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Rhodamine-based 'turn-on' fluorescent probe for Cu(II) and its fluorescence imaging in living cells

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ABSTRACT

A novel rhodamine spirolactam derivative 3′,6′-Bis(diethylamino)-2-(2-hydroxyethylamino) spiro[isoindoline-1,9′-xanthen]-3-one (**RO1**) was synthesized, and characterized by high-resolution mass spectrometry (HRMS), X-ray crystallography, Infrared spectroscopy (IR), and ^{1}H NMR and ^{13}C NMR spectroscopy. **RO1** exhibited highly sensitive and exclusively selective fluorescence response toward Cu^{2+} over other metal ions with a detection limit of 0.56 ppb in mixed aqueous solution. The fluorescence was pH-independent in the wide range pH 3.1–11.6. The turn-on fluorescence enhancement of the probe is based on Cu^{2+} induced ring-opening mechanism of the rhodamine spirolactam. Moreover, by means of fluorescence microscopy experiments, it was demonstrated that **RO1** could monitor trace Cu^{2+} changes by live cell imaging.

The design and development of a fluorescent probe with high selectivity and sensitivity targeting heavy and transition metal cations are of intense research activity, since they allow nondestructive and prompt detection of metal cations by a simple fluorescence enhancement (turn-on) or quenching (turn-off) response.[1–4] Among transition metal ions, copper is the third most abundant trace element in the human body and many living organisms, and is involved in redox processes of a number of biomolecules.[4] Besides the beneficial effects, excess copper in the human body has been reportedly linked to serious health threats such as cellular or organ damage and neurodegenerative disease, like Wilson disease, and Alzheimer's disease.[5] Accordingly, the U.S. Environmental Protection Agency (EPA) has set the maximum allowable level of copper in drinking water at 1.3 ppm (~20 μM).[6] Nevertheless, copper is present in waste effluents generated by various industries (i.e., electroplating, wood, painting, textile, and paper industries), and can be accumulated in the environment and food chain, especially in fish. It is, therefore, of considerable significance to develop highly sensitive and selective probes for Cu^{2+} determination. In recent years, many fluorescent probes for copper ion selective detection have been explored.[7–12] However, some of them suffered from drawbacks of practical application in terms of complicated organic synthesis, low sensitivity, cross-sensitivity toward other metal cations, a narrow pH span, a low fluorescence quantum yield, and cytotoxicities of ligand. In addition, fluorescence detecting of Cu^{2+} is particularly challenging since Cu^{2+} generally acts as quencher via the electron transfer and facilitated intersystem crossing (isc) processes.[1]

As a fluorogenic unit (fluorophore), rhodamine derivatives are one of the most promising dyes due to their pretty good photophysical properties, such as long absorption and emission wavelength, large extinction coefficient, high fluorescence quantum yield, and high light stability. Recently, many fluorescent probes based on rhodamine B, which were driven by visible light excitation have been studied. They can show turn-on response to the targeted HTM cation,[13] such as Cu^{2+},[14–19] Hg^{2+},[20,21] Fe^{3+},[22] Zn^{2+},[23] Cr^{3+},[24] Pd^{2+}.[25] The cation-sensing mechanism of these probes was based on the change in structure between spirocyclic and opencyclic forms. Without cations, these probes existed in a spirocyclic form, which was colorless and nonfluorescent. Addition of metal cation lead to the ring-opening of the corresponding spirocycle, and gave rise to a strong fluorescence enhancement and a color change.

Fluorescence imaging technology was regarded as a significant approach for visualizing subcellular distribution of Cu^{2+} in physiological process by virtue of its outstanding properties.[26] To image intracellular copper ions, highly sensitive, selective, and cell membrane-penetrable probes that exhibit an enhanced visible fluorescent emission in aqueous media need to be developed. In recent years, a lot of fluorescence 'off–on' probes for imaging various

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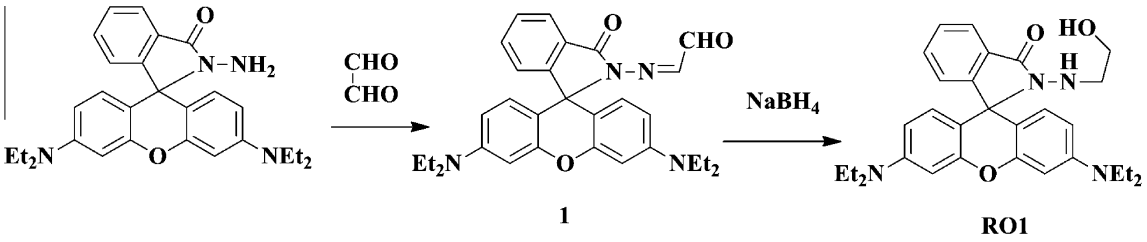

Scheme 1. Synthesis route of probe **RO1**.

intracellular metal ions have been developed. However, just a few probes reported for imaging Cu^{2+} in living cells.[27–36] Thus, it is still very necessary to design small-molecule fluorescent probes for Cu^{2+} with favorable photophysical properties suitable for imaging of copper ions in living cells.

Our research work involves the design, synthesis, spectroscopy, and biological applications of fluorescent probes for selective sensing of ions as well as evaluation of their switching behavior.[3] In this article, we have designed and synthesized a new rhodamine derivative **RO1**. The hydroxyethylhydrazine moiety is introduced into **RO1** due to its good binding affinity for transition metal ions.[18] The catalytic hydrolysis of hydroxyethylhydrazine moiety in **RO1** was selectively induced by Cu^{2+} ions to produce irreversible fluorescence turn-on changes ascribed to a ring opened form of the rhodamine. Especially, **RO1** showed excellent sensitivity and selectivity with a detection limit of 0.56 ppb in mixed aqueous media. **RO1** was pH-independent, but Cu^{2+} induced large increases in fluorescence intensity of **RO1** between pH 3.10 and 11.6. Herewith, we also present the bioapplication of **RO1** as a fluorescent probe to detect Cu^{2+} in cultured cells.

As depicted in Scheme 1, Compound **1** was synthesized according to the literature.[37] Compound **1** was dissolved in absolute ethanol. Sodium borohydride was then added slowly. The mixture was stirred under N_2 atmosphere at room temperature for 2 h. The crude product was purified to give **RO1** as a white solid in 89.9% yield.

The single crystal suitable for X-ray analysis was obtained by slow evaporation of the CH_2Cl_2/*n*-hexane solution. Table S1 summarizes the crystal data, data collection and refinement parameters for **RO1**. The crystal structure of **RO1** clearly reveals the unique spirolactam ring formation,[38] with the lactam and xanthene moiety form vertical planes (Fig. S1), which breaks the conjugation of the whole system, thus leading to the non-fluorescence of the molecule.

Spectral changes of **RO1** in CH_3CN/H_2O (1/1, v/v, 10 mM Tris–HCl buffer, pH = 7.15) solution were tested (Fig. S2) upon addition of various competitive metal ions, such as Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Cd^{2+}, Cr^{3+}, Co^{3+}, Ni^{2+}, Ag^+, Pb^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+}, Cu^+, Cu^{2+}. From UV/vis spectra of **RO1** (10 μM) (Fig. S2A), we can clearly observe a new absorption band centered at 562 nm in the presence of 5 equiv of Cu^{2+}. In contrast, other metal ions did not lead to any distinct spectral changes. On the other hand, fluorescence spectra (Fig. S2B) also showed a similar result, which was well consistent with that of UV/vis spectra. Addition of only 5 equiv Cu^{2+} resulted in a dramatic change of fluorescence intensity peaked at 589 nm (off–on), while other metal ions including Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Cd^{2+}, Cr^{3+}, Co^{3+}, Ni^{2+}, Ag^+, Pb^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+}, Cu^+ did not give rise to any fluorescence increases. Moreover, in the presence of miscellaneous competitive cations, the Cu^{2+} still resulted in the similar fluorescence changes (Fig. S3). In addition, the increases of fluorescence intensity resulting from the addition of the Cu^{2+} were not influenced by the subsequent addition of miscellaneous cations. More importantly, **RO1** is selective for Cu^{2+} over Cu^+, showing that this probe has metal and redox specificity. All of these results indicated that the selectivity of **RO1** for the Cu^{2+} over other competitive cations in the water medium was remarkably high, which was well consistent with that of UV/vis spectra.

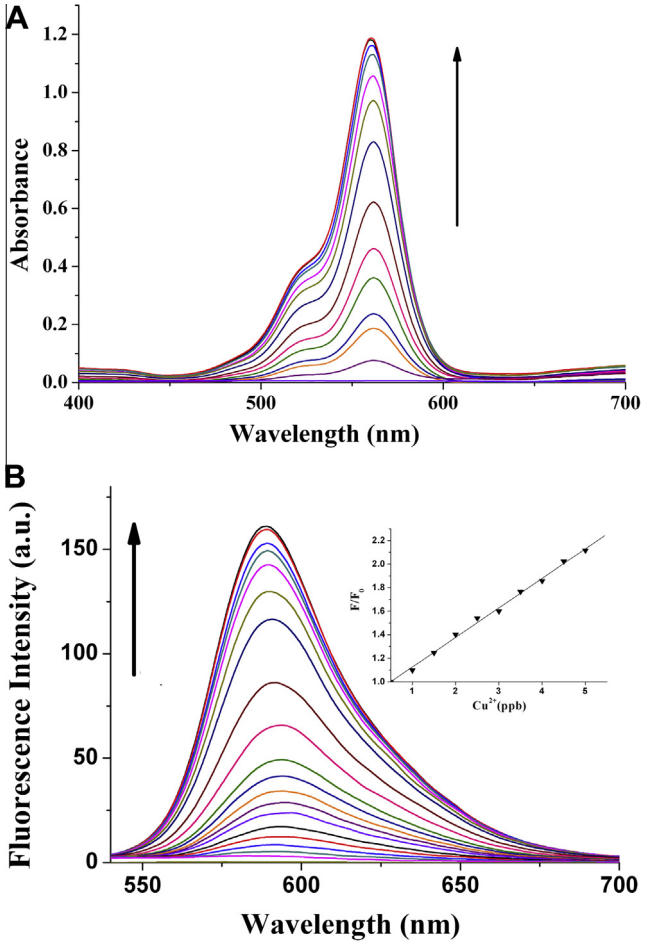

Figure 1. (A) Absorption spectra of **RO1** (10 μM) in CH_3CN/Tris–HCl buffer (5:5, v/v, pH 7.15) upon addition of different amounts of Cu^{2+}. (B) Fluorescence spectra of **RO1** (10 μM) in CH_3CN/Tris–HCl buffer (5:5, v/v, pH 7.15) upon addition of different amounts of Cu^{2+} (λ_{ex} = 510 nm). Inset: the changes of fluorescence intensity of **RO1** (10 μM) upon addition of Cu^{2+} (1–5 ppb).

Figure 1 shows a spectral variation of **RO1** upon the gradual addition of Cu^{2+}. The UV–vis titration spectra of Cu^{2+} was conducted using 10 μM of **RO1** in acetonitrile aqueous solution at pH = 7.15. Upon the addition of increasing concentrations of the Cu^{2+} (0–70 μM), a new absorption band centered at 562 nm appeared with increasing intensity, which induced a clear color change from colorless to pink (Fig. 1A). On the other hand, for the fluorescence titration spectra of **RO1**, in the presence of Cu^{2+} there was also a new emissive peak at 589 nm (Fig. 1B), which was in good consistency with the results of UV–vis spectra. Both UV–vis and fluorescence data lead to a significant off–on signal. From the molecular structure and spectral

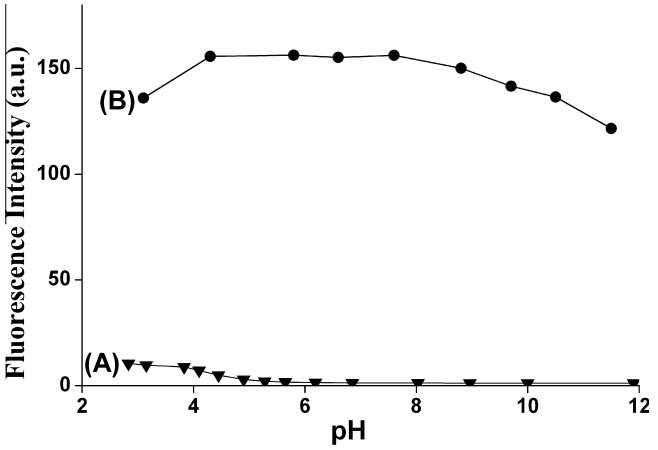

Figure 2. Fluorescence intensity (589 nm) of free **RO1** (10 μM) (▼) and after addition of 50 μM of Cu^{2+} (●) in the CH_3CN/H_2O (1:1, v/v, 10 mM Tris–HCl buffer, pH 7.15) solution as a function of different pH values. Excitation at 510 nm.

results of **RO1**, it is probably concluded that the addition of Cu^{2+} induced a ring-opening of the spirolactam in rhodamine framework, observed by the distinct color change and fluorescence off–on. The F/F_0 was proportional to the amount of Cu^{2+} added in ppb level with a detection limit of 0.56 ppb (Fig. 1B inset). The stoichiometry of a binding event between **RO1** and Cu^{2+} was also determined. The results obtained from Job's plot show the 1:1 stoichiometry between **RO1** and Cu^{2+} (Fig. S4).

The binding interaction of **RO1** with Cu^{2+} was investigated. **RO1** is a colorless, nonfluorescent compound. Upon the addition of Cu^{2+}, a pink color was observed, and a bright fluorescence developed simutaneously (Fig. S5). The above resulting colored solution was subsequently treated with excess EDTA, and the strong fluorescence of the **RO1**–Cu^{2+} was almost not affected by the addition of EDTA solution, indicating that the interaction of **RO1** with Cu^{2+} is chemically irreversible. The formation of rhodamine B as product was confirmed by comparison of the product's TLC data with that of authentic rhodamine B. The formation of rhodamine B as product was also confirmed by HRMS: m/z $[M+H]^+$ = 443.2337 (authentic rhodamine B: m/z $[M+H]^+$ = 443.2343) (Fig. S6). These results clearly showed that rhodamine B was formed in the reaction. The turn-on response of fluorescence in this system may be proposed to proceed through the following route (Scheme S1): **RO1** binds Cu^{2+}, and the subsequent complexation of Cu^{2+} promotes hydrolytic cleavage of the amide bond, leading to the release of rhodamine B and thus the retrievement of fluorescence.

It is well known that pH-insensitivity of probes is extremely valuable for practical applications.[37] Therefore, the proper pH environment of the probe **RO1** was also evaluated (Fig. 2). The acid titration control experiments revealed that no obvious fluorescence change of **RO1** could be observed between pH 3.10 and 11.6, suggesting that **RO1** is insensitive to pH and that the spirolactam form is still preferred in this range. Furthermore, the effect of pH on the fluorescence intensity of **RO1** (10 μM) in the CH_3CN/H_2O solution in the presence of Cu^{2+} (50 μM) was performed. 5 equiv of Cu^{2+} induced large increases in fluorescence intensity of **RO1** between pH 3.10 and 11.6. These results indicated that **RO1** would provide the potential applications in some environmental and physiological regions for the detection of Cu^{2+} in the wide pH range.

To demonstrate the practical applicability of the probe in biological samples, fluorescence imaging experiments were carried out in living cells. Hela cells were incubated with **RO1** (10 μM) for 0.5 h at 37 °C, then followed by the addition of Cu^{2+} (50 μM) and incubated for another 30 min. The cells were washed with 1% PBS buffer solution, and their fluorescence images were recorded before and after addition of Cu^{2+} (Fig. 3). In the absence

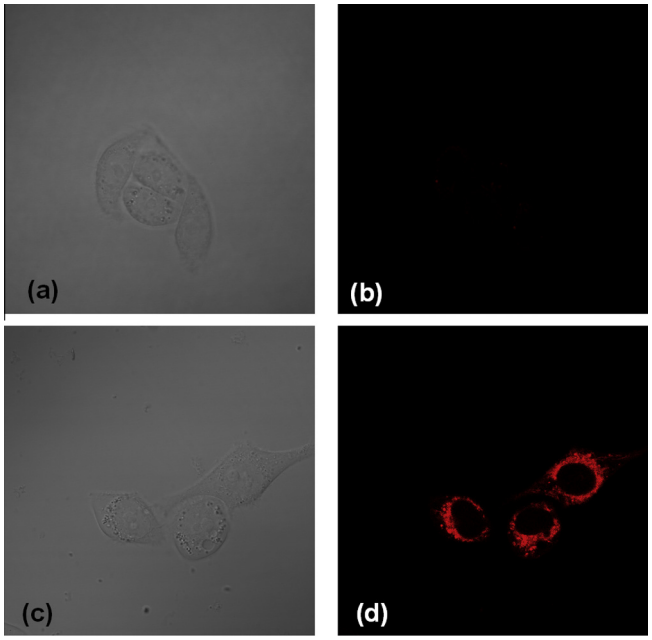

Figure 3. Fluorescence images of Cu^{2+} ion level using **RO1** in living Hela cells; (a) brightfield image of living Hela cells treated with **RO1** (10 μM); (b) fluorescence image of cells in panel (a); (c) brightfield image of **RO1** (10 μM) loaded cell exposed to 50 μM Cu^{2+}; (d) fluorescence image of cells in panel (c). The excitation wavelength was 515 nm and the emission was collected at 575–620 nm.

of Cu^{2+}, free **RO1** showed no detectable fluorescence signal in living cells. After incubation with Cu^{2+}, a bright fluorescence was observed in living cells. The results suggested that probe **RO1** can penetrate the cell membrane and be applied for in vitro imaging of Cu^{2+} in living cells.

In summary, a new rhodamine spirolactam based fluorescence probe **RO1** for Cu^{2+} has been synthesized and structurally characterized. Fluorescence changes of **RO1** are remarkably specific for Cu^{2+} in the presence of other commonly coexistent metal ions. Furthermore, the parts per billion level fluorescent detection limit suggests the possibility of practical applications in biology and environmental sciences. In addition, **RO1** shows pH-independent in the wide range 3.10–11.6. Especially, the probe **RO1** can be employed as a Cu^{2+}-selective probe in the fluorescence imaging of living cells. Such a sensor would become valuable in revealing the roles of Cu^{2+} in biological systems under either in vitro or in vivo conditions. The development of new rhodamine-based probes by incorporating various ligand groups is in progress in our laboratory.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.052.

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38. Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as SUPPLementary publication CCDC NO. 931232,, Copies of the data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data centre, 12 Union Road, Cambridge CN21EZ, UK; fax: +44 1223 33603 3; e-mail: deposit@ccdc.cam.ac.uk).