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A rhodamine-based "turn-on" fluorescent chemodosimeter for Cu^{2+} and its application in living cell imaging

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ABSTRACT

A new fluorescent probe 1, N-(Rhodamine-6G)lactam-hydrazinecarbothioamide, was synthesized as a fluorescent and colorimetric chemodosimeter in aqueous solution for Cu^{2+}. Following Cu^{2+}-promoted ring opening, redox and hydrolysis reactions, comparable amplifications of absorption and fluorescence signals were observed upon addition of Cu^{2+}; this suggests that chemodosimeter 1 effectively avoided the fluorescence quenching caused by the paramagnetic nature of Cu^{2+}. Importantly, 1 can selectively recognize Cu^{2+} in aqueous media in the presence of other trace metal ions in organisms, abundant cellular cations and the prevalent toxic metal ions in the environment with high sensitivity (detection limit <3 ppb) and a rapid response time (<2 min). In addition, the biological imaging study has demonstrated that 1 can detect Cu^{2+} in the living cells.

1. Introduction

Production of fluorescent devices for the sensing and reporting of chemical events are currently of significant importance for both chemistry and biology [1,2]. Recently, the development of selective and sensitive imaging tools capable of rapidly monitoring heavy and transition metal ions (HTM), such as Hg^{2+}, Zn^{2+}, and Cu^{2+} ions has attracted considerable attention due to the environmental and biological relevance of such metal ions [3–15]. In this regard, sensors directed toward the detection of divalent copper have enjoyed particular attention. Copper is third in abundance (after Fe^{2+} and Zn^{2+}) among the essential heavy metal ions in the human body and plays a pivotal role in a variety of fundamental physiological processes in organisms ranging from bacteria to mammals [16]. On the other hand, copper is a widely used industrial metal, and its cation is toxic at high concentration and is involved in brain diseases such as Alzheimer's, Parkinson's, and Prion at a trace amount [17–21]. Owing to the Janus-faced properties of copper in organisms, many fluorescent probes for Cu^{2+}-selective detection were reported and have been used with some success in biological applications [22–26]. However, most of them are not qualified for practical application with their shortcomings such as slow response, low water solubility, cross-sensitivities toward other metal cations and cytotoxicities of ligand. In this paper, a highly sensitive and selective fluorescent chemodosimeter based on rhodamine for Cu^{2+} in aqueous solution was developed. The imaging study of Cu^{2+} in living cells was also successfully demonstrated.

To date, rhodamine derivatives have attracted much attention in the design of chemosensors or chemodosimeters for metal ions. Rhodamine derivatives are colorless and nonfluorescent, whereas ring-opening of the corresponding spirolactam gives rise to a strong fluorescence enhancement and a color change. Recently, a rhodamine-based fluorescent chemodosimeter RH9, which responded to Hg^{2+} stoichiometrically, rapidly, and irreversibly at room temperature through desulfurization and cyclization reactions was reported by Tae et al. [27]. Inspired by such a chemodosimeter for Hg^{2+}, we surmised that a marked increase of the electronegativity of the S atom could lead to similar reactions, being induced by other sulfophilic elements, such as copper.

2. Materials and methods

2.1. Chemicals and instrumentations

^1H and ^{13}C NMR spectra were taken on a Varian mercury-400 spectrometer with TMS as an internal standard and $CDCl_3$ as solvent. Absorption spectra were determined on a Varian UV-Cary100 spectrophotometer. Fluorescence spectra measurements were performed on a Hitachi F-4500 spectrofluorimeter. All pH measurements were made with a pH-10C digital pH meter. HRMS were determined on a Bruker Daltonics APEXII 47e FT-ICR spectrometer.

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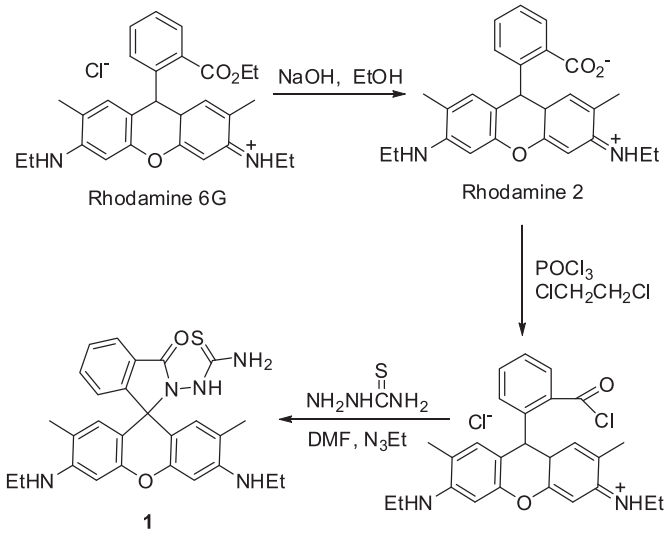


Scheme 1. Synthesis of chemosensors 1.

All the materials for synthesis were purchased from commercial suppliers and used without further purification. Methanol for spectra detection was HPLC reagent without fluorescent impurity.

2.2. Synthesis of 1

The synthesis of the rhodamine derivative 1 is shown in Scheme 1. The Rhodamine 2 was prepared according to the known procedure [28]. Rhodamine 6G (R6G) (3 g, 6.3 mmol) was added to the solution of NaOH (4.8 g, 0.12 mol) in ethanol (30 mL) and water (60 mL). After refluxed for 16 h, the reaction mixture was cooled and ethanol was evaporated in vacuo, then adjusted pH to 6–7 using hydrochloric acid (2 M). The formed precipitate was filtered, washed several times with water and dried to give 2.23 g 2 as a red solid (yield 86%).

To a stirred solution of 2 (0.2 g, 0.48 mmol) in 1, 2-dichloroethane (5 mL), phosphorus oxychloride (0.4 mL) was added dropwise. After refluxed for 6 h, the reaction mixture was cooled and evaporated in vacuo. The crude acid chloride was dissolved in DMF (15 mL), and the resulting solution was then added dropwise to a mixed solution of thiosemicarbazide (0.2 g, 2.2 mmol) and triethylamine (1.2 mL) in DMF (3 ml) at room temperature. After stirring over night, the solvent was removed under reduced pressure. The crude product was purified by silica-gel column chromatography with CH_2Cl_2 as eluent, affording 0.038 g of 1 (yield 18.3%). ^{1}H NMR ($CDCl_3$, 400 MHz, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), δ (ppm): 8.00–8.02 (d, 1 H, J = 7.2 Hz), 7.58–7.63 (m, 2 H), 7.18–7.20 (d, 1 H, J = 7.2 Hz), 6.84 (s, 1 H), 6.37 (s, 2 H), 6.20 (s, 2 H), 5.76 (s, 2 H), 3.53 (q, 2 H), 3.19–3.25 (m, 4 H), 1.91 (s, 6 H), 1.33 (t, 6 H, J = 6.8 Hz). ^{13}C NMR (CDCl3, 100 MHz) δ (ppm): 184.58, 166.83, 152.59, 150.59, 148.02, 134.32, 128.98, 128.71, 127.03, 124.71, 123.82, 118.38, 104.29, 97.23, 67.02, 53.40, 38.28, 16.77, 14.68. ESI-MS m/z = 488.20 $[M+H]^+$, calc. for $C_{27}H_{29}N_5O_2S$ = 487.20.

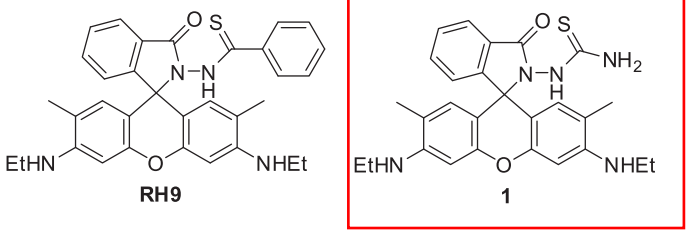


Fig. 1. Chemical structures of RH9 and 1.

2.3. Crystallography

Table 1 summarizes the crystal data, data collection and refinement parameters for 1. A red crystal of 1 (0.12 mm × 0.08 mm × 0.09 mm) was selected for the X-diffraction analysis. Data collection was performed on a Rigaku RAXIS-RAPID diffractometer using a MoKα radiation (λ = 0.71073) at 273(2) K. The intensity data were collected by the w scan mode within 2.34° < θ < 18.44° for hkl ($-13 \le h \le 13$, $-20 \le k \le 20$, $-21 \le l \le 15$) in the triclinic system. The structure was solved by direct methods. The positions of non-hydrogen atoms were determined from successive Fourier syntheses. The hydrogen atoms were placed in their geometrically calculated positions. The positions and anisotropic thermal parameters of all non-hydrogen atoms were refined on F^2 by full-matrix least-squares techniques with the SHELX-97 program package (G. M. Sheldrick, Bruker AXS, Madison, WI, 2001).

Table 1
Crystal and experimental data.

Formula	$C_{56}H_{60}Cl_{16}N_{10}O_4S_2$
Formula weight	1213.96
Temperature	293(2) K
Crystal system/space group	P-1
Unit cell dimensions	a = 10.889(8) Å, α = 106.058°(10)
	b = 16.748(12) Å, β = 99.892°(10)
	c = 17.700(13) Å, γ = 104.459°(9)
Volume	2901 Å^3
Z	2
Density (calculated)	1.390 mg/m^3
Absorption coefficient	0.423 mm^{-1}
F(0 0 0)	1264.0
Crystal size	0.12 × 0.08 × 0.09 mm
θ range for data collection [°]	2.03 to 25.5
Reflections collected/unique	18,850/10,600
Absorption correction	Empirical
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	10,600/0/711
Goodness-of-fit on F^2	0.943
Final R indices [I>2σ(I)]	R1 = 0.0791, wR = 0.2456
Largest diff. peak and hole	0.421 and −0.483 e·Å^{-3}
CCDC	777720

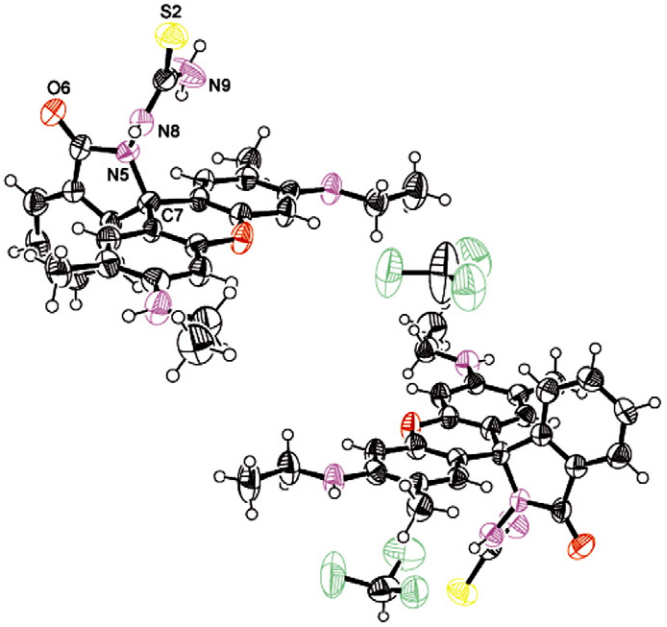


Fig. 2. ORTEP diagram of the compound 1 (30% probability level for the thermal ellipsoids).

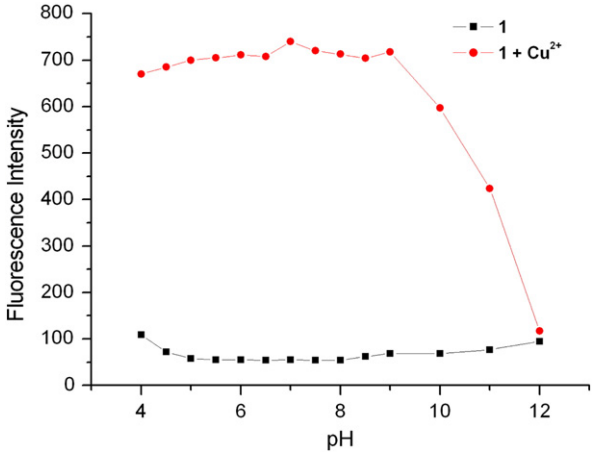


Fig. 3. Fluorescence spectra of 1 (10 μM) with the addition of Cu^{2+} (10 equiv) in 50% (v/v) water/CH_3CN (10 mM Tris–HCl buffer, pH = 7.2) at various pH values (Ex. 480 nm; Em. 554 nm).

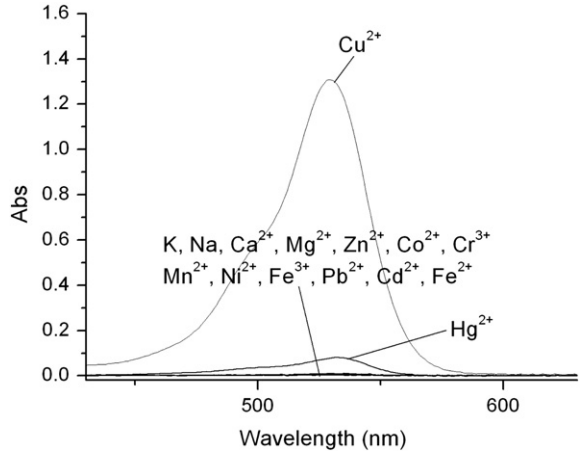


Fig. 5. Changes in the absorption spectra of 1 (20 μM) in the presence of different metal ions (200 μM) in 50% (v/v) water/CH_3CN (10 mM Tris–HCl buffer, pH = 7.2).

2.4. Fluorescence imaging

Stock solutions of the metal ions (2.5 mM) were prepared in deionized water. A stock solution of 1(1 mM) was prepared in DMF: CH_3CN (1:1 v/v). The solution of 1 was then diluted to 20 μM with 50% (v/v) water/CH_3CN (10 mM Tris–HCl buffer, pH = 7.2). In titration experiments, each time a 2 mL solution of 1 (20 μM) was filled in a quartz optical cell of 1 cm optical path length, and the Cu^{2+} stock solution was added into the quartz optical cell gradually by using a micro-pippet. Spectral data were recorded at 2 min after the addition. In selectivity experiments, the test samples were prepared by placing appropriate amounts of metal ion stock into 2 mL solution of 1 (20 μM). For fluorescence measurements, excitation was provided at 495 nm, and emission was collected from 508 to 650 nm.

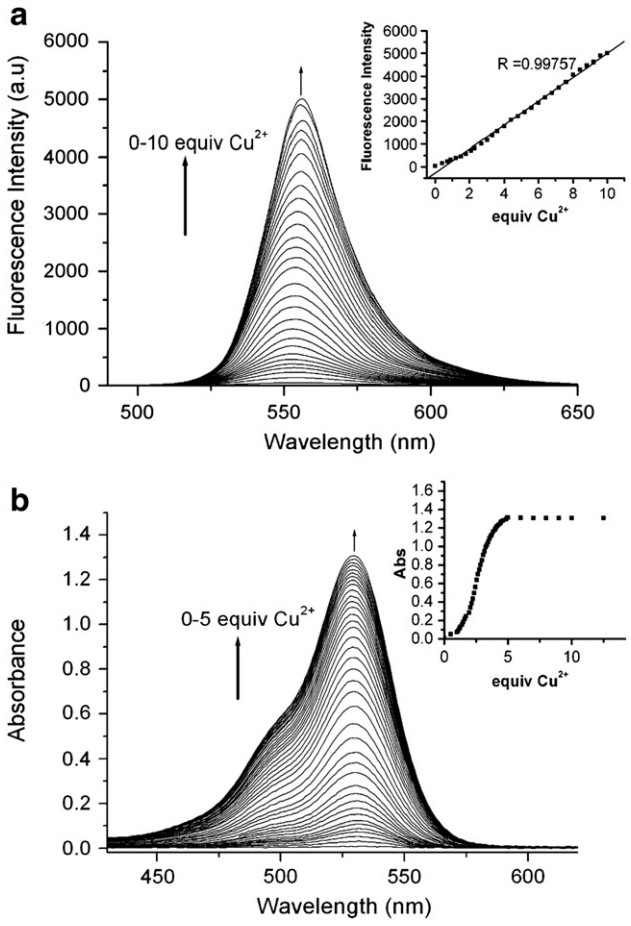


Fig. 4. a) Fluorescence spectra of 1 (20 μM) in Tris–HCl (10 mM, pH = 7.2) buffer containing 50% (v/v) water/CH_3CN solutions upon addition of Cu^{2+} (0–10 equiv) (λ_{ex} = 480 nm). Inset shows fluorescence intensity at 554 nm of 1 as a function of Cu^{2+} concentration. b) Absorption spectra of 1 (20 μM) in the presence of Cu^{2+} (0–5 equiv). Inset shows the absorbance at 529 nm of 1 as a function of Cu^{2+} concentration.

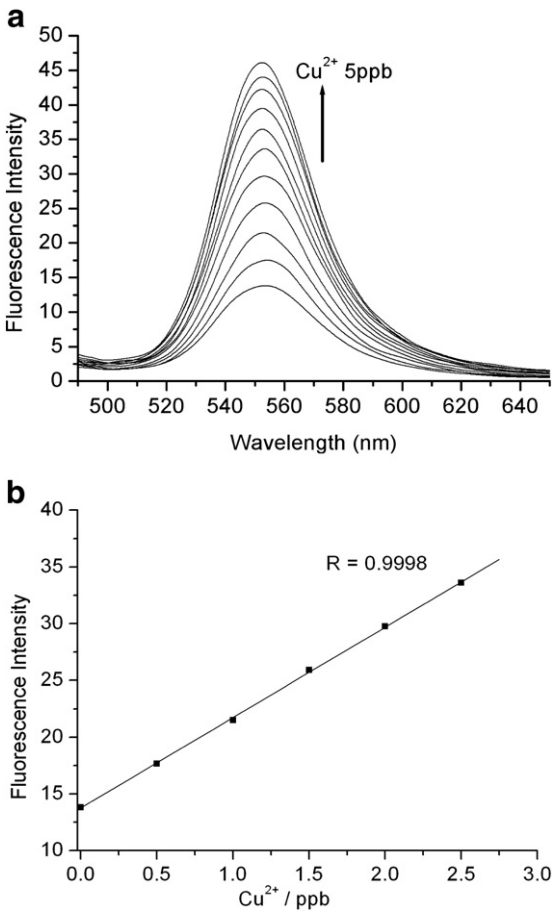


Fig. 6. a) Fluorescence responses of 1 μm 1 upon addition of 0–5 ppb Cu^{2+} in 50% water/CH_3CN (10 mM Tris–HCl, pH = 7.2) solution. b) shows the fluorescence intensity at 529 nm of 1 as a function of Cu^{2+} concentration 0–5 ppb.

Fluorescent pictures were taken on Zeiss Leica inverted epifluorescence/reflectance laser scanning confocal microscope. Excitation of 1-loaded cells at 488 nm was carried out with a HeNe laser. Emission was collected using a 560 nm long-pass filter. Emission was collected from 570 to 625 nm.

3. Results and discussions

Herein, we report that a new rhodamine derivative 1 (Fig. 1), containing a thiosemicarbazide moiety, has been demonstrated as a chemodosimeter for Cu^{2+}. The rhodamine derivative 1 was prepared from rhodamine 6G by a three-step procedure in a yield of 18%. The detailed experimental procedures, ^{1}H and ^{13}C NMR spectra are explained in the Supporting Information. Sensor 1 was further confirmed by X-ray analysis (Fig. 2).

3.1. Detection range of pH value

Compound 1 remained colorless and weak fluorescence in 50% (v/v) water/CH_3CN (10 mM Tris–HCl buffer, pH = 7.2). This indicates that the spirolactam form of 1 predominantly existed in the CH_3CN/Tris–HCl buffer (v/v, 1:1) solutions. The characteristic peak of the 7-carbon of 1 near 67 ppm in the ^{13}C NMR spectrum also supports this consideration [29]. In addition, no obvious fluorescence emission of 1 was observed between pH 4 and 12, suggesting that the compound is insensitive to pH and that the spirolactam form is still preferred in this condition. In the range pH 4–10, a marked fluorescence enhancement was observed upon addition of Cu^{2+} (Fig. 3). These data suggest that 1 could act as a fluorescent probe for Cu^{2+} under physiological pH conditions.

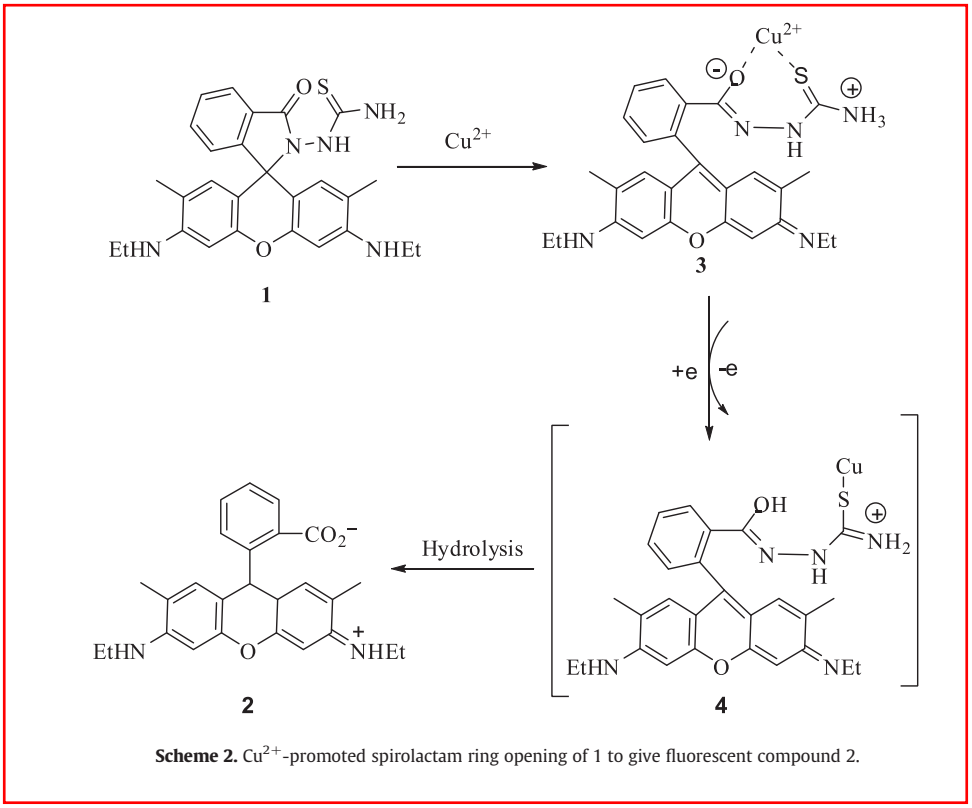
3.2. Response studies of 1 to Cu^{2+}

Electronic spectra of 1 (20 μM) in 50% (v/v) water/CH_3CN (10 mM Tris–HCl buffer, pH = 7.2) exhibited only a very weak band above 500 nm, which could be attributed to the presence of a trace amount of the ring-opened form of 1. Binding ability of 1 toward various metal ions (Cu^{2+}, Hg^{2+}, Fe^{3+}, Cr^{3+}, Fe^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}) was checked. Upon the addition of 5 equiv Cu^{2+}, a significant enhancement (358-fold) of absorbance at 529 nm was observed, suggesting the clear formation of the ring-opened amide form of 1. (Fig. 4b). Other cations of interest gave no such response. Only Hg^{2+} displayed a mild enhancement (21-fold) at the same concentration (Fig. 5).

To get a practical application view, the fluorescence sensing behavior of 1 for Cu^{2+} in (1:1 v/v) water/CH_3CN (10 mM Tris–HCl buffer, pH = 7.2) solution was also investigated. On addition of Cu^{2+} (0–10 equiv) to the solution of 1, a significant enhancement (180-fold) in fluorescence intensity at 554 nm was observed following excitation at 480 nm (Fig. 4a). Furthermore, to assess the possibility of detecting Cu^{2+} at a low concentration, fluorescence titrations were conducted with 1 μM 1. As shown in Fig. 6, the fluorescence intensity of the solution of 1 was nearly proportional to the amount of Cu^{2+} added.

From the molecular structure and spectral results of 1, we can see that a Cu^{2+}-promoted ring-opening reaction of 1 occurs instantly upon the addition of Cu^{2+}, owning to the strong binding ability of the O, N, and S atoms towards Cu^{2+}. The reaction with Cu^{2+} in aqueous media predictably affects a redox hydrolysis of 1 leading to rhodamine 6G (2) itself in three processes (Scheme 2) [30]. The formation of rhodamine 6G as product is confirmed by ESI mass spectrometry. The ESI mass spectrum of 1 with 0.5 equiv Cu^{2+} showed a peak at m/z 552.1484 (calcd for 552.1489) (Fig. S8, 9 in Electronic supplementary material), corresponding to an intermediate 4. In the case of 1 with two equiv Cu^{2+}, a peak at m/z 415.2015 (calcd for 415.2016) assigned to 2 (namely rhodamine 6G) was clearly observed. Moreover, addition of Cu^{2+} chelating agents, such as EDTA, does not decrease the intensity of the fluorescence signals, confirming the irreversible character of this process (Fig. S4 in Electronic supplementary material).

An important feature of the chemodosimeter is its high selectivity toward the analyte over the other competitive species. To validate the selectivity of 1 in practice, some other metal ions including alkali, alkaline earth, and transition-metal ions were added into a solution of 1 under the same conditions (water/CH_3CN, 1:1, v/v, 10 mM Tris–HCl buffer, pH = 7.2). As shown in Fig. 7, only Hg^{2+}, Cr^{3+} elicited a slight fluorescence intensity enhancement, while the other metal ions did not



Scheme 2. Cu^{2+}-promoted spirolactam ring opening of 1 to give fluorescent compound 2.

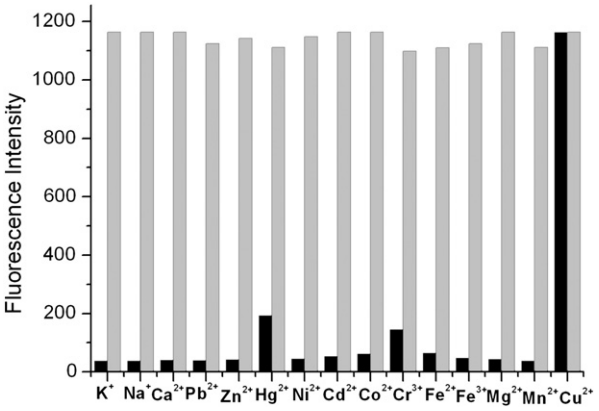


Fig. 7. Fluorescence intensities of 20 μm 1 upon the addition of various metal ions in 50% water/CH_3CN (10 mM Tris–HCl, pH = 7.2) solution. Black bars represent the fluorescence response of 1 to the metal ion of interest (5 mm for K^+, Na^+, Ca^{2+} and Mg^{2+}; 75 μm for other metal ions). Gray bars represent the addition of Cu^{2+} (75 μm) to the solution. (λ_{ex} = 480 nm).

cause any discernible changes. In addition, the increases of fluorescence intensity resulting from the addition of the Cu^{2+} ion were not influenced by the subsequent addition of miscellaneous cations. All of these results indicate that the selectivity of 1 for the Cu^{2+} ion over other competitive cations in the water medium is remarkably high.

3.3. Biological imaging studies using living cells to monitor Cu^{2+}

To test the sensitivity of the interaction between 1 and Cu^{2+}, we also investigated the time course of the response of 1 (20 μM) in the presence of 5 equiv of Cu^{2+} in (1:1 v/v) water/CH_3CN (10 mM Tris–HCl buffer, pH = 7.2). It was found that the obvious spectral change was observed within 1 min (Fig. S3 in Electronic supplementary material) upon addition of 5 equiv Cu^{2+}. Therefore, this system could be used for real-time tracking of Cu^{2+} in cells and organisms.

Then, we studied bioimaging applications of 1 for monitoring of Cu^{2+} ions in biological systems. As determined by laser scanning confocal microscopy, staining cells treated with 10 μM solution of 1 for 20 min give no intracellular fluorescence (Fig. 8a). EJ (lung cancer) cells were supplemented with 10 μM 1 for 20 min and washed with PBS buffer (pH 7.4) to remove the remaining chemodosimeter, and then the treated cells were incubated with $CuCl_2$ (50 μM) in culture medium for 20 min at 37 °C, after that a significant fluorescence increase from the intracellular region was observed (Fig. 8c). Bright-field measurements after treatment with Cu^{2+} and 1 confirmed that the cells were permeable throughout the imaging experiments (Fig. 8b). As shown in Fig. 8d, the overlay of fluorescence and PTS images reveal that the fluorescence signals are localized in the perinuclear area of the cytosol, indicating a subcellular distribution of Cu^{2+}.

4. Conclusions

In summary, a new fluorescent chemodosimeter based on rhodamine 6G for Cu^{2+} in aqueous solution was synthesized. The sensing mechanism involves Cu^{2+}-promoted ring-opening, redox and hydrolysis reactions, which may be attributed to the highly electron-rich S atom in 1. The selectivity of this system for Cu^{2+} over other metal ions is remarkably high, and its sensitivity is below 2 ppb in aqueous solutions. Fluorescence imaging of Cu^{2+} in living cells is also successfully demonstrated. We expect that the present fluorescent

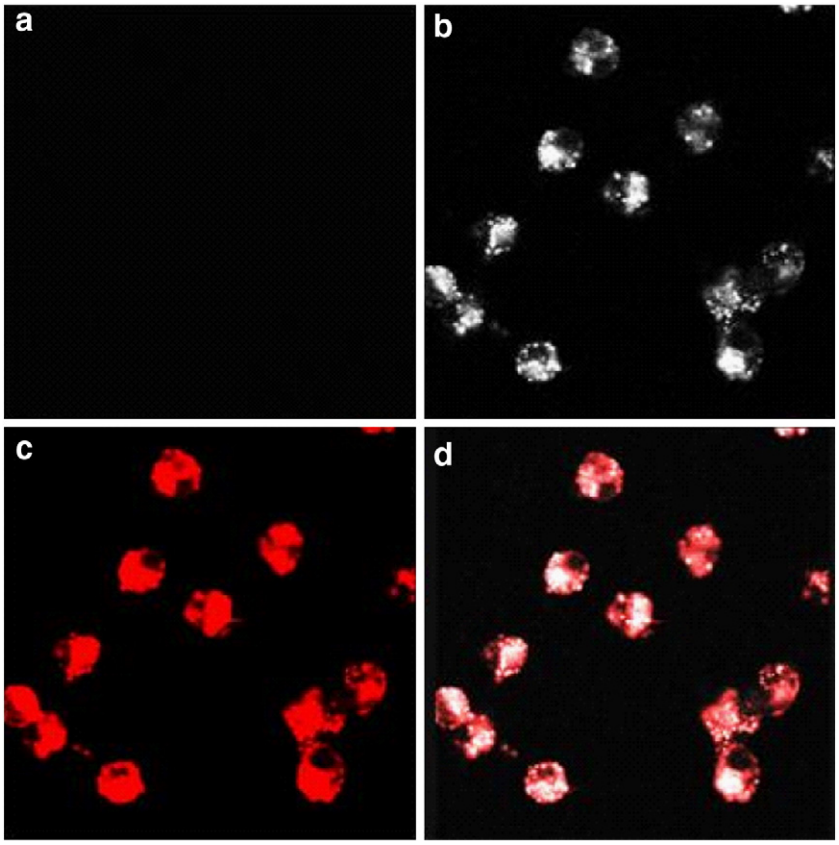


Fig. 8. Confocal fluorescence, brightfield, and overlay images of EJ (cystic cancer) cells. a) Fluorescence image of EJ cells treated with 1 (10 μM) in the absence of $CuCl_2$ (control). b) Brightfield image of cells shown in panel c. c) Cells supplemented with 10 μM 1 in the growth media for 20 min and then incubated with 50 μm $CuCl_2$ for 20 min at 37 °C. The overlay image of b) and c) is shown in d) (λ_{ex} = 488 nm).

probe could serve as a new tool in copper ion-related chemical and biological studies.

Abbreviations

1	N-(Rhodamine-6G)lactam-hydrazinecarbothioamide
2	(E)-2-(6-(ethylamino)-3-(ethylimino)-2,7-dimethyl-3H-xanthen-9-yl)benzoic acid
HTM	transition metal ions
HRMS	High Resolution Mass Spectrometer
TMS	Temperature Measurement Society
DMF	N, N-dimethyl formamide

Acknowledgments

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Appendix A. Supplementary data

Experimental procedures for the synthesis, spectral data, and copies of ^{1}H NMR and ^{13}C NMR of 1, data for UV–vis, fluorescence of 1-Hg^{2+}, and other data are available.

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2011.02.012.

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