Preparation of novel imidazo[1,2-a]indole fluorophore and its application for detecting extreme pH of fungus

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Abstract

A novel pH fluorescent probe imidazo[1,2-a]indole derivative is reported. The probe is highly selective to strong acidic pH (pKa = 3.56) with high sensitivity and a fast response time (within 30 s). It is hardly interfered by ordinary metal ions and has good reversibility under strong acid conditions. The probe transfers charge under different pH conditions, and the response mechanism depends on the change of ICT. It can also be used for imaging in strong acidic Saccharomyces cerevisiae and detection of intracellular H⁺ as well.

Keywords: Imidazo[1,2-a]indole; pH fluorescent probe; Saccharomyces cerevisiae; ICT; Imaging
1. Introduction

As an important parameter reflecting the acid-base strength of the solution, pH keeps a stable state in cells and organisms, maintaining the normal shape and function of cells [1-2]. Normal human body's pH is maintained at 6.5-7.1, but the pH of different parts of the cell varies. For example, the local pH range of lysosomes and endosomes is from 4.5 to 6.8 [3-6], the pH of mitochondria is about 8 [7-9] and the cytoplasm can maintain cell viability at a pH of about 6.8-7.4 [10-11].

Changes in pH will affect the proliferation, differentiation and apoptosis of cells [12-13], muscle contraction [14-15], ion transport [16-17], and the stability of the internal environment. Some diseases may arise from abnormal pH such as cystic fibrosis, cancer and neurodegenerative disorders [18-20]. Significant changes in pH in the human body can cause cell metabolism disorders and physiological changes. Various techniques such as absorption spectroscopy, electrochemistry and nuclear magnetic resonance have been reported to measure pH [21-23]. Because of the advantages of high sensitivity, good selectivity, ease of use, and low cost, fluorescent probes have been widely used in molecular biology, biochemistry, medicine and other fields [24]. So far, many small molecule pH fluorescent probes suitable for acidic organelles (lysosomes, pH 4.5-5.0,) or neutral organelles (mitochondria, pH 6.8-7.4) have been used [25-36]. Unfortunately, the application of pH fluorescent probes in the extremely acidic range (pH <4) has received relatively little attention. On the one hand, strong acidity is lethal to most organisms. Bacteria such as acidogenic bacteria and Helicobacter pylori can live in the stomach of strongly acidic mammals and can cause infections, which can be life threatening [37-38]. On the other hand, the secretory and endocytic pathways of certain eukaryotic cell organelles can only be carried out under acidic pH conditions. Hence, it is necessary to design a pH fluorescent probe with high sensitivity and photostability under strong acid conditions.

Indole derivatives are usually found in natural products, such as certain alkaloids, auxins, essential oils, coal tar, etc., all contain indole and its derivatives [39]. In
addition, indole derivatives are often used as preferred structures in drug discovery and synthesis [40-42]. Although they show important biological activities, there are few reports on their optical properties due to the limitation of synthetic methods[43-46].

In this article, we report a new type of imidazo[1,2-a]indole derivative YH-1, which is a novel simple small molecule fluorescent probe that can be used in extreme acidic conditions, and its response mechanism is based on ICT. The advantage of this probe compared with other pH fluorescent probes [47-49] is that it can measure pH in a short time (within 30s) with high sensitivity. In addition, fluorescence imaging experiments of bacteria have been conducted to prove the value of this probe in Saccharomyces cerevisiae.

2. Experimental section

2.1. Materials

Except for special instructions, all reagents were purchased online and were used directly without further processing. In order to avoid the interference of impurities, all deionized water was used throughout the experiment. The chloride salt was dissolved in deionized water to prepare the metal ion solution to avoid interference of other metal ions. The sample solutions used in the experiment were all prepared under natural conditions, shaken for 15 seconds, and then allowed to stand for 10 minutes to mix well. Then UV-vis and fluorescence measurements were performed. The Britton-Robinson buffer solution (B-R) used in the experiment was obtained by mixing 40 mM acetic acid, phosphoric acid and boric acid in deionized water. The pH of the solution was adjusted with dilute NaOH or HCl solution.

2.2. Instruments

UV-2600 spectrophotometer (Shimadzu) was used for absorption measurement. FS5 fluorescence spectrophotometer was used for recording the fluorescence spectrum. Bruker Avance 400 (400 MHz) spectrometer was used to measure $^1$H NMR and $^{13}$C NMR spectra, DMSO-$d_6$ was used as the solvent, and tetramethyl silane (TMS) was used as the internal standard material. FE28-standard pH meter (Shanghai
Mettler) was used to measure pH. The laser confocal microscope Ti 2 (Nikon, ECLIPSE) performed cell imaging under excitation at 350 nm.

2.3. Fungus imaging

Saccharomyces cerevisiae (abbrev. S. cerevisiae, a kind of fungus used to make bread, steamed bread and brewing) was extracted in yeast at 30°C with peptone glucose (YPD) medium (tryptophan 2%, yeast extract 1%, glucose 2 %) and then stirred in a table concentrator (ZHI) at 200 rpm for 12 hours. The cultured Saccharomyces cerevisiae solution was placed in a 2 mL Eppendorf tube and centrifuged at 4500×g for 2 minutes to collect the Saccharomyces cerevisiae cells. Resuspend the pellet in 1 mL Britton-Robinson buffer with different pH (3.0, 5.0, 7.0). Then the tube was placed in the bench top concentrator. The pH probe was dissolved in DMSO. After 2 hours, the probe solution was added to each tube containing buffer solution to make the probe concentration reach 5 μM and then incubate continuously for 30 minutes. Finally, it was coated on a glass slide and observed by a laser confocal microscope Ti 2 (Nikon, ECLIPSE) at a wavelength of 350 nm.

![Scheme 1. Synthetic route of the probe YH-1](image)

2.4. Synthesis and characterization of probe 9-((benzyloxy)carbonyl)-1-methyl-1H-
imidazo[1,2-a]indole-3-carboxylic acid (YH-1).

Compound 1 1-fluoro-2-nitrobenzenewas achieved commercially. The synthesis of compounds 2-7 has been mentioned in the literature [50].

Ethanol (20ml) and water (10mL) were mixed together, and then compound 7 (0.96 g, 2.56 mmol) and NaOH (0.12 g, 3mmol) were added to the mixed solution. The mixture was reacted for 4 hours at 80°C. The crude product solution was added to 40mL of water, and then hydrochloric acid was added to adjust the pH = 2, and it was left to be filtered with suction. After drying in the oven, a yellow solid was obtained with a yield of 82% (0.78g). mp: 216-218 °C. 

$$\text{H}^1\text{NMR (400 MHz, DMSO-}d_6\text{) }\delta\text{ }13.27 (s, 1H), 8.65 (d, J = 8.2 \text{ Hz, } 1H), 8.15 (s, 1H), 8.07 (d, J = 8.0 \text{ Hz, } 1H), 7.49 (d, J = 7.3 \text{ Hz, } 2H), 7.41 (t, J = 7.3 \text{ Hz, } 2H), 7.35 (d, J = 7.1 \text{ Hz, } 1H), 7.25 (t, J = 7.5 \text{ Hz, } 1H), 7.11 (t, J = 7.6 \text{ Hz, } 1H), 5.34 (s, 2H), 4.06 (s, 3H).$$

$$\text{C}^{13}\text{NMR (100 MHz, DMSO-}d_6\text{) }\delta\text{ }163.66, 160.92, 144.88, 137.77, 133.56, 131.18, 128.96, 128.50, 128.30, 126.81, 123.61, 120.71, 119.60, 115.22, 114.96, 80.89, 64.78, 37.15.$$

HRMS ([M+H]⁺): Calcd for C_{20}H_{17}N_{2}O_{4}: 349.1188; found: 349.1185.

3. Results and discussion

3.1. Synthesis of the probe YH-1

Scheme 1 shows the general synthetic route of the probe. The structure of the probe was characterized by HRMS, H NMR and C NMR.

3.2. Spectral characteristics of probe N-1 and its optical response to pH

All samples were dissolved in Britton-Robinson buffer solution (B-R)/DMSO (8/2, v/v) solution in the fluorescence experiment and measured after 10 minutes. Probe YH-1 is highly fluorescent, and it can be seen from the Fig. 1 that the fluorescence intensity is unchanged when the pH is higher than 4.4. When the pH value is in the range of 2.0-4.4, as the pH value decreases, the fluorescence intensity decreases significantly. The fluorescence intensity at 450 nm increased significantly from 29346.2 at pH 2.0 to 100201.8 at pH 4.4. We calculated that the quantum yield at pH of 4.4 was 0.115 (Quinine sulphate dehydrate in 0.1 N H_{2}SO_{4} was used as the main standard, ϕ=0.546, λ_{ex}=350 nm).
Fig. 1. The fluorescence spectrum of the probe YH-1 (1μM) dissolved in the B-R/DMSO (8/2, v/v) solution in the pH range of 2.0-7.0 (λex=350 nm).

Fig. 2. (a) The fluorescence titration pH value of 2.0 to 7.0 at 450 nm fluorescence intensity. (b) The linear relationship between the fluorescence intensity of the probe YH-1 at 450 nm and the pH value (pH 3.2-4.4) (R² =-0.9975).

Fig. 3. The linear regression relationship between the pH value and “(log [(Fmax-FX)/(FX-Fmin)])”.

In Fig. 2a, we can see that the X axis and Y axis represent pH value and
fluorescence intensity respectively, and they are arranged in a "Z" arrangement (emission wavelength 450 nm). In Fig. 2b, When the pH is from 2.3 to 4.4, the fluorescence intensity and pH forms an ideal linear relationship ($R^2 = -0.9975$). Britton-Robison buffer/DMSO (8/2) can be used to determine the pKa of the probe. In Fig. 3, according to the acid-base balance formula (Henderson-Hasselbach equation) 

$$\log \left( \frac{F_{\text{max}} - F}{F - F_{\text{min}}} \right) = \text{pKa} - \text{pH},$$

F in the formula is the probe's emission wavelength at 450 nm fluorescence emission intensity), pKa calculated was equal to 3.56, which is very valuable for measuring the pH of strong acids. When the pH value is from 2.3 to 4.4, the relationship between the pH value and "log $[(F_{\text{max}}-Fx)/(Fx-F_{\text{min}})]$" can be expressed by a very good linear relationship ($R^2 = 0.9790$). Through the regression curve, we get the following formula $\text{pH} = -0.8497X + 3.5605$. In the linear relationship formula, X means "log $[(F_{\text{max}}-Fx)/(Fx-F_{\text{min}})]$". Therefore, we can use this formula to calculate any sample with a pH range of 2.3 to 4.4.

![Graph showing reversibility of fluorescence emission intensity](image)

**Fig. 4.** The reversibility of the fluorescence emission intensity of the probe **YH-1** between pH 2.6 and pH 5.0.
Fig. 5. The fluorescence intensity of the probe YH-1 changes with time in 0-10 minutes.

Fig. 6. Changes in the fluorescence intensity of probe YH-1 in the solution (8/2, B-R/DMSO, v/v) under the influence of different metal ions and amino acids at (a) pH 2.6 and (b) pH 5.0 (probe (1μM), Zn$^{2+}$ (5μM), Fe$^{3+}$ (5μM), Cu$^{2+}$ (5μM), Mg$^{2+}$ (5μM), Ca$^{2+}$ (10μM), Na$^{+}$ (10μM), K$^{+}$ (10μM), H$_2$O$_2$ (5μM), GSH (5μM), Cys (5μM), Hcy (10μM), $\lambda_{ex} = 350$ nm, $\lambda_{em} = 450$ nm).

Scheme 2. The mechanism of the change in fluorescence intensity of YH-1 after addition of H$^+$. 

In Fig. 4, the fluorescence emission intensity of the probe at 450nm is reversible when changing between pH 2.6 and 5.0, which means it can be used to detect acidic systems with different pH values. In addition, Fig. 5 shows that under different conditions, the response time of the probe to pH does not exceed 30 s. In addition,
probe has basically no change in fluorescence intensity under the interference of different metal ions and amino acids, and the probe can respond to the excellent selection of H⁺ (Fig. 6). Hence, it is preliminarily judged that the probe can detect the internal pH of Saccharomyces cerevisiae.

![Fig. 7. The ¹H NMR spectrum of probe YH-1 in DMSO-d₆ under neutral conditions and acid conditions (CF₃COOH).](image)

3.3. The mechanism of pH detection

The probes were compared by ¹H NMR under neutral and acid conditions (CF₃COOH) (Fig. 7). It can be seen from the figure that no hydrogen has a significant chemical shift change, so the nitrogen bridgehead is not protonated, and no protonation process occurs on the indole ring. Under neutral conditions, 1-Nitrogen has a rich electron density, and it binds protons from the carboxyl group as a basic part, so there is a good push-pull system. However, under acidic conditions, carboxylic acid groups can better attract electrons. Compared with the protons under neutral conditions, the protons in imidazoinole absorb under a higher electric field in acidic conditions. Compared to neutral, the intramolecular charge transfer should change under acidic conditions. Scheme 2 shows the process of protonation.
In order to verify whether the probe can be used in biology, we tested it in bacteria with strong acidic conditions. In order to simulate the presence of a strong acid environment in S. cerevisiae, pH 3.0, 5.0 and 7.0 buffers were used to cultivate S. cerevisiae. Then, we added YH-1 and imaged it. In the image taken by a fluorescent confocal microscope (Fig. 8), we can see that there is almost no fluorescence of bacteria in a highly acidic medium with a pH of 3.0. As the intracellular pH value increases, the fluorescence intensity increases significantly. These results indicate that the probes can image biological systems with very low pH.

4. Conclusions

In short, an imidazo[1,2-a]indole derivative YH-1 was synthesized, which is a new kind of simple pH fluorescent probe for pH detection under strong acid conditions. This is the first time that imidazo[1,2-a]indole derivatives have been used as fluorophore for pH detection. Through the analysis of YH-1 $^1$H NMR under neutral and acidic conditions, the response of the probe to pH depends on the ICT. In addition, the probe responds quickly to H$^+$ (within 30 s), and has high selectivity, sensitivity and good reversibility. More importantly, the experiment of saccharomyces cerevisiae proved that the probe could image in bacteria well, and it had a good effect on the imaging of strong acid in S. cerevisiae. We believe that research on chemical and biological systems will be beneficial.
**Author Declarations**

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**Competing Interests**  The authors declare that there are no conflicts of interest.

**Supplementary Information**  The online version contains supplementary material available at https://

**Author Contributions**  All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yanhao Xu, and Ruikang Duan. Data analysis were performed by Hao Liu, and Chengcai Xia. The first draft of the manuscript was written by Guiyun Duan and Yanqing Ge. All authors read and approved the final manuscript.

**Data Availability**  The authors declare that the data supporting the findings of this study are available in the article and the supplementary materials.

**Ethics Approval**  For this type of study, the ethical approval was not required, because this study does not involve cell or animal manipulation.

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