

A novel far-red fluorescence bioimaging probe based on rhodamine 101 derivative for Cu²⁺ with high sensitivity and selectivity

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Abstract. A novel rhodamine 101 derivative has been synthesized and employed as a fluorescent off-on probe for monitoring Cu²⁺ in aqueous solutions and living cells. Under physiological conditions, it exhibits a remarkable increase in absorbance at 587 nm and an enhanced fluorescence at 615 nm toward Cu²⁺ in a highly sensitive, selective and rapid manner. The probe can be applied to the quantification of Cu²⁺ with a linear concentration range covering from 0.4 M to 16 M ($R^2 = 0.9955$) and a detection limit of 40 nM. Furthermore, the proposed probe has been successfully applied for fluorescence imaging of Cu²⁺ in living cells.

Introduction

Copper, the third most abundant element in human bodies among the essential heavy metals, plays a crucial role as a catalytic cofactor for a vast variety of metalloenzymes, such as superoxide dismutase, cytochrome c oxidase and tyrosinase^[1]. However, the overload of copper ions in living systems can cause adverse health effect^[2,3]. In addition, copper is a widely used pollutant. Therefore, measurement of Cu²⁺ with high sensitivity, selectivity and rapid response is of significant interest in the field of chemical, environmental and biological science.

Although numerous fluorescent probes for sensing of Cu²⁺ have been developed recently^[4-11], there still has a great space to develop novel ones which can overcome these limitations, including only work in pure organic solvents^[6], ultraviolet excitation and shorter emission (below 550 nm)^[8], operation at non-physiological pH for fluorescence imaging, turn-off manner in emission spectra upon Cu²⁺ binding due to the fluorescence-quenching nature of paramagnetic Cu²⁺.^[7, 8, 10]

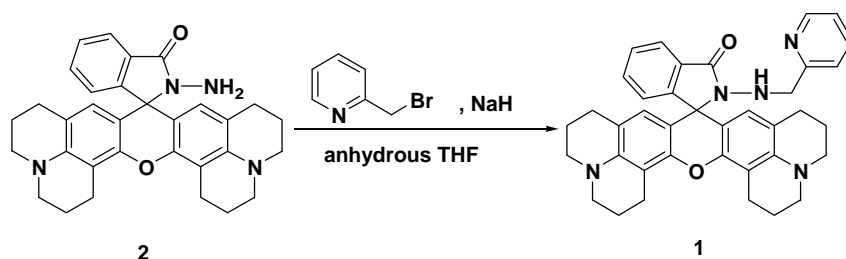
Rhodamine spirolactam is non-fluorescent and colorless, whereas its ring-opening form gives rise to strong fluorescent emission and a pink color. By utilization of this unique ring-opening process, many metal ions (including Cu²⁺)-amplified fluorescent probes have been developed^[12, 13]. However, rhodamine B or 6G is mainly used as scaffold for such fluorescent probes. Compared with rhodamine B or 6G, rhodamine 101 has two amino groups rigidly linked to the xanthene skeleton at 3 and 6-position by multiple n-propylene bridges, and its maximal emission wavelength reaches well into the far-red region (> 600 nm), which is more suitable for biological application. Herein, rhodamine 101 derivatives (probe **1**) was designed, synthesized, and applied for Cu²⁺ detection in vitro and in vivo under physiological conditions with satisfactory results.

Experimental

Reagents and apparatus. Before used, tetrahydrofuran (THF) was freshly distilled from sodium chips under argon with benzophenone/ketyl as indicator. All other reagents used were of analytical reagent grade. Twice distilled water was used throughout all experiments.

¹H NMR spectra were recorded on a Bruker Avance II 400 spectrometer operating at 400 MHz, respectively. All chemical shifts are reported in the standard δ notation of parts per million. LC-MS analysis was performed using an LXQ Spectrometer (Thermo Scientific, USA) operating on ESI. UV-vis absorption spectra were recorded on a Shimadzu UV-2600 spectrophotometer. All fluorescence measurements were conducted on a Thermo Scientific Lumina fluorescence spectrometer

with excitation slit set at 5.0 nm and emission at 5.0 nm. Fluorescence images of HepG2 cells were carried out with an inverted fluorescence microscope (Carl Zeiss, Axio ObserverA1). The pH measurements were carried out on a PB-10 digital pH meter (Sartorius, Germany) with a combined glass-calomel electrode.



Scheme 1. Chemical structure and synthetic route of compound **1**.

Synthesis of compound 1. A stirred suspension of NaH (60% dispersion in oil, 0.060 g, 1.5 mmol) in anhydrous THF (3 ml) was added to a solution of rhodamine 101 hydrazide^[14] (compound **2**, 0.252 g, 0.5 mmol) in anhydrous THF (2 ml), and the resulting mixture was stirred at room temperature for 0.5 h. 2-(bromomethyl) pyridine hydrobromide (0.129 g, 0.75 mmol) was added in portions, and then refluxed under N₂ for 3 h. The mixture was cooled, filtered, and diluted with CHCl₃. It was then successively washed with water, brine and dried over anhydrous Na₂SO₄. The crude product was purified as a white solid by column chromatography (CH₂Cl₂/C₂H₅OH/Et₃N, 50/1/0.3, v/v/v). The yield of compound **1** was 50%.

¹H NMR (CDCl₃, 400 MHz) δ (ppm): 1.97-2.08 (m, 8H), 2.46 (t, 4H, J=6.4 Hz), 2.90 (t, 4H, J=6.8 Hz), 3.06-3.17 (m, 8H), 3.96 (s, 2H), 4.79 (m, 1H), 6.00 (s, 2H), 7.05-7.09 (m, 2H), 7.20 (d, 1H, J=7.6 Hz), 7.44-7.47 (m, 2H), 7.48-7.54 (m, 1H), 7.92-7.94 (m, 1H), 8.40-8.41 (m, 1H); ESI-MS: [M+H]⁺=596.30, calculated for C₃₈H₃₇N₅O₂: [M+H]⁺=596.54, [M+Na]⁺=618.58.

Results and discussion

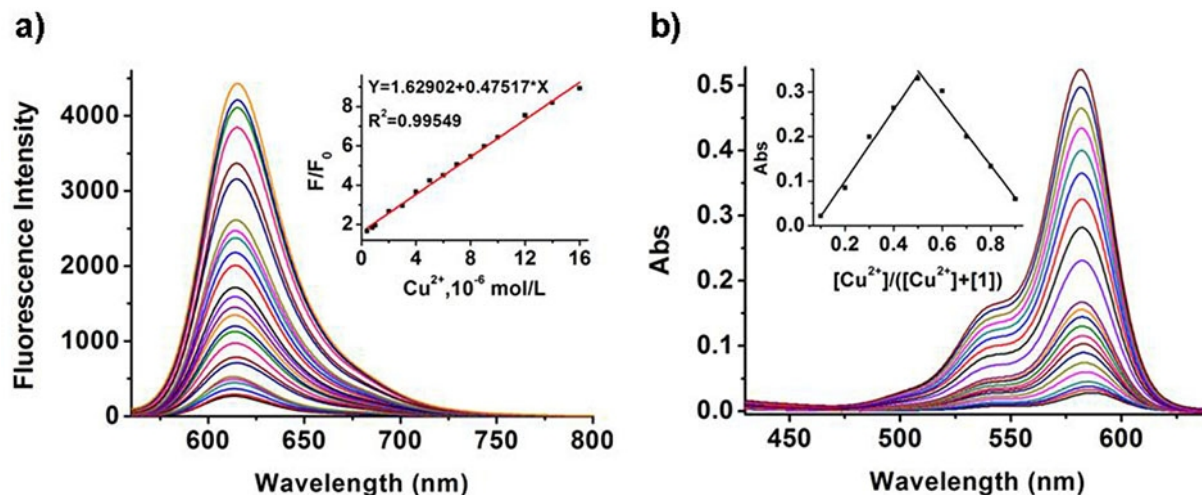


Fig.1 Changes in fluorescence emission (a, λ_{ex} =540 nm) and absorption (b) spectra of **1** (10 μ M) upon addition of increasing concentrations (0~100 μ M, from bottom to top) of Cu²⁺ in test system. Inset (a): The plot of F/F_0 as a function of the concentration of Cu²⁺. Inset (b): Job's plots for **1** in test system (the total concentration of **1** and Cu²⁺ was 20 μ M).

Spectral Characteristics. The fluorescence titration of the Cu²⁺ ion was performed with a solution of 10 μ M of probe **1** in test system (pH = 7.4 Tris-HCl / CH₃CN, 7:3, v/v). On the titration of Cu²⁺, as shown in Fig. 1a, an emission band centered at 615 nm (λ_{ex} = 540 nm) increases. With the concentration of Cu²⁺ up to 10 equiv of probe **1**, a 16.7-fold fluorescence enhancement was observed. The fluorescent response of probe **1** toward Cu²⁺ was calculated to cover a linear range from 0.4 to 16 μ M of Cu²⁺ (Fig.

1a, inset), with a detection limit of 40 nM (based on $S/N=3$). The absorption spectra were carried out in the same conditions mentioned above (Fig. 1b). In the absence of Cu^{2+} , the solution exhibits weak absorption in the visible wavelength range, indicating that **1** is predominantly in the form of spirolactam. Upon the gradual addition of Cu^{2+} to the solution, a new absorption peak at 587 nm emerged with increasing intensity, and the solution displayed a clear change from colorless to pink simultaneously, which can be ascribed to variation from a spirocyclic form to a ring-opened amide form. The Job's plot (Fig. 1b, inset) was conducted when $[\mathbf{1} + \text{Cu}^{2+}] = 20 \mu\text{M}$, and the turn point clearly appears at 0.5, indicating a 1:1 stoichiometry ratio of probe **1** to Cu^{2+} .

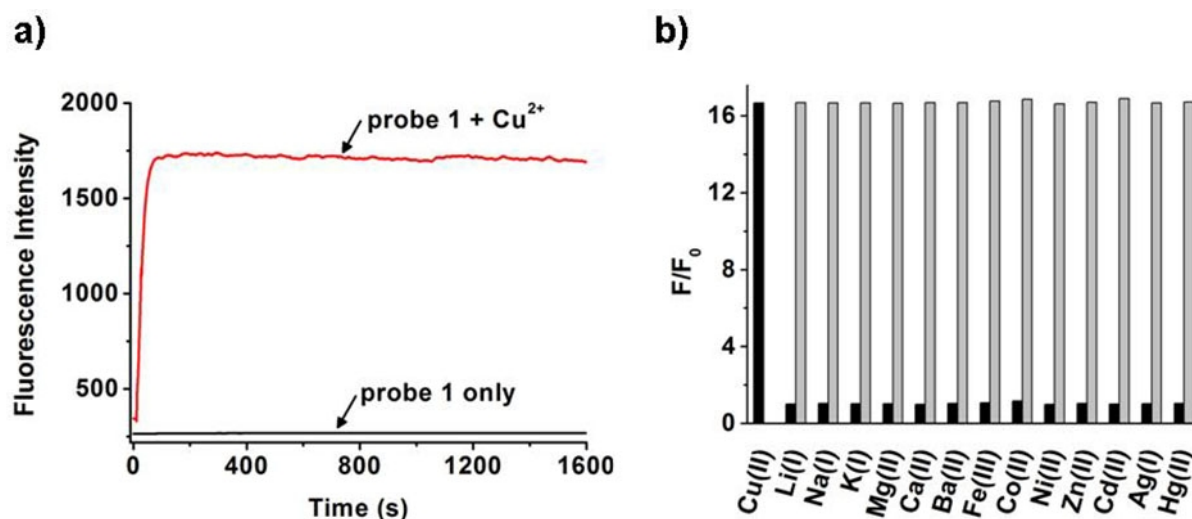


Fig.2. **a)** Time dependent fluorescence intensity (615 nm) changes of probe **1** (10 μM) in the absence or presence of 10 μM Cu^{2+} . **b)** Fluorescence response of **1** (10 μM) to 100 μM metal ions (the black bar portion) and to the mixture of 10 μM of other divalent metal ions with 100 μM of Cu^{2+} (the gray bar portion).

Response Time. A short response time is necessary for a fluorescent probe to monitor Cu^{2+} in aqueous samples and in living cells. The kinetic characteristics of probe **1** chelation with Cu^{2+} were investigated by fluorescence spectroscopy. As shown in Fig. 2a, the reaction of 10 μM of probe **1** with 10 μM of Cu^{2+} was completed within 1 min, denoting the rapid chelation of probe **1** with Cu^{2+} . Thus, probe **1** can be potential utilized in real-time determination of Cu^{2+} in living cells. No change in fluorescence was observed in the absence of Cu^{2+} .

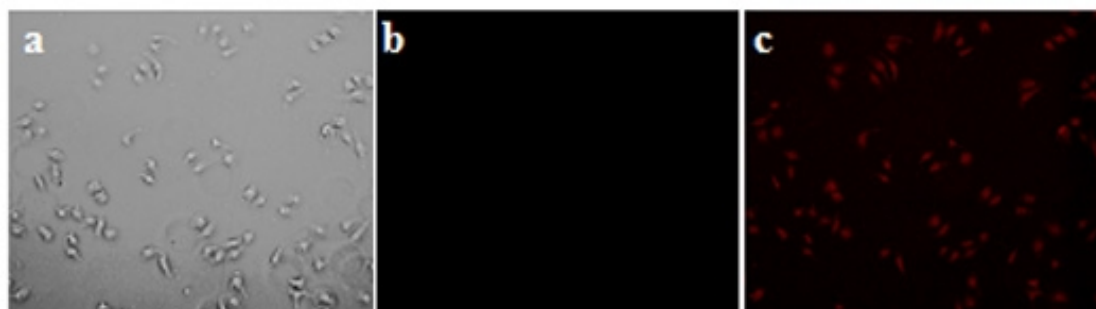


Fig. 3. Bright-field transmission image (a) and fluorescence image (b) of HepG2 cells incubated with **1** (10 μM) for 30 min. (c) Fluorescence image of the cells above further incubated with 10 μM Cu^{2+} for another 30 min.

Selectivity. Selectivity is another very important aspect to evaluate the performance of a fluorescence probe. From Fig. 2b, one can see that no significant fluorescence intensity change of **1** (10 μM) occurred in the presence of other metal ions (100 μM). In contrast, upon the addition of Cu^{2+} (100 μM) into **1** (10 μM) containing the interfering metal ions (100 μM for each), a remarkable fluorescence intensity centered at 615 nm was observed.

Detection of Cu^{2+} in living cells. Taking into account the advantage of real-time response,

remarkably high sensitivity and selectivity under physiological condition, probe **1** was employed for *in vitro* Cu²⁺ detection in living cells on an inverted fluorescence microscope. No significant fluorescence could be observed in the absence of Cu²⁺ (Fig. 3b). After incubation with Cu²⁺, however, a bright fluorescence was observed in living cells (Fig. 3c). The results suggest that probe **1** can penetrate the cell membrane and respond to Cu²⁺ in living cells.

Conclusions

In summary, we have developed a new fluorescent probe which exhibits a fluorescence response toward Cu²⁺ in aqueous solution with high sensitivity and selectivity. The probe can be used to detect Cu²⁺ in far-red region. Most importantly, the proposed probe cannot only monitor Cu²⁺ in solutions, but also be used for fluorescent imaging Cu²⁺ in living cells with satisfying results.

Acknowledgements

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