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A colorimetric and ratiometric fluorescent pH probe based on ring opening/closing approach and its applications in monitoring cellular pH change

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A colorimetric and ratiometric fluorescent pH probe with a pK_a value of 6.0 based on ring opening/closing approach of *N*,*O*-disubstituted hemiaminal ether has been developed. The probe exhibits fast response toward pH change with high selectivity and low cytotoxicity and was applied in HK-1 cells to visualize pH change.

Intracellular pH, an important metabolic and cellular factor, plays critical roles in cellular events, such as proliferation and apoptosis,[1-5] ion transport and homeostasis,[6-14] endocytosis[3] and muscle contraction.[15,16] The pH of intracellular fluid is 7.4,[17] while human cytosolic pH ranges between 6.8 – 7.4, and is usually higher if a cell is growing.[18] Different types of organelles have distinct pH due to their unique functions, e.g., endosomes have intracompartmental pH of 4 – 6,[19] pH value of lysosomes ranges from 4.5 – 5.5, while mitochondrial matrix has a pH of about 7.8.[20] Beyond these pH range may cause serious cellular dysfunction, the abnormality in pH could be triggered by many diseases such as cancer[21] and Alzheimer's disease.[22-24] Thus, the precise measurement of intracellular pH is of great significance.

Fluorescence technique has been an important tool in molecular imaging for the measurement of intracellular pH due to its noninvasiveness to cells and its ability displaying spatial-temporal patterns.[25] Most commonly used measurement is based on fluorescent OFF–ON method. However, this method may be easily influenced by environmental factors such as incident laser power, temperature, viscosity, solvent polarity and concentration of analytes.[26] A more attractive method relies on ratiometric measurement, which records the fluorescence output signal at two wavelengths simultaneously and their ratio is calculated for correlating with the concentration of analytes. Therefore, ratiometric measurement can provide better accuracy with the built-in correction of two emission bands in the detection rather than that obtained by single-emission measurement.

Recently, we have developed a series of pH probes based on ring-opening/ring-closing of spiropyran platform.[27,28] By exploiting the excited-state intramolecular electron transfer (ESIPT) mechanism, ratiometric pH probes in near infrared (NIR) range were designed and developed. However, this type of pH probes has scarce application in the biomolecular imaging. On the other hand, Bakker *et al.*[29] reported that oxazinoindolines dyes could serve as fluorescent H^+ turn-on chromoionphores based on ring-opening approach and further was applied in the development of polymeric ion-selective membranes. A consequence of the presence of nitro group strongly stabilized the ring-opening form and significantly increased their pK_a values. The pK_a values of these dyes in the range 9.80 – 12.95 prevent their application in the intracellular pH measurement. These types of ring opening/closing mechanism induced by H^+/OH^- inspired us to develop a series of ratiometric pH probes with high quantum yield, good photostability and ability to function under acidic condition. We envisioned that the change of nitro group in oxazinoindolines dyes would significantly alter their pK_a values. Herein, we reported a fluorescent pH probe **1** with a pK_a value of 6.0 and its ring opening/closing mechanism was confirmed by ^{1}H NMR titration of compound **2** with a similar structure of probe **1**. Furthermore, probe **1** could be applied in monitoring the intracellular pH fluctuations of living cells.

Probe **1** was constructed by connecting a coumarin fluorophore and *N*,*O*-disubstituted hemiaminal ether moiety through a C=C double bond. Hemiaminal ether moiety of **1** was labile under acidic condition and would be cleaved to liberate indolenium moiety. The open form molecule extended the π-conjugation system of coumarin fluorophore, displaying an emission with a bathochromic shift to the NIR range (Fig. 1). Furthermore, under neutral and basic conditions, the nucleophilicity of the free phenol group is strong enough to cyclize back to the original *N*,*O*-disubstituted hemiaminal ether which only emits a short green emission reminiscent to that emitted by coumarin fluorophore.

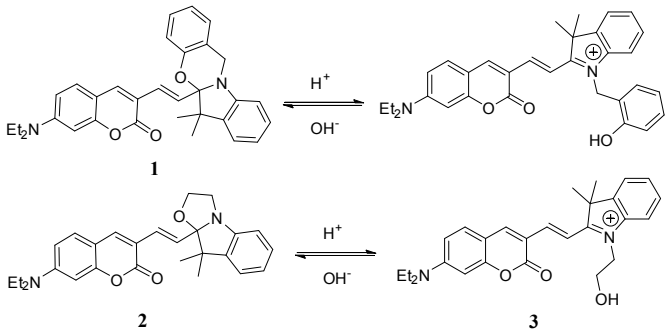
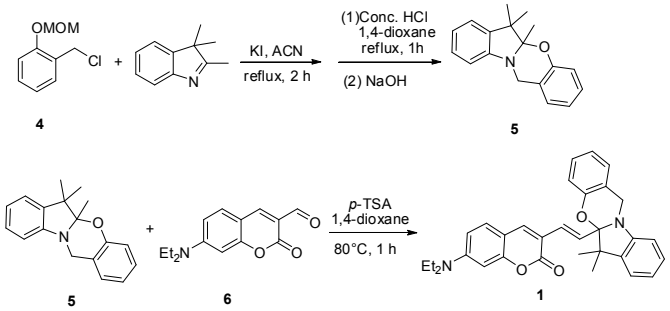


Fig. 1 The proposed reversible pH sensing mechanism of probes **1** and **2**.

The synthesis of probe **1** was started from the substitution of **4** with 2,3,3-trimethylindoline in the presence of potassium iodide, followed by the deprotection of methoxymethyl (MOM) group under strong acidic conditions and subsequent intramolecular cyclisation mediated by NaOH yielded key intermediate **5**. Then condensation of **5** with aldehyde **6**[30] in the presence of catalytic amount of *para*-toluenesulfonic acid (*p*-TSA) gave the corresponding probe **1**. The final compound was fully characterized by ^{1}H NMR, ^{13}C NMR, HRMS analysis and X-ray diffraction experiment (Fig. S2–S14).



Scheme 1 Synthesis of probe **1**.

The photophysical properties of probe **1** were studied in Britton-Robinson buffer solution (40 mM, containing 50% acetonitrile, ACN) (Fig. 2 ~ Fig. 4). Under basic condition (pH = 10), probe **1** showed an absorption maximum at 415 nm ($\varepsilon = 3.53 \times 10^4$ M^{-1} cm^{-1}) and an intense emission maximum at 498 nm (Φ_F = 0.58, fluorescein in 0.1 M NaOH as standard, Φ_F = 0.95)[31]. These results were similar to the photophysical properties of 7-diethylaminocoumarin fluorophore. When the solution pH decreases from 10.0 to 3.0, absorption/fluorescence maximum at 415/498 nm decreases with concomitant of increases of that at 585/657 nm ($\varepsilon = 7.34 \times 10^4$ M^{-1} cm^{-1}, Φ_F = 0.08, cresyl violet in MeOH as standard, Φ_F = 0.53),[31] as a result of ring-opening process of hemiaminal ether structure to indolenium moiety. A clear isosbestic/isoemission point was observed at 464/626 nm. The pK_a of probe **1** was deduced from the titration curve of absorption ratio (A_{415}/A_{464}) (Fig. 2 inset) and emission ratio (F_{498}/F_{626}) (Fig. 4), respectively. A sigmoidal plot of absorption ratio and emission ratio versus pH gave pK_a value 5.7 and 6.0, respectively[32-35]. Experiments revealed that the color of solution changed from light yellow to blue and the fluorescence of solution changed from cyan to dark red in response to the decreased pH in the solution (Fig. 4).

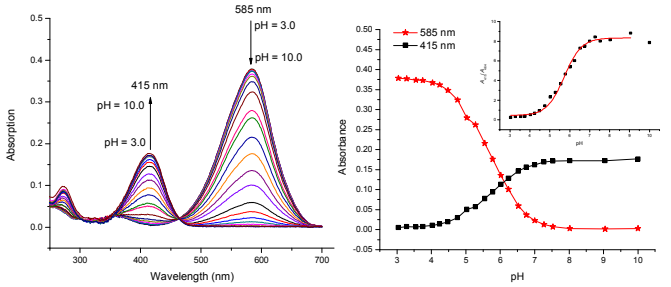


Fig. 2 Left: Absorption spectra of probe **1** (5 μM) in Britton-Robinson buffer (40 mM, containing 50% ACN) at various pH. Right: Plot of absorbance at 415 nm and 585 nm as a function of pH, respectively. Inset: Calibration curve of A_{415}/A_{464} (absorbance at 415 nm *vs* absorbance at 464 nm).

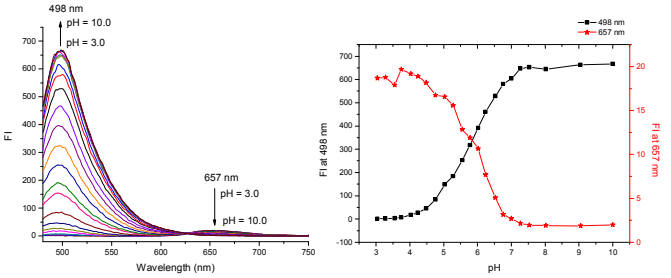


Fig. 3 Left: Fluorescence spectra of probe **1** (5 μM) in Britton-Robinson buffer (40 mM, containing 50% ACN) at various pH. Excitation wavelength was set at 460 nm. Slits = 3 nm. Right: Plot of fluorescence intensity at 498 nm and 657 nm as a function of pH, respectively.

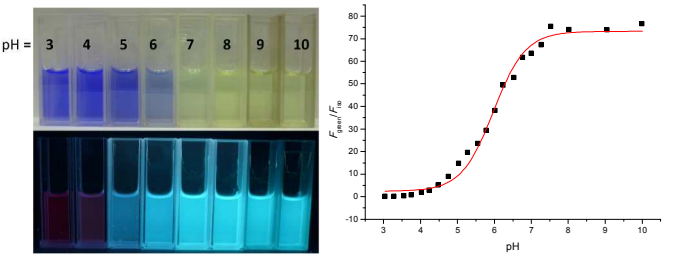


Fig. 4 Left: Visual (upper) and fluorescence (bottom) color change of probe **1** (10 μM) at various pH (from left to right: pH = 3, 4, 5, 6, 7, 8, 9, 10, respectively). Right: Calibration curve of F_{498}/F_{626} (intensity at 498 nm *vs* intensity at 626 nm).

To assess the selectivity profile of probe 1, physiologically abundant cations (Na^+, K^+, Mg^{2+}, Ca^{2+}: 10 mM) and transition metal ions (Ag^+, Li^+, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}: 0.1 mM) were studied in Britton-Robinson buffer (40 mM, containing 50% ACN) at pH 4.6 and 7.4, respectively (Fig. 5). The results showed that these substances hardly influence the fluorescence signal ratio (F_{498}/F_{626}) of probe **1**.

Probe **1** also showed a fast response time and excellent stability in Britton-Robinson buffer (40 mM, containing 50% ACN) at pH 5.0, 6.0 and 7.0, respectively (Fig. S15). Probe **1** quickly reaches its equilibrium and gives a steady emission output within 2 h of experiment time. These results confirmed that probe **1** is an excellent candidate for the pH measurement in a biological environment.

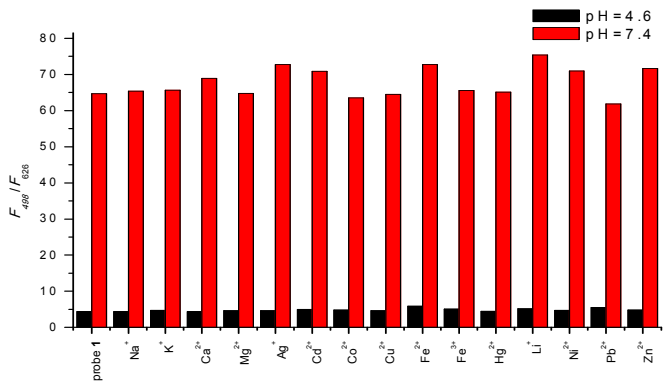


Fig. 5 Interference profiles (F_{498}/F_{626}) of probe **1** (5 μM) upon addition of various analytes at pH 4.6 (black bar) and 7.4 (red bar), respectively.

To corroborate the ring-opening mechanism of probe **1** under acidic condition, two probes **2** and **3** with similar structure of probe **1** were also synthesized. ^{1}H NMR titration of the probe **2** against the addition of TFA was conducted in ACN-d_3 solution (Fig. S16–S17). Two well-separated singlets at δ 1.11 and 1.38 ppm ascribed to *gem*-dimethyl group indicated probe **2** mainly existed in spirocyclic form. After addition of 1 equiv of TFA, these two singlets gradually disappeared and coalesced to a single downfield signal at δ 1.78 ppm. Meanwhile, two multiplets in the range of δ 3.34 – 3.75 ppm attributed to ethylene group moved downshift and formed two well-separated triplets at δ 4.01 and 4.51 ppm, respectively. These results indicated the disappearance of chiral carbon center. Two doublets at δ 6.60 and 6.73 ppm ascribed to *trans*-protons of C=C double bond also downshifted to δ 7.95 and 8.18 ppm without change in coupling constant (J = 15.9 Hz). The large downfield shift implied the extended π-conjugation system with electron-withdrawing group. On the other hand, after the addition of 1 equiv of TFA to the solution of probe **2**, the spectrum matched well with that of probe **3**. All of these results corroborate the ring-opening mechanism depicted in Fig. 1.

To disclose the cell imaging ability of probe **1**, we first studied the cell permeability of the probe in HK-1 cells using fluorescence microscopy. When HK-1 cells were incubated with probe **1** (20 μM), they gave fluorescence in both green and red channels (Fig. 6). The results showed that the probe stained the cytoplasm, indicating a good permeability of probe. It is noteworthy that a scarce overlap of green and red fluorescence window (Fig. 6c) confirmed the different pH distribution within cytoplasm.

Also, probe **1** could be used to image intracellular pH change of living cells. To modify the intracellular pH with the external pH buffered solutions for internal calibration purpose, nigericin (K^+/H^+ ionophore)-treated HK-1 cells pre-labeled with probe **1** were incubated with buffered solutions at pH 5.0, 6.0 and 7.0, respectively (Fig. 6).[36-39] Compared with normal untreated living cells, an obvious decrease of green fluorescence and increase of red fluorescence was observed when the pH of the cells was altered from 7.0 to 6.0 and 5.0. Moreover, a rough pH correlation curve was generated (Fig. 7a).

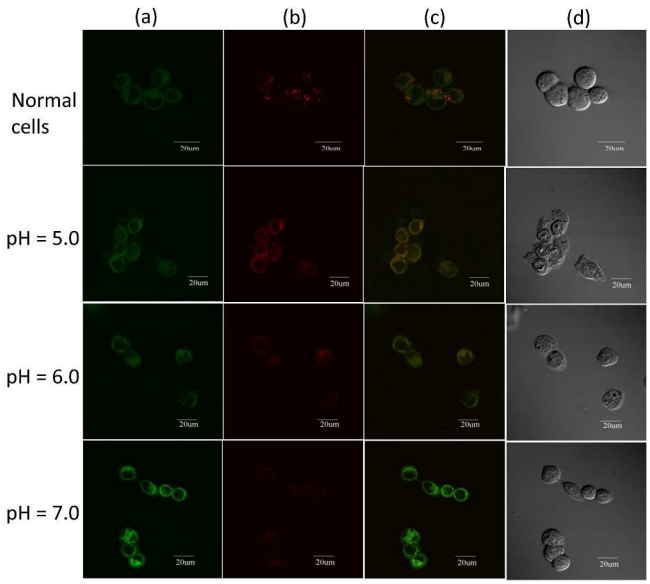


Fig. 6 Pseudocolored ratiometric (I_{green}/I_{red}) images of normal cells and K^+ ionophore-treated HK-1 cells labeled with **1** (20 μM) for 2 hours at pH 5.0, 6.0 and 7.0, respectively. Excitation wavelength was set at 488 nm. Scale bar: 20 μm. From left to right: (a) emission collected in 496 – 536 nm (I_{green}); (b) emission collected in 630 – 700 nm (I_{red}); (c) merge of images from (a) and (b); (d) bright image of the control.

It has been reported that chloroquine could cause comparable increases in lysosomal pH just like the effect initiated by tributyl amine, ammonium chloride and methylamine.[40] When HK-1 cells were incubated with probe **1** followed by the incubation of chloroquine (100 μM), a significant decrease of red fluorescence was observed (Fig. 7d).

To test the cytotoxicity of **1** toward cells, HK-1 cells were treated with high concentrations of the probe using MTT assay (Fig. 7b). After 2 h of cellular internalization of **1** at a concentration of 20 μM, the result indicated that the probe is not toxic to the cells under these experimental conditions (more than 97% cells were viable).

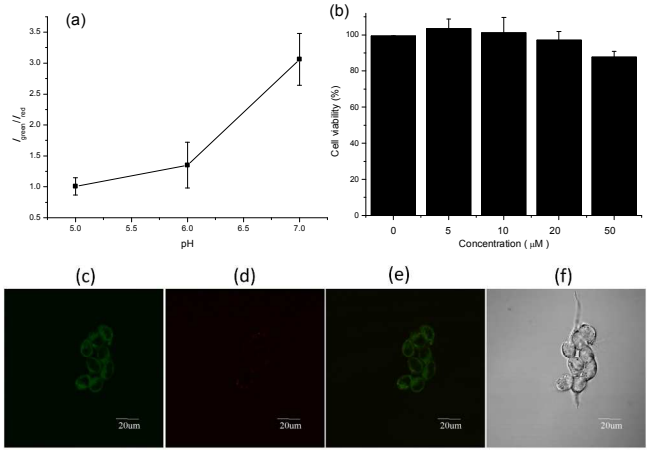


Fig. 7 (a) Plot of fluorescence ratio I_{green}/I_{red} as a function of pH in HK-1 cells. (b) Viability of the treated cells. (c-f) Pseudocolored ratiometric (I_{green}/I_{red}) images of chloroquine-treated HK-1 cells labeled with **1** (20 μM) for 2 hours. Excitation wavelength was set at 488 nm. Scale bar: 20 μm. From left to right: (c) emission collected in 496 – 536 nm (I_{green}); (d) emission collected in 630 – 700 nm (I_{red}); (e) merge of images from (c) and (d); (f) bright image of the control.

In summary, a colorimetric and ratiometric pH probe **1** operative on ring opening/closing mechanism of *N*,*O*-disubstituted hemiaminal group have been designed and investigated. The pK_a of probe makes it suitable for the monitoring of pH fluctuation of acidic organelle. These probes exhibit fast response to pH change, high selectivity, high photostability, low cytotoxicity, good cell permeability and distinct emission pattern when cells were subjected to pH change in the range from 5.0 to 7.0. Furthermore, probe **1** was applied in the visualizing intracellular pH change triggered by chloroquine. On the other hand, it should be pointed out that the corresponding phenolic and hydroxyl group in the ring-opening form of probes **1** and **2** offer viability to conjugate with other functional group to provide an excellent analyte-induced switchable platform for the design of other ratiometric sensors.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [experimental procedures, ^{1}H NMR and ^{13}C NMR spectra, MALDI-TOF HRMS spectra, time-course spectrum, X-ray spectrum and crystal data]. See DOI: 10.1039/b000000x/

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