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A novel ratiometric fluorescent probe for the detection of HOCl based on FRET strategy

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ABSTRACT

A novel ratiometric fluorescent probe (**RIM**) for HOCl was designed on imidazo[1,5-*a*]pyridine-rhodamine FRET platform. Upon addition of HOCl, the imidazo[1,5-*a*]pyridine fluorescence increased and the rhodamine fluorescence increased simultaneously. The ratios (I_{585}/I_{465}) displayed brilliant HOCl-dependent performance and responded linearly to HOCl. **RIM** could respond to HOCl rapidly (within 20 s) with a low detection limit of 24 nM **RIM** also exhibited high selectivity, and low cytotoxicity. Moreover, it has been successfully applied in fluorescence imaging in living cells and the results confirmed that the probe could selectively stain lysosomes with excellent photostability.

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1. Introduction

Hypochlorous acid (HOCl)/hypochlorite ($^{-}$OCl) is one of the most significant reactive oxygen species (ROS), which is produced from the myeloperoxidase (MPO)-mediated peroxidation of chloride ions [1]. In innate immunity, HOCl acts as an oxidizing agent with powerful antibacterial activity once microbial invasion occurs [2,3]. Furthermore, it has been extensively employed in daily life, including the bleaching of paper and textile, as well as the disinfection of drinking water [4–6]. Despite its contributions to the destruction of bacteria in *vivo*, the abnormal level of HOCl causes oxidative stress and is involves in various disorders such as atherosclerosis, arthritis, cardiovascular diseases and even cancers [7–10]. Therefore, the precise measurement of HOCl has become an important area of research.

For quantitatively detecting HOCl in this respect, fluorescence-based techniques have been well established for both sensing and imaging applications. Fluorescent probes have become promising tools owing to their superb sensitivity and selectivity, simple operation and capability of real-time monitoring [11–16]. Many probes rely on the fluorescence changes in single-emission windows, the signal tends to be perturbed by temperature, solvent polarity and probe concentration. By contrast, ratiometric probes are more advantageous since they could eliminate the above interferences, thus providing more accurate detection of intracellular HOCl [17–22].

For designing ratiometric probes in general, constructing a fluorescence resonance energy transfer (FRET) system is considered to be an efficient approach, since FRET-based probes could reduce detection error and self-quenching [23,24]. However, the substantial overlap between the donor emission and acceptor absorption is a prerequisite for an efficient FRET process, which would limit the development of fluorescent probes on the basis of FRET strategy. Hence, it is of great significance to explore new fluorophores for the fabrication of FRET-based probes. Imidazo[1,5-*a*]pyridines have been largely investigated in the pharmaceutical area, including the aromatase inhibitors in estrogen-dependent diseases, thromboxane A2 synthetase inhibitors as well as cardiotonic agents [25–27]. Yet, their spectroscopic properties have been rarely studied perhaps for the lack of facile synthetic methods of imidazo[1,5-*a*]pyridines from easily accessible precursors [28].

Our group recently reported an FRET-based fluorescent probe for Cu^{2+}, comprising of imidazo[1,5-*a*]pyridine donor and rhodamine acceptor [29]. The results revealed that the emission spectrum of imidazo[1,5-*a*]pyridine and the excitation spectrum of rhodamine have large overlap, giving effective fuorescence energy transfer between energy donor and acceptor. Inspired by this, we designed an FRET-based fluorescent probe (**RIM**) based on imidazo[1,5-*a*]pyridine-rhodamine platform, which was capable of detecting HOCl. In comparison with other HOCl probes, **RIM**

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showed much lower detection limit (24 nM) [24,30,31]. In addition, **RIM** has been successfully applied to image endogenous HOCl in RAW264.7 cells with low cytotoxity.

2. Experimental

2.1. Apparatus and chemicals

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were acquired on a Bruker Avance spectrometer, with d_6-DMSO used as a solvent and tetramethylsilane (TMS) as an internal standard. High-resolution mass spectroscopy (HRMS) was collected on a Q-TOF6510 spectrograph (Agilent). UV–vis spectra were measured by a Hitachi U-3900 spectrophotometer. Fluorescent measurements were performed on a Perkin-Elmer LS-55 luminescence spectrophotometer. The pH was determined with a model PHSJ-3F pH meter (LeiCi, Shanghai, China). Quartz cuvettes with a 1-cm path length and 3-mL volume were used for all measurements.

All reagents and solvents were purchased from commercial sources and used without further purification. The solutions of metal ions were prepared from nitrate salts dissolved in deionized water.

2.2. Preparation of test solutions

Probe **RIM** was dissolved in ethanol to afford the stock solution (10^{-4} M). All UV–vis and fluorescence samples were prepared by displacing the stock solution into a 10 mL volumetric flask. The solution was diluted to 10 mL in a mixture solution of EtOH-PBS (pH 6) = 3:7, v/v. Appropriate aliquots of each testing species solution were then added. The resulting solutions were shaken well and incubated for 30 min at room temperature before UV–vis and florescence determination.

HOCl solution was prepared by diluting the commercially available sodium hypochlorite solution. ROS and reactive nitrogen species (RNS) solutions were prepared by the literature [24,32,33].

2.3. Synthesis of probe *RIM*

The synthetic route of probe **RIM** was displayed in Scheme 1.

Compound **1** and **3** were prepared according to our previous work [29]. Compound **1** (0.469 g, 1.0 mmol) and Lawesson's reagent (0.404 g, 1.0 mmol) were dissolved in 50 mL dry toluene. The solution was heated and kept reflux for 2 h under N_2 atmosphere, and then toluene was removed under reduced pressure to give violet solid (compound **2**). The crude product was used for next step without purification.

Compound **3** (0.277 g, 1.1 mmol) was added into thionyl chloride (10 mL) and kept stirring at room temperature for 4 h. After the reaction completed, thionyl chloride was removed under reduced pressure to give faint yellow solid (compound **4**) for next step without purification.

Compound **2** (1.0 mmol) was dissolved in 10 mL dry dichloromethane under an ice bath. A solution of compound **4** (1.1 mmol) and triethylamine (1 mL) in 10 mL dry dichloromethane was added dropwise over a period of 30 min at 0 °C. The mixture was stirred at room temperature for 4 h under N_2 atmosphere, and then diluted with 100 mL dichloromethane. The organic layer was washed with water (100 mL × 3), dried over anhydrous Na_2SO_4 and filtered. Then the solvent was evaporated under reduced pressure and the resulting crude product was purified by column chromatography on silica gel using dichloromethane/methanol (50/1, v/v) as the eluent to afford **RIM** as a yellow solid (150 mg, yield: 21%). ^{1}H NMR (400 MHz, d_6-DMSO), δ (ppm): 0.89 (t, 3H, J = 7.4 Hz, CH_2 $CH_2CH_2CH_3$), 1.06 (t, 6H, J = 6.8 Hz, NCH_2CH_3), 1.29-1.38 (m, 2H, CH_2), 1.65-1.72 (m, 2H, CH_2), 2.92 (t, 2H, J = 7.4 Hz, CH_2), 3.25 (br, 4H, $N(CH_2CH_2)_2$), 3.28-3.35 (m, 4H, NCH_2CH_3), 3.64 (br, 4H, $CON(CH_2CH_2)_2$), 5.36 (s, 2H, NNH_2), 6.18 (d, 1H, J = 8.4 Hz, ArH), 6.23 (d, 1H, J = 8.4 Hz, ArH), 6.36 (d, 2H, J = 9.2 Hz, ArH), 6.65 (d, 1H, J = 9.2 Hz, ArH), 6.73 (d, 2H, J = 6.0 Hz, ArH), 7.03 (d, 1H, J = 6.8 Hz, ArH), 7.50 (m, 3H, ArH), 7.88 (d, 1H, J = 7.2 Hz, ArH), 8.26 (d, 1H, J = 7.2 Hz, ArH). ^{13}C NMR (100 MHz, d_6-DMSO), δ (ppm): 181.3, 167.1, 152.7, 152.5, 151.7, 149.1, 148.7, 138.4, 135.8, 132.1, 128.8, 127.7, 125.9, 123.8, 123.2, 122.9, 122.3, 118.2, 115.6, 111.9, 111.7, 108.6, 102.9, 101.9, 97.3, 72.4, 63.0, 43.7, 41.5, 28.6, 25.2, 21.7, 13.7, 12.4. HRMS m/z: calcd for $C_{40}H_{43}ClN_7O_2S$ $[M+H]^+$: 720.2887; found: 720.2896.

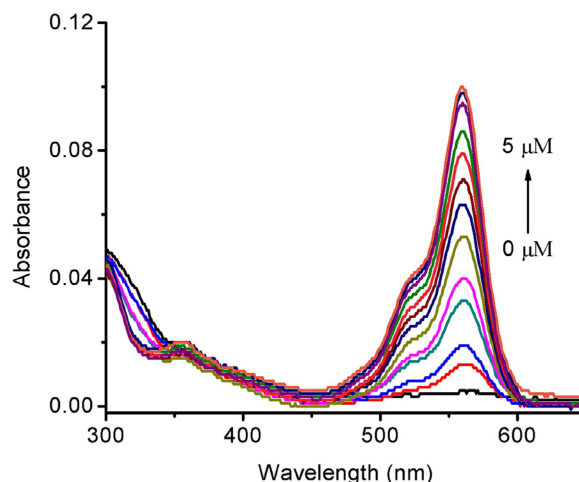

Fig. 1. Absorption spectra of probe **RIM** with the addition of HOCl. Conditions: [**RIM**] = 2 μM, [HOCl] = 0–5 μM, EtOH-PBS (pH 6) = 3:7, v/v.

2.4. Cell culture and imaging

RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum in an atmosphere containing 5% CO_2 and 95% air at 37 °C. The cells were imaged using confocal fluorescence microscopy (Leica SP8)

3. Results and discussion

3.1. Design and synthesis of probe *RIM*

Since the emission spectrum of imidazo[1,5-*a*]pyridine and the excitation spectrum of rhodamine largely overlapped, the imidazo[1,5-*a*]pyridine-rhodamine FRET pair was chosen here, thus ensuring the efficient energy transfer. Probe **RIM** was synthesized via a simple process (Scheme 1) and characterized by ^{1}H NMR, ^{13}C NMR and HRMS (Fig. S1-S3, ESI†).

3.2. Spectroscopic response of probe *RIM* to HOCl

The absorption spectra of **RIM** were recorded upon the addition of HOCl (Fig. 1). The probe only exhibited an absorption band centered at 360 nm, which represented the absorption of imidazo[1,5-*a*]pyridine moiety. However, the addition of HOCl resulted in the emergence of a novel absorption peak at 560 nm and the absorbance increased gradually along with the added HOCl. Meanwhile, the absorbance at 360 nm remained almost unchanged. The phenomena could be ascribed to the structural formation of ring-opened form of rhodamine moiety in the probe.

We next investigated the fluorescence properties of **RIM** with various concentrations of HOCl upon excitation at 370 nm. As shown in Fig. 2a, probe **RIM** only exhibited fluorescence emission

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