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A Highly Selective and Reversible Fluorescent Cu^{2+} and S^{2-} Probe in Physiological Conditions and in Live Cells

Cite this: DOI: 10.1039/xoxxoooooox

Quan Zhou, Yong Zhu, Pengtao Sheng, Zeming Wu, Qingyun Cai*

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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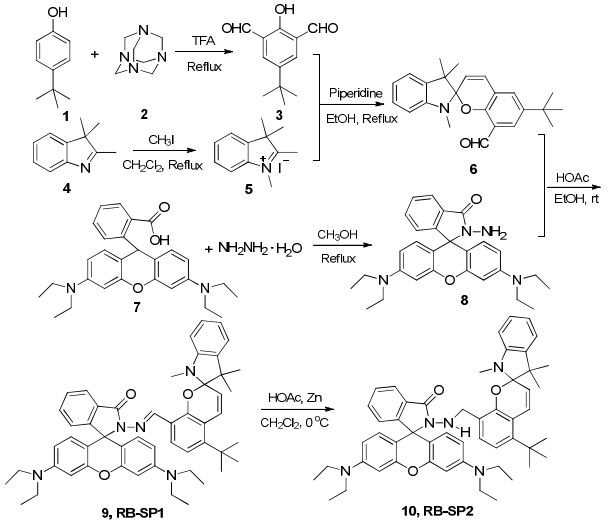
A new spiropyran functionalized rhodamine derivative RB-SP2 has been synthesized and applied to detect Cu^{2+} and S^{2-}. RB-SP2 was then used as an imaging probe for detection of these ions in HeLa cells at the physiological pH.

The fluorescent chemosensor capable of sensing specific analytes has potential applications in chemistry and biology,[1] as it generally allows detection of analytes present in ultratrace quantity. Such detection of heavy metal ions is of high importance due to the high toxicity of these metal ions toward human health.[2] Fluorescent sensors for the detection of Cu^{2+} are actively investigated, as it is a significant environmental pollutant and also an essential element for humans.[3] Meanwhile, development of selective and efficient signaling units for detection of various chemically and biologically important anions has also attained significant interest.[4] Being one of the biologically and environmentally important anions, sulfide is largely used in industrial processes.[5] Consequently, there are high risks for the sulfide ions to be exposed to drinking water. Sulfide can damage mucous membranes and can cause unconsciousness and respiratory problems.[6] Therefore, development of a quick and sensitive fluorescence probes for the detection of S^{2-} and Cu^{2+} in aqueous media and in biological systems is of high interest.

Although a significant number of rhodamine-based fluorescent probes have been developed for different metal ions (Zn^{2+}, Fe^{3+}, Hg^{2+}),[7] a few ones have been reported for copper.[8] In this work, a new fluorescent probe **RB-SP2** (Scheme 1) is proposed. **RB-SP2** is capable of detection both Cu^{2+} and S^{2-} at physiological pH.

Compound **RB-SP2** was readily synthesized in six steps. Condensation of **1** with **2** afforded the intermediate **3**; which was then treated with intermediate **5** under the basic conditions to give the intermediate compound **6**; and **7** was treated with hydrazine hydrate affording the intermediate **8**, then compound **6** with **8** afforded **9** (**RB-SP1**), which was then treated with znic powder under the acidic conditions to give the target compound **RB-SP2** (Scheme 1). Details about the synthesis and characterization of **RB-SP2** are presented in the ESI†



Scheme 1 Synthesis of compound RB-SP2

The compound **RB-SP2** is synthesized by reducing **RB-SP1** which is composed of spiropyran and rhodamine. The N and O atoms of rhodamine can coordinate with metal ions inducing the ring-open of spiropyran, and consequently an increase in fluorescence. Considering that the Schiff base of **RB-SP1** may respond to many transition metal ions, and in order to enhance the selectivity of the fluorescent probe, **RB-SP1** was reduced to **RB-SP2**, and the next experiment has proved this point.

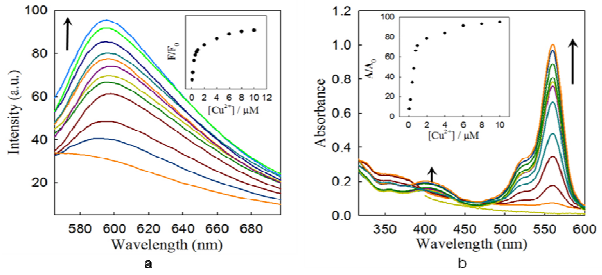
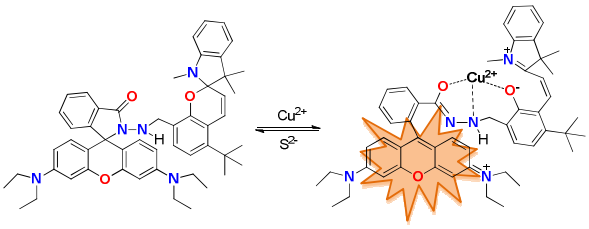


Fig. 1. Changes in the (a) fluorescence (λ_{ex} = 500 nm) and (b) UV-vis absorption spectra of **RB-SP2** (10 μM) in the presence of increasing concentrations of Cu^{2+} (0-10 μM) in C_2H_5OH/aqueous PBS (1 mM, pH 7.4; 4:6 v/v; 1% DMSO) solutions. Inset: the Cu^{2+} concentration-dependent responses at room temperature.

As evident from Fig. 1a, excitation of the initial solution of **RB-SP2** at 500 nm wavelength did not show any significant emission over the range from 560 to 680 nm (orange line). This supports the fact that in absence of Cu^{2+}, **RB-SP2** remains in the ring-close form. Addition of Cu^{2+} to **RB-SP2** solution induces a significant switch ON fluorescence response near 590 nm, with a visual display of reddish fluorescence. The switch ON responses for the emission band at ~590 nm on binding to Cu^{2+} suggest the opening of the spirolactam ring in **RB-SP2** through the coordination with Cu^{2+}. The fluorescence intensity at ~590 nm increases with increasing the Cu^{2+} concentration, with about 300 % increase of its fluorescence intensity upon addition of only 10 μM Cu^{2+} ions. The minimum detectable concentration was 1×10^{-7} M. We have measured the liner equation detecting the Cu^{2+} (Figure S1, see ESI†) which was y=1.25x+1.24 with a liner range from 0.1 μM to 1.0 μM, the limit of detection was calculated as 0.05 μM from the 3s method.

UV-vis spectra recorded for **RB-SP2** in C_2H_5OH/aqueous PBS indicated the maximum absorption at 324 nm (Fig. 1b), which may be attributed to the intra-molecular π–π* charge transfer (CT) transition. According to previous studies some transition-metal ions can selectively bind with suitable derivatives of rhodamine,[9] inducing the opening of the spirolactam ring and generation of the xanthene form. This structural change is manifested in the electronic and fluorescence spectral patterns. Significant change in UV-vis spectrum was observed in the presence of Cu^{2+} (Fig. 1b). The absorption band appeared around 550 nm increases with increasing Cu^{2+} concentration from 0 μM to 10 μM (Fig. 1b), and the solution turned from colorless to pink (Fig. S2, see ESI†). It should be noted that a small adsorption band between 400 and 450 nm slightly increased with the increase of the band around 550 nm, which clearly indicated that Cu^{2+} caused the ring-open of not only RB but also SP.

We have examined mechanism using FTIR techniques. The IR spectra of RB-SP2 revealed that the peak of C=O amide bond at 1687 cm^{-1}, the characteristic stretching frequency for the C=O amide bond of the rhodamine unit, shifted to 1636 cm^{-1} in presence of 2 equiv of the Cu^{2+} ion (Figure S3, see ESI†). Such shift in the stretching frequency of C=O amide bond of the rhodamine unit on binding to a metal ion has been reported earlier[9c], indicating that the amide carbonyl group is involved in the interactions with Cu^{2+}. This is the key to the spiro ring-opening and fluorescence turn-on of the rhodamine dye. Meanwhile, the pronounced down field shifts of the methylene protons of **RB-SP2** in the ^1H NMR spectra indicate that the binding interactions among the metal center, hydrazide and phenolate hydroxy group (Fig. S4, see ESI†). Furthermore, in order to verify Cu^{2+}-triggered spiro ring-opening process, we studied mass spectrum of the **RB-SP2−Cu** system, which shows a molecular-ion peak at m/z 898.3, corresponding to [**RB-SP2** + Cu + Cl] (Fig. S5, see ESI†). Taken these results together, a likely sensing mechanism based on the Cu^{2+}-triggered spiro ring-opening process is proposed in Scheme 2. Based on the relationship between the fluorescence intensity and concentration of Cu^{2+}, the binding constant was calculated to be logK = 6.11 (Fig. S6, see ESI†).



Scheme 2 Proposed mechanism for the fluorescent changes of sensor **RB-SP2** upon the addition of Cu^{2+} and S^{2-}

As other metal ions may also coordinate with **RB-SP2**, the responses to other metal ions (Au^{3+}, K^+, Mg^{2+}, Hg^{2+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, Ba^{2+}, Co^{2+}, Mn^{2+}, Na^+, Fe^{3+}, Cu^+, Zn^{2+} and Ag^+) were investigated (Fig. S7, see ESI†). There are not any noticeable changes in fluorescence spectrum with addition of these tested ions, suggesting that the generation of xanthene moiety is highly selective toward Cu^{2+} ions. This conclusion was further confirmed with the UV-vis absorption spectra analysis, no noticeable changes in UV-vis absorption were observed with the addition of these tested ions in **RB-SP2** solution (Fig. S8, see ESI†).

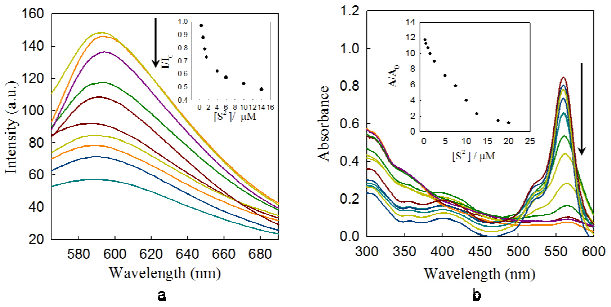


Fig. 2. Changes in the (a) fluorescence (λ_{ex} = 500 nm) and (b) UV-vis absorption spectra of **RB-SP2-Cu** complex (Adding 10 μM Cu^{2+} to RB-SP2 10 μM) in the presence of different concentrations of S^{2-} (0-20 μM) in C_2H_5OH/aqueous PBS (1 mM, pH 7.4; 4:6 v/v; 1% DMSO) solution. Inset: the S^{2-} concentration-dependent response at room temperature.

The above-mentioned studies show that **RB-SP2** selectively binds with Cu^{2+} to form **RB-SP2-Cu** complex resulting in considerable changes in the spectral properties. One may think that if the **RB-SP2−Cu** complex dissociates, the fluorescence would be quenched. Herein sulfide was introduced to quench the fluorescence by forming CuS. Fig. 2a shows that the fluorescence intensity of 10 μM **RB-SP2-Cu** decreases with increasing the concentration of S^{2-}. In the presence of 20 μM S^{2-} anion the intensity of **RB-SP2-Cu** decreases by about 280%. Similar results are observed on the UV–vis spectral pattern (Fig. 2b) which is in a reverse direction to the spectral pattern as shown in

Fig. 1b. These results further confirmed the conclusion that the **RB-SP2−Cu** complex results in the switch ON of fluorescence response (Scheme 2). Their corresponding fluorescence changes were also shown in Figure S9 (see ESI†), implying the reproducibility and reversibility of this experiment.

The spectral responses of the **RB-SP2-Cu** complex were also investigated in the presence of other anions including F^-, Cl^-, Br^-, I^-, CN^-, $H_2PO_4^-$, NO_3^-, ClO^-, ClO_4^-, SO_4^{2-} and HSO_3^-. These investigated anions show little fluorescence quenching effect except the S^{2-} (Fig. S10, see ESI†). The reason is that these anions cannot form stable precipitate/complex or the formed precipitate/complex is with less stability than **RB-SP2-Cu.** As expected, same results were observed on the UV-vis absorption spectral analysis, which also demonstrate high selectivity toward S^{2-} ions (Fig. S11, see ESI†).

The fluorescence intensity of **RB-SP2** probe is independent on pH in the range of pH 5.0-9.0 either in the absence or presence of Cu^{2+} (Fig. S12 see ESI†), indicating that the probe was suitable for the detection of Cu^{2+} at physiological pH.

Based on the above experiment, it was conceived that compound **RB-SP2** could be exploited for fluorescence imaging of live cells, particularly for sensitive detection of intracellular Cu^{2+}. To pursue this goal, it was pertinent to assess the cytotoxic effect of compound **RB-SP2** on live cells. Various concentrations of compound **RB-SP2** and **RB-SP2-Cu** complex were thus chosen, and their cytotoxic effects on HeLa cells were ascertained following an exposure period of 24 h. The well-established MTT assay, which is based on mitochondrial dehydrogenase activity of viable cells, was adopted. It is quite evident from Fig. 3a that compound **RB-SP2** does not exert any effect on the viability of HeLa cells. However, exposure of HeLa cells to 100 μM **RB-SP2-Cu** complex resulted in a decline in cell viability. In the presence of higher concentrations of **RB-SP2-Cu** complex, the cytotoxic effect was more prominent as a result of cytotoxic and antiproliferative effects of Cu^{2+} complex on cancer cells.[10]

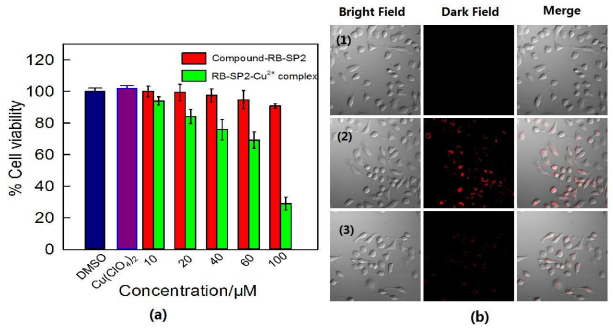


Fig. 3. (a) MTT assay to determine the cytotoxic effect of compound **RB-SP2** and **RB-SP2-Cu** complex in HeLa cells. (b) Fluorescence microscopic images of HeLa cells: (1) after treating with 10 μM **RB-SP2** (under green light); (2) after adding 10 μM of Cu^{2+} (under green light) to the **RB-SP2** treated cells, and (3) after adding 20 μM S^{2-} (under green light) to the (**RB-SP2-Cu**$^{2+}$) treated cells.

Fluorescence microscopic studies reveals that HeLa cells treated with probe **RB-SP2** alone show no fluorescence (Fig. 3b. 1). Upon incubation with 10 μM $Cu(ClO_4)_2$ for 1 h, a striking switch-ON fluorescence is observed inside HeLa cells, indicating the formation of **RB-SP2-Cu** complex (Fig. 3b. 2), as observed earlier in solution studies. Further, an intense red fluorescence was conspicuous in the perinuclear region of HeLa cells. Interestingly, sulfide sensing inside HeLa cells by **RB-SP2-Cu** complex could also be pursued as evident from the remarkable switch-OFF of the red fluorescence inside cells following incubation with Na_2S solution (Fig. 3b. 3). Essentially, the fluorescence microscopic analysis strongly suggested that probe **RB-SP2** could readily cross the membrane barrier, permeate into HeLa cells, and rapidly sense intracellular Cu^{2+} and S^{2-}. It is significant to mention here that brightfield images of treated cells did not reveal any gross morphological perturbations, which suggested that HeLa cells were viable. This finding is encouraging for future in vivo biomedical applications of the probe.

Conclusions

A highly selective and reversible fluorescent probe **RB-SP2** was designed for the detection of Cu^{2+} and S^{2-} by incorporating both spiropyran and rhodamine. **RB-SP2** can be employed to image Cu^{2+} and S^{2-} in living cells. Notably, the reduction of **RB-SP1** to **RB-SP2** guaranteed the reversible as well as selective response to Cu^{2+}. This work opens an avenue for development of reversible fluorescent probe from irreversible chemo dosimeters with high selectivity.

We are grateful for the financial support from the NSFC (2009CB421601)

Notes and references

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, China; Tex/Fax: +86 731 88822170; E-mail: qycai0001@hnu.edu.cn

Electronic Supplementary Information (ESI) available: Experimental details, synthesis and characterization of RB-SP2, optimized procedure. See DOI: 10.1039/c000000x/

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