A dual near-infrared pH fluorescent probe and its application in imaging of HepG2 cells†

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A dual near-infrared pH fluorescent probe has been designed, synthesized and applied to HepG2 cells, with a pK_a value of 5.14 under acidic conditions and 11.31 under basic conditions, which is valuable for studying acidic organelles in living cells and pH changes in chemical systems.

H^+ concentration, as an important metabolic and cellular parameter, plays a central modulating role in many cellular events.[1–4] Reports have suggested a connection between some diseases and abnormal pH values in cytoplasm or acidic organelles.[5–7] As a result, the precise measurement of intracellular pH is of great importance. A number of methods are currently available for the measurement of pH including microelectrodes,[8] nuclear magnetic resonance (NMR),[9] absorption[10] and fluorescence spectroscopy.[11–13] Among these methods, fluorescence spectroscopy offers significant advantages due to its nondestructive character, high sensitivity and specificity, and the availability of a wide range of indicator dyes.[14] Moreover, the fluorescence microscopic imaging technique allows us to map the spatial and temporal distribution of H^+ within living cells.

Nowadays, two classes of fluorescent pH probes have been developed, that is probes for cytosol which work at a pH of 6.8–7.4,[15–20] and probes for acidic organelles such as lysosomes and endosomes which function over the pH range of 4.5–6.0.[21,22] As minor variations of intracellular pH may induce cellular dysfunction, desirable pH fluorescent probes should be able to respond sensitively to a minor change of pH, and to avoid interference from native cellular species. However, limitations of the currently available pH probes include low sensitivity, and/or excitation profiles in the ultraviolet region which can damage living samples and cause interfering autofluorescence from native cellular molecules. Additionally, very few acidic fluorescent probes are considered to be desirable ones for studying acidic organelles, which is really a bottleneck in cell biological or medical studies. Thus, the focus of this study is to design a near-infrared fluorescent probe with good selectivity, high sensitivity, good photostability and the ability to work within the acidic pH range.

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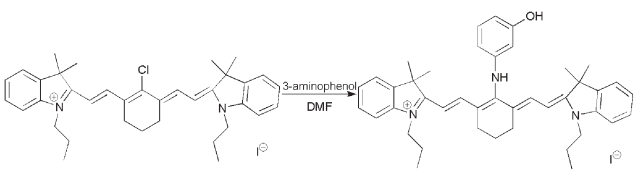
Fluorescent pH probes are generally composed of two moieties: a fluorophore with desirable photophysical properties and a pH-sensitive fluorescence modulator. Our strategy for designing a fluorescent pH probe is based on photoinduced electron-transfer (PET) between fluorophore and modulator to induce the change of fluorescence with pH. We chose tricarbocyanine, a near-infrared fluorescent dye with high extinction coefficients,[23] as the fluorophore and 3-aminophenol as the modulator. The probe, AP-Cy, was synthesized through a one-step reaction of tricarbocyanine with 3-aminophenol under mild conditions with a large throughput (about 50%) (Scheme 1) and was characterized with ^{1}H NMR and MS (see Supporting Information). As expected, AP-Cy exhibits high sensitivity, good photostability and excellent cell membrane permeability. The probe can sensitively respond to H^+ changes within the pH range of 4.0–6.5. Interestingly, it can also respond to H^+ within the pH range of 10.5–11.8, which firstly realized dual measurements for H^+ within different pH ranges at different excitation and emission wavelengths. Furthermore, as the first near-infrared fluorescent probe for intracellular acidic pH imaging, it is especially suitable for studying acidic organelles.

We first examined the spectral properties of the probe. AP-Cy in acetonitrile shows dual absorption at 431 nm and 750 nm, and emission at 800 nm (λ_{ex} = 750 nm, Fig. 1). In 40 mM buffer solution containing 40 mM acetic acid, phosphoric acid, boric acid (5% acetonitrile cosolvent, 0.1 M NaCl), it is interesting that AP-Cy undergoes distinct color changes from yellow to blue,



Scheme 1

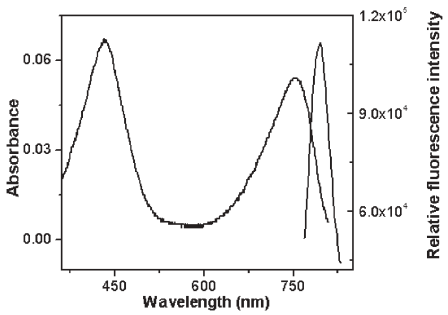


Fig. 1 Absorption and fluorescence spectra of AP-Cy in acetonitrile.

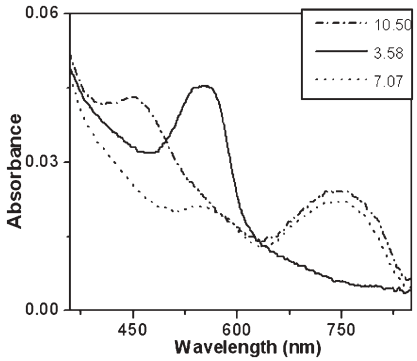


Fig. 2 Absorption spectra of AP-Cy in buffer solutions with different pH values.

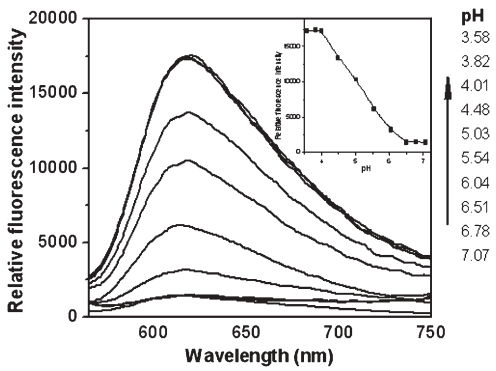


Fig. 3 Fluorescence spectra (λ_{ex} = 558 nm) and pH titration curve of 10 μM AP-Cy in acidic buffer solutions with different pH values.

finally to purple along with the pH titration. The absorption spectra at different pH values (Fig. 2) show absorption at 558 nm at pH 3.58, 750 nm at 7.07, 453 nm and 750 nm at pH 10.50.

Furthermore, we performed standard fluorescence pH titration analysis in buffer solution at a probe concentration of 10 μM. Fig. 3 shows the fluorescence emission spectra under acidic conditions (λ_{ex} = 558 nm). Unlike other fluorescent pH probes such as fluoresceins and rhodamines in which the fluorescence is strongly quenched at lower pH,[11] the fluorescence intensities of AP-Cy increase significantly at lower pH due to the protonation of N atom which prevents the PET process. We also noted a more than 10-fold increase in the emission intensity (λ_{em} = 615 nm) within the pH range of 4.0–6.5. The analysis of fluorescence intensity changes as a function of pH by using the Henderson–Hasselbach-type mass action equation[24] yielded a pK_a of 5.14 (Fig. 3), which is valuable for studying acidic organelles. Under basic conditions, we chose 468 nm as the excitation wavelength because the fluorescence intensity is much stronger when the probe is excited at 468 nm than those at other wavelengths. The fluorescence emission spectra at 517 nm of AP-Cy under basic conditions show that the intensities increase gradually under the higher pH values (Fig. 4). The pK_a value of AP-Cy was determined to be 11.31 within the pH range of 10.5–11.8, which is valuable for studying pH changes in chemical systems.

On account of the complexity of the intracellular environment, we performed an additional examination to determine whether other ions were potential interferents. It is well known that amines can bind to many metal cations in solution.[25–27] Among them,

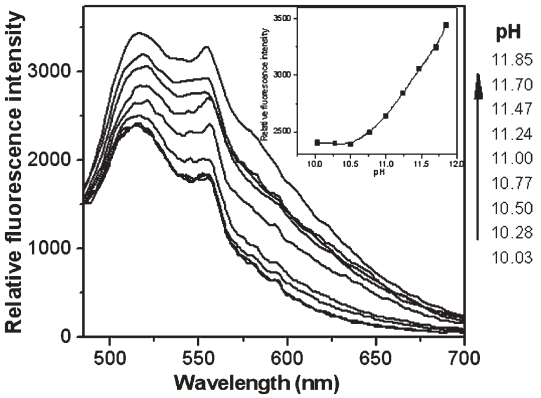


Fig. 4 Fluorescence spectra (λ_{ex} = 468 nm) and pH titration curve of 10 μM AP-Cy in basic buffer solutions with different pH values.

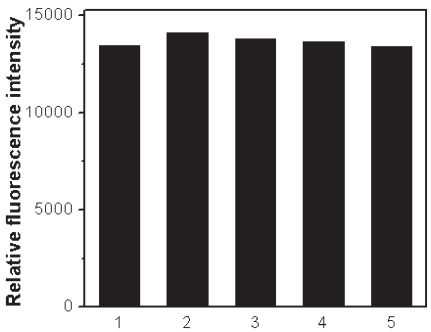


Fig. 5 Relative fluorescence intensity at 615 nm of AP-Cy in the absence or presence of 200 μM Cu^{2+}, Zn^{2+}, Ca^{2+} or Mg^{2+} ions in buffer solution at pH 4.48 (λ_{ex} = 558 nm). 1: H^+; 2: $H^+ + Cu^{2+}$; 3: $H^+ + Zn^{2+}$; 4: $H^+ + Ca^{2+}$; 5: $H^+ + Mg^{2+}$.

Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} are the types of species likely to be targeted by free amines. The relative fluorescence intensity of AP-Cy in the absence or presence of an excess of Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} ions at pH 4.48 is shown in Fig. 5, which indicates that the effect of such metals on pH measurement is negligible.

Finally, we applied AP-Cy to HepG2 cells (human hepatocellular liver carcinoma cell line) to examine whether it can work in biological systems. The cells were incubated with AP-Cy (10 μM) for 1 h at 37 °C and then washed three times with PBS buffer (0.1 M, pH 7.4). The distribution of the probe within the cells was observed by fluorescence microscopy following excitation at 532 nm. As shown in Fig. 6, AP-Cy is membrane-permeable, and

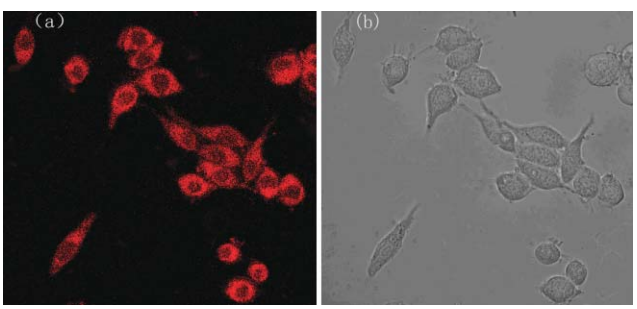


Fig. 6 Confocal fluorescence images of live HepG2 cells. (a) Cells incubated with 10 μM AP-Cy at 37 °C for 1 h. (b) Bright field image of live HepG2 cells to confirm their viability.

the fluorescence intensities are different within single cells, which is consistent with intracellular different pH distribution. Bright field transmission measurements after AP-Cy incubation confirm that the cells are viable. We anticipate that this simple, sensitive near-infrared fluorescent probe will be of great benefit to biomedical researchers for investigating the effects of H^+ in biological systems.

In summary, we have presented the synthesis, property analysis, and biological applications of AP-Cy, a dual near-infrared fluorescent probe for optical imaging of intracellular H^+. The synthesis of AP-Cy is facile, and the probe exhibits strong pH dependence, which can respectively respond to H^+ under acidic or basic conditions at different excitation and emission wavelengths. The pH titrations indicate a more than 10-fold increase in fluorescence intensity within the pH range of 4.0–6.5 with a pK_a value of 5.14 which is valuable for studying acidic organelles in living cells, and a pK_a value of 11.31 within the pH range of 10.5–11.8. Furthermore, we have demonstrated the value of AP-Cy by monitoring intracellular H^+ within HepG2 cells. We believe that such a dual near-infrared pH fluorescent probe will have great potential in investigating the pivotal role of H^+ in a biological context, especially in acidic organelles through direct intracellular imaging. Further studies on the biological applications of AP-Cy are in progress.

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