Optimized pH-responsive cyanine fluorochromes for detection of acidic environments†

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Modulation of pH-responsive cyanine dye pK_a values *via* heteroatom substitution allows for design of fluorescent reporters that are tuned for potential imaging of biologically relevant acidic environments.

The emergence of near-infrared (NIR) fluorescence imaging techniques using fluorescent probes has spurred the need for the design of new environment-responsive dyes to visualize biological processes *in vivo*. For efficient *in vivo* imaging, dyes that absorb and emit in the far-red or NIR are necessary due to the increased optical transparency and lower tissue autofluorescence in this regime.[1] A variety of NIR probes for interrogation of enzyme activity,[2,3] nitric oxide,[4] zinc,[5] and proton concentrations[6–9] have been reported. Imaging local pH is a promising target for the development of new probes. Acidic environments are known to be associated with solid tumors,[10] cystic fibrosis,[11] asthma,[12] and a variety of renal conditions. For example, intratumoral pH was recently reported to range from 6.3 to 7.0,[10,13] and lung airway pHs as low as 5.2 have been recorded in asthmatics.[12] Acid responsive probes may also find application for cellular imaging of acidic intracellular vesicles, such as endosomes, lysosomes, and phagosomes, where pH can range from 4.5 to 6.5.[14] Existing far-red or NIR pH-responsive dyes are not optimized for *in vivo* imaging of acidic environments due to their poor optical properties, low aqueous solubility, or sub-optimal pK_a values, often greater than 7.0,[7,9] that are not matched for visualization of physiologically relevant acidic conditions. For example, a dye with a pK_a of 7.4 would be 50% activated at pH 7.4, this translates into high background fluroescence and a two-fold maximum signal increase as the pH of the local environment is lowered. There is therefore a need to design novel bright, water-soluble, pH-sensitive fluorochromes that are tuned for imaging acidic pH *in vivo*.

Cyanine dyes are a well known platform, which may be modified to confer pH responsive properties.[15] If one or both of the indole nitrogen atoms on a cyanine dye are not alkylated, the dye becomes sensitive to pH (Fig. 1). In acidic environments, protonation of the indole nitrogen atoms gives rise to long-wavelength absorption and strong fluorescence. Deprotonation of the nitrogen atoms results in a blue-shifted absorption band and little or no observable fluorescence emission. In this report, we detail the synthesis and characterization of new water-soluble pH-responsive fluorochromes that are optimized for visualization of biological acidic environments.

The traditional synthesis of cyanine dyes involves the base-catalyzed condensation of indolium salts with malonaldehyde dianilide or gluconaldehyde dianilide in pyridine.[16,17] Under these conditions, the nitrogen atoms of the indolenine derivatives, used in the synthesis of pH-sensitive cyanines, are deprotonated. This decreases the acidity of the indolenine 2-methyl hydrogens and deactivates them for reaction with malonaldehydes. We have found that dye condensation can be effected under mildly acidic conditions. The pH sensitive fluorochromes can be prepared without the need for any base catalyst using commercially available substituted malonaldehydes (Scheme 1). The reaction of two equivalents of 2,3,3-trimethylindolenine-5-sulfonic acid, which is readily prepared from 4-hydrazinobenzenesulfonic acid, with one equivalent of the desired malonaldehyde in methanol generates the crude pH sensitive dyes as dark blue solutions. The desired pH-responsive dyes can also be synthesized from the corresponding malonaldehyde dianilide, however in lower yields. Purification by reverse-phase C18 chromatography followed by cation exchange chromatography affords the pure fluorochromes as bright blue solids after lyophilization. Using this synthetic route, it is possible to prepare cyanine derivatives with different electron withdrawing substituents on the polymethine backbone of the dyes, which allows for modulation of the fluorophore pK_a.

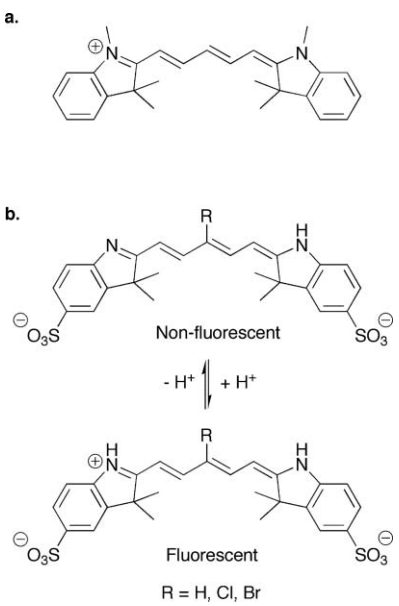

Fig. 1 The core structure of a pH insensitive pentamethine cyanine dye (a) and pH dependent equilibrium of the pH-responsive cyanine dyes (b).

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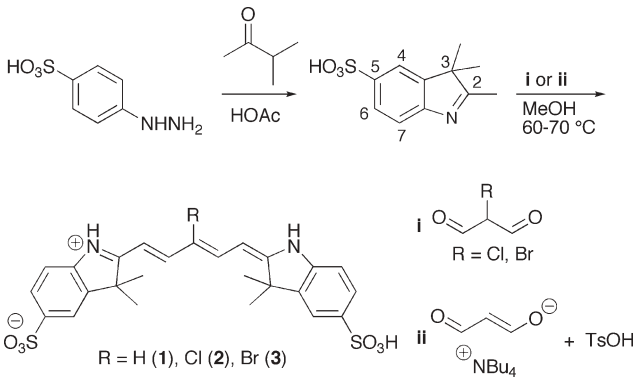

Scheme 1 Synthesis of the pH-responsive dyes.

Table 1 Optical properties of the pH-responsive fluorochromes in aqueous media

Dye	λ_{abs}[a]/nm	λ_{em}[a]/nm	ε[a]/M^{-1} cm^{-1}	Θ[a] (%)	pK_a[b]
1	638	664	141 000	12.9	6.3
2	642	663	167 000	6.4	5.7
3	639	659	146 000	3.5	5.7

[a] Measured in 50 mM glycine buffer pH 3.0. [b] Determined by pH titration in 50 mM phosphate–citric acid buffer.

The pH-responsive dyes prepared in this report (Table 1) are water-soluble and show strong long-wavelength fluorescence emission in acidic media (Fig. 2). The two sulfonate groups on the fluorochromes are also sufficient to prevent the formation of dye–dye aggregates up to concentrations of at least 5 μM (Fig. S7, ESI†), enabling their use in aqueous environments without the need of organic co-solvents. Typical fluorescence reflectance imaging (FRI) instruments only require concentrations in the nanomolar to low micromolar range for visualization of NIR fluorochromes. All three dyes have similar absorption and emission properties (Table 1). When protonated, the dyes show strong absorption near 640 nm with extinction coefficients greater than 140 000 M^{-1} cm^{-1}, and display strong fluorescence emission near 660 nm. The quantum yields Θ of the dyes are 12.9, 6.4 and 3.5% for **1**, **2** and **3**, respectively (Table 1). These values compare favorably with indocyanine green, a clinically approved non-pH sensitive NIR imaging agent, which has a quantum yield of 2.7%

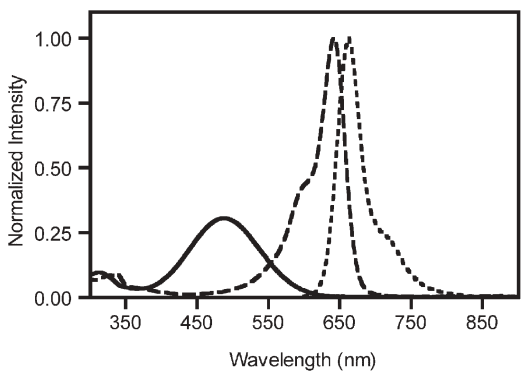

Fig. 2 Absorption spectrum of **2** in 50 mM phosphate–citric acid buffer at pH 9.0 (solid line). Absorption (dashed line) and emission (dotted line) spectra of **2** in 50 mM phosphate–citric acid buffer at pH 3.5.

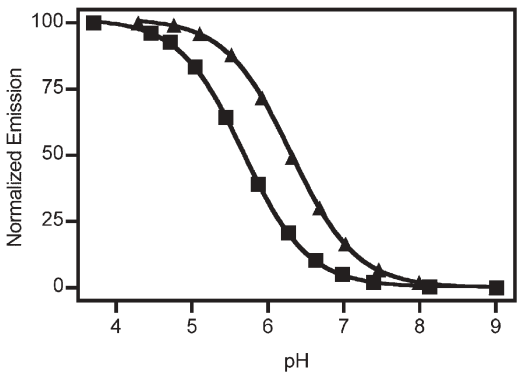

Fig. 3 Fluorescence-based pH titrations of **1** (triangles) and **2** (squares) at various pH values in 50 mM phosphate–citric acid buffer. The pK_a values for **1** and **2** are 6.3 and 5.7, respectively. Excitation is at 590 nm and the fluorescence intensity is integrated from 620 to 850 nm.

in water.[18] Following the expected heavy-atom effects, the fluorescence quantum yields for the dyes show a sequential decrease as the size of the substituent heteroatom increases in the order H > Cl > Br. The deprotonated conjugate bases of the dyes have absorption maxima at 480, 487 and 489 nm and weak fluorescence emission at 648, 643 and 637 nm for **1**, **2** and **3**, respectively.

The addition of electron-withdrawing halogen substituents on the pentamethine core of the dyes results in depression of the indole nitrogen pK_a values. Unsubstituted dye **1** has a pK_a of 6.3; whereas the chloride and bromide substituted derivatives have pK_a readings of 5.7. As a result of the low pK_a values for dyes **2** and **3** at pH 7.4 less than 2% of the dye molecules are in their protonated, fluorescent form. Dye **1** only displays minimal background fluorescence at physiological pH, showing 7% activation (Fig. 2). As with other pH sensitive fluorophores, these dyes can be used to determine a wide range of pH values, but have maximum sensitivity approximately ±0.9 pH units from the pK_a of the dye, which corresponds to 10–90% protonation (Fig. 3). Thus, dye **1** has optimal sensitivity between pH 5.4 and 7.2, and is well suited for potential visualization of the acidic environments associated with tumors, which range from pH 6.3 to 7.0.[10] Fluorochromes **2** and **3** with a more acidic pK_a of 5.7 show strong fluorescence response between pH 4.8 and 6.6, indicating these dyes are well matched for imaging acidic intracellular vesicles or renal diseases with pH values between 4.5 and 6.5.[14]

To demonstrate the potential utility of these pH-responsive dyes, preliminary experiments using biological samples were undertaken. Disorders in the urinary tract, such as renal tubular acidosis,[19] are characterized by altered urine pH. In order to image the urinary tract *in vivo*, one must be able to differentiate between urine, which is slightly acidic, and blood. Fig. 4 clearly demonstrates that dye **2**, which has low background fluorescence emission at physiological pH, can distinguish between samples of mouse urine and blood by fluorescence reflectance imaging.

In summary, new long-wavelength pH-responsive cyanine dyes were prepared by a simple synthetic route. These fluorophores show an increase in emission as the pH of their local environment decreases. The pK_a values for the dyes can be modulated by variation of the heteroatom substituents on the polymethine-conjugated system of the molecules. The dyes are water-soluble,

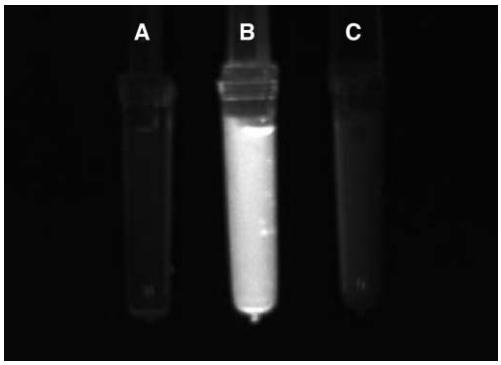

Fig. 4 Phantoms containing 1 μM dye **2** in: (A) PBS, pH 7.40; (B) mouse urine, pH 6.78; and (C) mouse blood, pH 7.69. The fluorescence reflectance image was collected with excitation at 590 nm, emission at 660+ nm, and a 575 ms exposure time.

have large extinction coefficients, good quantum yields, and show no tendency to aggregate in aqueous solution. The fluorochromes prepared in this work have potential application for *in vivo* imaging of pH in the renal and pulmonary systems where specific targeting is not necessary for probe delivery. Additional functionalization of these fluorochromes with carboxylic acid groups will allow for conjugation of the pH sensitive dyes to biomolecules and ultimately may enable targeting to specific biological tissues of interest.

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