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A new fluorescent pH probe for extremely acidic conditions

Yu Xu[a,c], Zheng Jiang[b,c], Yu Xiao[a,c], Fu-Zhen Bi[a], Jun-Ying Miao[b,*], Bao-Xiang Zhao[a,*]

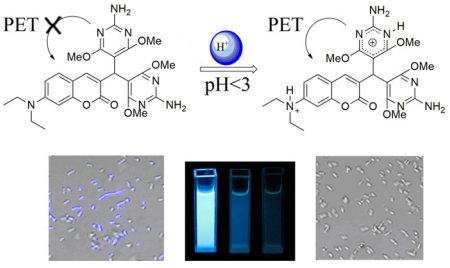
[a] School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, PR China
[b] School of Life Science, Shandong University, Jinan 250100, PR China
[c] Taishan College, Shandong University, Jinan 250100, PR China

HIGHLIGHTS

- A new fluorescence probe for very low pH was synthesized and characterized.
- The probe can monitor pH in solution and bacteria.
- The two-step protonation of N atoms of the probe leads to fluorescence quenching.

GRAPHICAL ABSTRACT

A new coumarin-based fluorescent probe can detect highly acidic conditions in both solution and bacteria with high selectivity and sensitivity.



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ABSTRACT

A novel turn-off fluorescent probe based on coumarin and imidazole moiety for extremely acidic conditions was designed and developed. The probe with $pK_a = 2.1$ is able to respond to very low pH value (below 3.5) with high sensitivity relying on fluorescence quenching at 460 nm in fluorescence spectra or the ratios of absorbance maximum at 380 nm to that at 450 nm in UV–vis spectra. It can quantitatively detect pH value based on equilibrium equation, $pH = pK_a - \log[(I_x - I_b)/(I_a - I_x)]$. It had very short response time that was less than 1 min, good reversibility and nearly no interference from common metal ions. Moreover, using ^1H NMR analysis and theoretical calculation of molecular orbital, we verified that a two-step protonation process of two N atoms of the probe leaded to photoinduced electron transfer (PET), which was actually the mechanism of the fluorescence quenching phenomenon under strongly acidic conditions. Furthermore, the probe was also applied to imaging strong acidity in bacteria, E.coli and had good effect. This work illustrates that the new probe could be a practical and ideal pH indicator for strongly acidic conditions with good biological significance.

1. Introduction

Proton, as a familiar cation, plays key roles in biological system. The fluctuation of pH has obvious effect on numerous cellular events, such as cellular metabolism [1–3], cellular growth [4], signal transduction [5], chemotaxis [6], apoptosis [7] and autophagy [8]. Therefore, monitoring pH changes inside living cells is crucial for exploring cellular functions and understanding physiological and pathological processes in organisms.

Most of the known pH fluorescent indicators belong to two categories. Some of them respond to neutral pH range from 6 to 8 [9–11]. Others can detect weak acidic pH in the range from 4 to 6 [12–14]. But very few have been applicable for more acidic conditions with pH below 4 [15–17]. Thus, monitoring the very low intracellular pH conditions is still challenging. In spite of the fact that the majority of living organisms could hardly survive in

* Corresponding authors. Tel.: +86 531 88366425; fax: +86 531 88564464.
E-mail addresses: miaojy@sdu.edu.cn (J.-Y. Miao), bxzhao@sdu.edu.cn, sduzhao@hotmail.com (B.-X. Zhao).

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strong acidic environment, there still exists a considerable number of microorganisms, such as helicobacter pylori and "acidophiles", that particularly favor this harsh living conditions [15,16]. Moreover, in some eukaryotic cells, acidic pH has important effect on organelles along the secretory and endocytic pathways [18,19]. Enteric pathogen is another example, which is able to reach small intestine by passing through the highly acidic mammalian stomach, causing life-threatening infections [20]. Even for mammals, there are some parts with very low pH value, such as gastric juice, the pH level of which can also influence their physiological process remarkably [21]. Because of lacking effective ways to detect such acidic pH in living species, the precise pH values in these cellular compartments remain elusive [17]. Thus, it is necessary and meaningful to develop new fluorescent probe that can be applied in such strong acidic conditions in living systems.

Here, based on coumarin, we designed and synthesized a new pH probe with a pK_a 2.1 and applies it to bacteria.

2. Materials and methods

2.1. Materials

All reagents and solvents were purchased from commercial sources and used without further purification. The solutions of metal ions were prepared from nitrate salts which were dissolved in deionized water. Deionized water was used throughout the process of absorption and fluorescence determination. All samples were prepared at room temperature, shaken for 10 s and rested for 1 h before UV–vis and fluorescence determination. Britton–Robinson (B–R) buffer was prepared with 40 mM acetic acid, boric acid, and phosphoric acid. Dilute hydrochloric acid or sodium hydroxide was used for tuning pH values.

2.2. Instruments

Thin-layer chromatography (TLC) involved silica gel 60 F_{254} plates (Merck KGaA). Melting points were determined on an XD-4 digital micro melting point apparatus. ^{1}H NMR spectra were recorded on a Bruker Avance 300 (300 MHz) spectrometer and ^{13}C NMR spectra were recorded on a Bruker Avance 300 (75 MHz) spectrometer, using d_6-DMSO as solvent and tetramethylsilane (TMS) as an internal standard. IR spectra were recorded with an IR spectrophotometer VERTEX 70 FT-IR (Bruker Optics). HRMS spectra were recorded on a Q-TOF6510 spectrograph (Agilent). Fluorescent measurements were recorded on an F-7000 (Hitachi) luminescence spectrophotometer and UV–vis spectra were recorded on a U-4100 UV–Vis–NIR Spectrometer (Hitachi). The pH measurements were measured by use of a PHS-3C digital pH-meter (YouKe, Shanghai). Images of *E. coli* cells were captured with a laser confocal microscope (Carl Zeiss LSM-700, Germany). The single crystal was measured on a Bruker-AXS CCD single-crystal diffractometer with graphite-monochromated Mo Kα radiation source (λ = 0.71073 Å).

2.3. Synthesis of 3-(bis(2-amino-4,6-dimethoxypyrimidin-5-yl) methyl)-7-(diethylamino)-2H-chromen-2-one (L)

7-(Diethylamino)-2-oxo-2H-chromene-3-carbaldehyde **1** and 4,6-dimethoxypyrimidin-2-amine **2** was synthesized as described previously in the literature [22–24].

Compound **1** (0.735 g, 3 mmol) was dissolved in 20 mL ethanol, while 4,6-dimethoxypyrimidin-2-amine **2** (0.515 g, 3.3 mmol) was dissolved in 10 mL ethanol. Mix the two shares of solution and add 4 drops of glacial acetic acid. The mixture was then heated to reflux for 12 h. The solvent was evaporated and the crude product was purified by column chromatography using petroleum ether/ethyl acetate (1:2, v/v) as an eluent. The target compound **L** was obtained as light yellow crystals in 35% yield (400 mg). mp: 242–244 °C. IR (KBr), υ: 3436, 3348, 3235, 2975, 2896, 1685, 1563, 1451, 1373, 1306, 1246, 1189, 1127, 1011, 902, 860, 793, 697, 642, 597, 508, 441 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz), δ (ppm): 1.11 (t, 6H, NCH_2CH_3, J = 6.6 Hz), 3.39–3.41 (m, 4H, NCH_2CH_3), 3.63 (s, 12H, OCH_3), 5.32 (s, 1H, *tert*-CH), 6.27 (s, 4H, NH_2), 6.51 (s, 1H, coumarin-H), 6.59 (d, 1H, coumarin-H, J = 8.7 Hz), 6.96 (s, 1H, coumarin-H), 7.36 (d, 1H, coumarin-H, J = 8.7 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz), δ (ppm): 168.24 (4C), 161.24, 160.23, 154.71 (2C), 149.21, 136.72, 128.59, 122.94, 108.37 (2C), 96.27, 92.43, 53.21 (4C), 43.87 (2C), 30.16, 14.05, 12.31 (2C); HRMS: calcd. for $C_{26}H_{32}N_7O_6^+$ $[M+H]^+$ 538.2414, found: 538.2477. Additionally, the single crystal of **L** was obtained by volatilizing the mixed solvent (ethanol/ethyl acetate = 1:1, v/v) slowly at ambient temperature. The crystal structure was determined by X-ray single crystal diffraction.

2.4. Calculation methods

In this study, all the calculations were implemented with the Gaussian09 program package [25]. The structure of these molecules in ground state were optimized using the density functional theory (DFT) method, CAM-B3LYP, with the 6-31g** basis set. Vibrational frequency analyses were carried out to ensure the minimums of the ground state were reached on the potential energy surfaces. On the basis of these optimized structures, the absorption spectra were predicted by time-dependent (TD-DFT) method. The solvent effects were modeled with the polarizable continuum model (PCM) model.

2.5. Bacteria culture and imaging

E. coli (Trans 5a) was incubated at 37 °C in Luria-Bertani (LB) culture (Trptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) for 12 h in a table concentrator (ZHI CHENG ZHWY-2112B, China) at 180 rpm. Then the culture was centrifuged (Heal Force Neofuge-18R, China) in 10 mL Eppendorf tubes at 5000 rpm for 5 min to collect *E. coli* cells. The sediment was resuspended with hydrochloric acid at different pH (0.61, 2.07, 3.62), respectively. 5 min after resuspension, the pH probe dissolved in DMSO was added into every tube to make the final probe concentration to be 10 μM. *E. coli* cells with the probe were incubated in a table concentrator as mentioned above for 2 h, then smeared on slides and observed by laser confocal microscopy (Carl Zeiss LSM-700, Germany) at the wavelength of 405 nm.

3. Results and discussion

3.1. Synthesis of probe (L)

The general synthetic route of probe **L** is given in Scheme 1. The structure of probe **L** was characterized by IR, ^{1}H NMR, ^{13}C NMR, HRMS spectra and X-ray single crystal diffraction (CCDC No. 962732, Fig. 1).

3.2. Spectroscopic properties and optical responses to pH

Spectroscopic properties of probe **L** were studied. From Fig. 2, we can find that the fluorescence intensity was high and stable when the pH value of the buffer is above 3.98, while it decreased drastically when the buffer pH went down from 3.98 to 0.65. Meanwhile, the quantum yield (Φ) decreased from 0.65 to 0.06 as calculated by the following formula, according to the literature [26].

$$\Phi_u = \frac{(\Phi_s)(F_u)(A_s)(\eta_u)^2}{(F_s)(A_u)(\eta_s)^2}$$