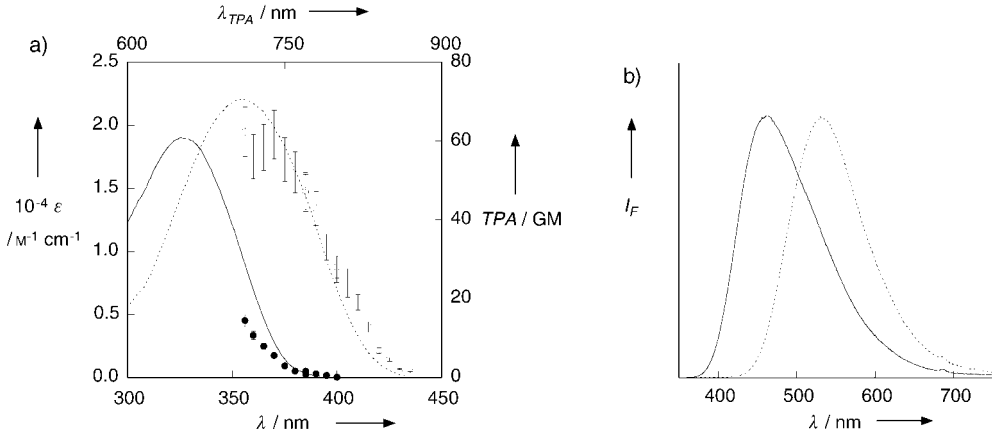


Figure 1. Photophysical properties of PYMPON **2** (in Britton–Robinson buffer)[20] at different pH values at 298 K; a) single-photon absorption (molar absorption coefficient ε in $\text{M}^{-1}\text{cm}^{-1}$; solid line = pH 8, dotted line = pH 3) and two-photon absorptivity (TPA, ● = pH 9, ○ = pH 2) spectra; b) normalized steady-state fluorescence emission I_F after one-photon excitation at $\lambda = 335$ nm (solid line = PYMPON **2**, dotted line = **2**H^+). 1 GM = 10^{-50} $\text{cm}^4\,\text{s}$ (photon molecule)$^{-1}$

spectra of **2**, as well as its excitation spectra after two-photon excitation at pH 3 and pH 8. PYMPON **2** exhibits a large one-photon absorption band ($\varepsilon \approx 2 \times 10^4\,\text{M}^{-1}\,\text{cm}^{-1}$) at $\lambda_{max} = 355$ nm (pH 3) and at $\lambda_{max} = 326$ nm (pH 8); (Figure 1a). The bathochromic shift observed upon decreasing the pH value is in line with the increase in the electron-withdrawing power of the pyridine ring upon protonation of the pyridyl nitrogen atom. PYMPON is highly fluorescent at 298 K (Figure 1b): At pH 3, the fluorescence emission maximum of the protonated PYMPON species $\mathbf{2H^+}$ is located at 530 nm with a quantum yield $\Phi_F = 0.7$. At pH 8, the emission maximum of **2** lies at 465 nm with $\Phi_F = 0.8$.

Figure 1a also displays the two-photon excitation spectra of PYMPON **2** recorded at pH 2 and 9 at 298 K. The power-squared dependence of two-photon-excited fluorescence was checked at several wavelengths in the investigated range and revealed satisfactory behavior over the 0–60 mW range. The two-photon absorption spectra compared well with the one-photon absorption spectra after division of the wavelength by a factor of 2: This suggests that the same excited states are reached regardless of the excitation mode. Such an observation is in agreement with other reports that make use of a comparable technique with unsymmetrical donor–acceptor compounds.[18,19] In the present case with **2**, the maximum two-photon absorptivity at 710 nm ($\delta_{max}(710)$) is 60 ± 10 GM at pH 2 and 15 ± 3 GM at pH 9 (1 GM $= 10^{-50}\,\text{cm}^4\,\text{s}\,(\text{photon-molecule})^{-1}$). These values compare well with the corresponding values for commonly employed pH probes (fluorescein at pH 11: $\delta_{max}(780) = 35$ GM, $\Phi_F = 0.9$; pyranin backbone: $\delta_{max}(750) = 4$ GM, $\Phi_F = 0.54$[19]).

The evolution of the fluorescence emission after one-photon excitation of an aqueous solution of PYMPON **2** (100 nM) as a function of pH is shown in Figure 2. The acid–base reaction depicted in Equation (1) yields $pK_a(\mathbf{2H^+}) = 5.7 \pm 0.1$ at 298 K; the same value was extracted from the pH-dependence of the one-photon absorption spectrum. The pK_a value confirms the earlier observation from the photophysical studies, that is, protonation of PYMPON **2** occurs at the nitrogen atom of the pyridine ring. The $pK_a(\mathbf{2H^+})$ value is shifted by $+1.7$ units relative to that of PYMPO ($pK_a(\mathbf{1H^+}) \approx 4$): this difference is similar to the corresponding shift in the pyridine series (pK_a(pyridine) $= 5.3$, pK_a(2-amino-pyridine) $= 6.9$[17]). Furthermore, one anticipates much lower pK_a values for acids that result from protonation of either the nitrogen atom of the oxazole ring ($pK_a \approx 0$) or the amino group on the pyridine ring ($pK_a < 0$).[17]

$$\text{PYMPON H}^+ \rightleftharpoons \text{PYMPON} + \text{H}^+ \qquad (1)$$

Figure 2b shows that PYMPON **2** can serve as a fluorescent probe to measure pH values in the range 3–8 by a ratiometric method by tuning the excitation and emission wavelengths [Eq. (3), see Experimental Section]. When one-photon excitation is performed at the isobestic point (339 nm), the ratio $\rho^{e,1}_{550/430}$ of the fluorescence emissions at 550 nm and at 430 nm varies by one order of magnitude in the pH 3.5–6.5 range. The same behavior is observed for $\rho^{e,2}_{550/430}$ after two-photon excitation at 712 nm between pH 4.5–7.5.

In conclusion, the present study suggests that the PYMPON platform **2** is appropriate to measure pH values in the range 3–8 by a ratiometric method that relies on fluorescence emission. This is especially attractive when two-photon excitation is used as it would allow local addressing on the femtoliter scale. Its similarity to the PYMPO backbone **1**, which was used to design an efficient probe,[8] suggests that derivatives of PYMPON **2** could find useful applications as intracellular pH meters.

Experimental Section

2: ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 8.19$ (d, $^3J = 5.4$ Hz, 1H), 7.65 (AA′XX′, $^3J = 8.8$ Hz, 2H), 7.36 (s, 1H), 7.29 (dd, $^3J = 5.4$ Hz, $^4J = 1.4$ Hz, 1H), 7.16 (m, 1H), 6.98 (AA′XX′, $^3J = 8.8$ Hz, 2H), 3.87 ppm (s, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 160.19$, 158.88, 158.61, 152.27, 148.90, 136.07, 126.02, 122.34, 120.34, 114.48, 110.55, 104.65, 55.40 ppm; HRMS: calcd for $C_{15}H_{14}N_3O_2$: 268.1086; found: 268.1084.

UV/Vis absorption and fluorescence spectra were recorded on a Kontron Uvikon-940 spectrophotometer and a Photon Technology International LPS 220 spectrofluorimeter, respectively. The two-photon excitation spectra were recorded with a homebuilt setup[18] by using the reported excitation spectrum of fluorescein for calibration.[19] All experiments were performed at 293 K in Britton–Robinson buffer solution (0.1 mol L^{-1}) prepared according to the literature.[20]

Theoretical expressions of the ratios of the absorbances and of the fluorescence emissions at two wavelengths with pH: Denoting AH for PYMPON H$^+$ and A for PYMPON, the ratio $\rho^{\alpha^i,i}_{\lambda_1,\lambda_2}$ of the absorbances of PYMPON solutions at two different wavelengths λ_1 and λ_2 after one ($i = 1$, $\alpha^i = \varepsilon$) or two ($i = 2$, $\alpha^i = \delta$) photon excitation can be written:

$$\rho^{\alpha^i,i}_{\lambda_1,\lambda_2} = \frac{\alpha^i_{AH}(\lambda_1)10^{-pH} + \alpha^i_{A}(\lambda_1)10^{-pK_a}}{\alpha^i_{AH}(\lambda_2)10^{-pH} + \alpha^i_{A}(\lambda_2)10^{-pK_a}} \qquad (2)$$

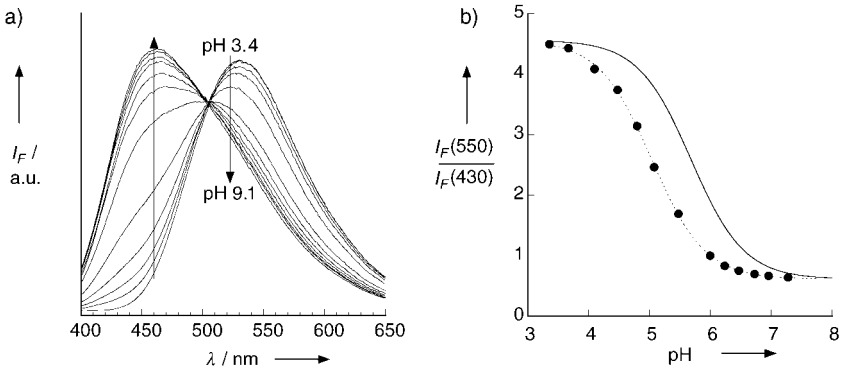


Figure 2. a) Dependence of the emission spectrum of PYMPON **2** (100 nM in Britton-Robinson buffer, 298 K) on pH: from acidic to basic conditions: pH 3.4, 4.5, 4.8, 5.1, 5.5, 6.0, 6.2, 6.5, 6.7, 7.0, 7.3, 9.1. b) Evolution of the ratio of the fluorescence emissions at 550 and 430 nm after one- ($\rho^{e,1}_{550/430}$, $\lambda_{exc} = 339$ nm; dotted line) and two- ($\rho^{e,2}_{550/430}$, $\lambda_{exc} = 712$ nm; solid line) photon excitations. Experimental data: ●; theoretical predictions: dotted and solid lines (calculated from Eq. (3) by using the parameter values measured during the present study).

At a given excitation wavelength λ_{exc}, the ratio $\rho^{e^l,i}_{\lambda_1,\lambda_2}$ of the fluorescence emissions of PYMPON solutions at two different wavelengths λ_1 and λ_2 can be written:

$$\rho^{e^l,i}_{\lambda_1,\lambda_2} = \frac{I_{AH*}(\lambda_1)\phi_{AH*}\alpha^i_{AH}(\lambda_{exc})10^{-pH} + I_{A*}(\lambda_1)\phi_{A*}\alpha^i_{A}(\lambda_{exc})10^{-pK_a}}{I_{AH*}(\lambda_2)\phi_{AH*}\alpha^i_{AH}(\lambda_{exc})10^{-pH} + I_{A*}(\lambda_2)\phi_{A*}\alpha^i_{A}(\lambda_{exc})10^{-pK_a}} \quad (3)$$

The terms ϕ and $I(\lambda)$ designate the quantum yield of fluorescence and the normalized emission spectrum of the considered species, respectively ($\int_0^\infty I(\lambda)d\lambda = 1$). The experimental curves were analyzed with Specfit software to extract the pK_a values.

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