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ORIGINAL PAPER

Fluorescent probe for copper(II) ion based on a rhodamine spirolactame derivative, and its application to fluorescent imaging in living cells

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Abstract A fluorescent probe for Cu(II) ion is presented. It is based on the rhodamine fluorophore and exhibits high selectivity and sensitivity for Cu(II) ion in aqueous methanol (2:8, *v/v*) at pH 7.0. The response is based on a ring opening reaction and formation of a strongly fluorescent 1:1 complex. The response is reversible and linear in the range between 50 nM and 900 nM, with a detection limit of 7.0 nM. The probe was successfully applied to fluorescent imaging of Cu(II) ions in HeLa cells.

Keywords Copper(II) ions · Fluorescent probe · Fluorescence enhancement · Rhodamine derivatives · Fluorescence imaging · Living cells

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Introduction

Copper ion is one of toxic and dangerous heavy metal ions at high concentration level, and meanwhile an essential trace element in biological systems [1–3]. The detection and estimation of Cu^{2+} either in vitro or in vivo always constitutes an active area of research. Currently, the common techniques for detection of Cu^{2+}, such as atomic absorption spectrometry (AAS) and inductively coupled plasma mass spectrometry (ICP-MS), are often expensive, complex, and unsuitable for on-site analyses. Therefore, the development of new analytical methods for the sensitive and selective determination of Cu^{2+} is highly desirable. Because of its operational simplicity, low cost, real time monitoring and high selectivity, fluorescent detection has become the promising strategy used for Cu^{2+} detection.

Even though considerable efforts have been undertaken to develop fluorescent probes for Cu^{2+}, many reported fluorescent probes generally undergo fluorescence quenching upon binding with its inherent paramagnetic nature [4–10], which is not as sensitive as a fluorescence enhancement response. Recently there are still a few examples reported wherein enhancement in the fluorescence intensity has been observed upon complexation with Cu^{2+} ions [11–25]. In addition, the fluorescence enhancement in most cases is small and usually suffers from a high background. Therefore, searching for Cu^{2+} probes based on fluorescence enhancement with high sensitivity is still an active field as well as a challenge for the analytical chemistry research effort.

Fluorescence bioimaging has provided a facile and less cell-damaging means of visualizing analytes of biological interest in living cells, by virtue of its highly sensitive and high-speed spatial analysis of cells. To image intracellular metal ions, highly sensitive and selective probes that can

exhibit an enhanced visible fluorescent emission in aqueous media urgently require to be developed.

The rhodamine framework is an ideal mode to construct fluorescent probes due to its excellent spectroscopic properties such as large molar extinction coefficient (ε), relatively long excitation and emission wavelengths, and high fluorescence quantum yield (Φ). On the basis of the spirolactam (non fluorescent) to ring-open amide (fluorescent) equilibrium of rhodamine, most of rhodamine fluorescent probes based on rhodamine hydrazide derivatives or rhodamine amine conjugates for various cations via chromogenical and fluorogenical signals have been successfully fabricated as the fluorescence enhancement type [26–29]. To date, many investigations are still in progress to search for cheaper, more stable, convenient and effective rhodamine derivatives. Herein we report the synthesis of a new compound of rhodamine–benzoyl hydrazine (1, Scheme 1) that behaves as a fluorescent Cu^{2+} probe with remarkably high sensitivity and selectivity in aqueous methanol (2:8, *v/v*) at pH 7.0. Moreover, probe 1 exhibits the feature of reversibility to Cu^{2+}. Furthermore, confocal fluorescence microscopy experiments have demonstrated that 1 can be used to image Cu^{2+} in living cells.

Experimental

Materials and instruments

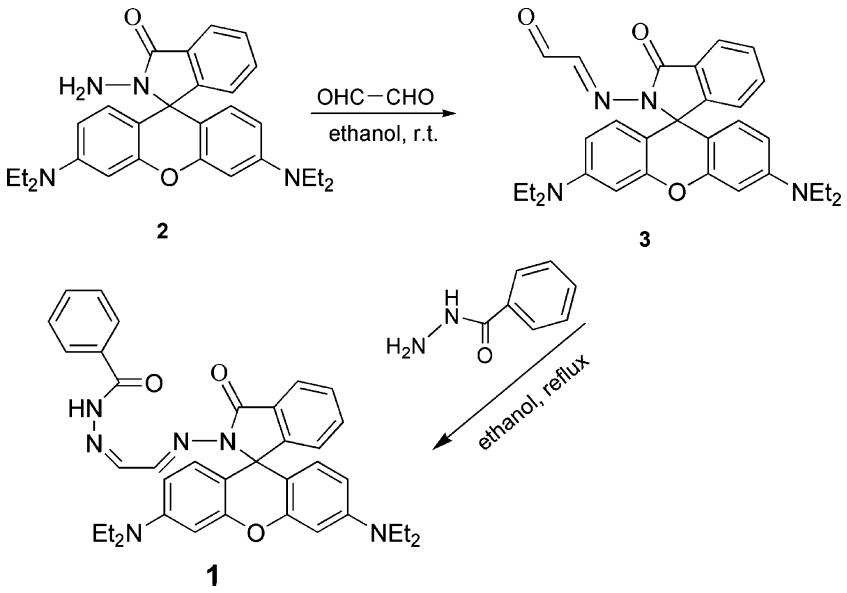
All the used chemicals are of analytical grade or of the highest purity available. Rhodamine B and benzoyl hydrazine were purchased from Aldrich (http://www.sigmaaldrich.com/china-mainland.html). Hydrazine hydrate (85%) and glyoxal were obtained from Shanghai Reagent Company (Shanghai, China) (http://www.reagent168.cn/). All solutions were prepared with double–distilled water.

NMR spectra were measured with a Bruker WM–300 spectrometer (500 MHz), using tetramethylsilane (TMS) as an internal standard. The pH measurements were carried out on a PHS-3C meter at 25 °C. Melting points of compounds were measured on a Shanghai WRS–1B digital melting point, and all melting points were uncorrected. Mass spectra (MS) were performed on a Thermo TSQ Quantum Mass Spectrometer. UV–Vis absorption spectra were recorded on a Beckman DU–800 spectrophotometer (1 cm quartz cell) at 25 °C. All fluorescence measurements were carried out on a Perkin Elmer LS55 luminescence spectrometer (1 cm quartz cell) at 25 °C, with excitation and emission slit widths of 10 nm and 3 nm, respectively, and excitation wavelength at 510 nm.

Synthesis of Compound 1

The synthesis route of Compound 1 is depicted in Scheme 1. Compound 2 and Compound 3 were synthesized according to the reported procedures [30, 31]. Briefly, Compound 3 (0.511 g, 1 mmol) and benzoyl hydrazine (0.16 g, 1.2 mmol) were firstly refluxed in absolute ethanol (30 mL) for 5 h under N_2; then the resulting solution was cooled to room temperature; and then the produced precipitate was filtered and washed with cold ethanol; finally, the crude product was purified by recrystallization from ethanol to afford Compound 1 as yellow solid (0.51 g, 85%).

An elemental analysis is provided as proof of purity of compound 1, i.e. anal. calcd. for $C_{37}H_{38}N_6O_3$ (614.30): C,



Scheme 1 Chemical structure and synthetic route of Compound 1

72.29; H, 6.23; N, 13.67; found: C, 72.18; H, 6.42; N, 13.50. ^{1}H NMR, ^{13}C NMR and ESI-MS charts of the Compound 1 are shown in Fig. S3–S5 (Supporting Information), briefly as follows. M.p. 230.4–231.5 °C. ^{1}H NMR (δ ppm, $CDCl_3$): 10.31 (s, 1H, HNC = O), 8.17–8.18 (d, 1H, Ar-H), 7.85 (s, 1H, N = CH), 7.84 (s, 1H, N = CH), 7.72–7.73 (d, 1H, Ar-H), 7.57–7.58 (d, 1H, Ar-H), 7.49–7.52 (t, 1H, Ar-H), 7.39–7.44 (m, 3H, Ar-H), 7.28–7.30 (t, 1H, Ar-H), 7.03–7.05 (d, 1H, Ar-H), 6.50 (s, 1H, Ar-H), 6.48 (s, 1H, Ar-H), 6.46 (d, 2H, Ar-H), 6.23–6.24 (d, 1H, Ar-H), 6.21–6.22 (d, 1H, Ar-H), 3.31–3.35 (m, 8H, CH_2), 1.16–1.18 (t, 12H, CH_3). ^{13}C NMR (δ ppm, $CDCl_3$): 166.43, 164.71 (C = O); 153.76, 152.32 (ArC), 149.21, 148.42 (C = N); 142.90, 134.31, 133.29, 131.97, 128.72, 128.04, 127.70, 126.58, 125.53, 123.82, 123.28, 108.05, 104.27, 98.96 (ArC); 65.91, 44.36 (CH_2); 12.68 (CH_3). MS (ESI) m/z: 615.33 $(M + H)^+$, 637.15 $(M + Na)^+$, and 1252.87 $(2M + H)^+$.

Preparation of the test solution

The stock solution of probe 1 was prepared at 1.0×10^{-4} M in dimethylsulfoxide (DMSO). The solutions of various testing cation species were prepared from $AgNO_3$, $CaCl_2$, $MgCl_2$, $CdCl_2\cdot1/2H_2O$, $CoCl_2\cdot6H_2O$, $CuCl_2\cdot2H_2O$, $HgCl_2$, $MnSO_4\cdot H_2O$, $NiCl_2\cdot6H_2O$, $Pb(NO_3)_2$, and $ZnCl_2$, and anion species from various salts such as NaClO, $NaNO_3$, Na_2CO_3, NaCl, NaAc, $NaClO_4$, KBr and Na_2HPO_4, in the twice-distilled water. The test solution of probe 1 (1 μM) in 10 mL of aqueous solution was prepared by placing 0.1 mL of the probe stock solution, 8.0 mL of methanol, 1.0 mL of twice-distilled water and 1.0 mL of 20 mM HEPES buffer (pH 7.0), i.e. the probe 1 in aqueous methanol (2:8, *v/v*) at pH 7.0. The resulting solution was shaken well before recording the spectra.

Cell incubation

HeLa cells grown on coverslips were washed with phosphate-buffered saline (PBS), followed by incubating with 1 μM of $CuCl_2$ in PBS for 1 h at 37 °C, and then washed with PBS three times. After incubating with 20 μM of probe 1 in PBS, which was attained by diluting the 0.01 M probe 1 solution in DMSO with PBS, for 30 min at 37 °C, the cells were washed with PBS three times again.

Fluorescence imaging

Confocal fluorescence imaging was performed with an Olympus FluoView FV1000 laser scanning microscope with 40× objective lens. Excitation of 1-loaded cells at 559 nm was carried out with a solid laser and emission was collected at 570–670 nm.

Results and discussion

Spectroscopic properties and optical responses to Cu^{2+}

Fluorescence titrations of 1 with Cu^{2+} in aqueous methanol (2:8, *v/v*) at pH 7.0 were performed and the responding spectra are shown in Fig. 1. Free 1 exhibited very slight fluorescence response in the range from 530 to 750 nm, since the spirolactam form of rhodamine prevailed. Upon addition of Cu^{2+}, an emission band peaked at 580 nm significantly increased in intensity, attributable to delocalization effects in the xanthene moiety of the rhodamine. And a continuous increase of fluorescence intensity could be observed with Cu^{2+} concentration increasing (Fig. 1). This fact means that 1 can act as an off–on fluorescent probe for Cu^{2+}.

In order to obtain a better insight into the response mechanism of 1 toward Cu^{2+}, the absorption spectra of 1 in the absence and presence of Cu^{2+} were recorded (Fig. 2). The absorption spectrum of probe 1 (10 μM) in aqueous methanol (2:8, *v/v*) at pH 7.0 solution displays a very weak band over 500 nm. Addition of 5 equiv Cu^{2+} immediately resulted in a significant enhancement of absorbance at 556 nm (ε=83500 L mol^{-1} cm^{-1}). Besides, an obvious color change from colorless to pink was observed by the naked eye in the presence of Cu^{2+}, clearly indicating the ring-opening process of rhodamine in probe 1.

Operation principle

Binding analysis using the method of continuous variations (Job's plot) was measured. As seen from

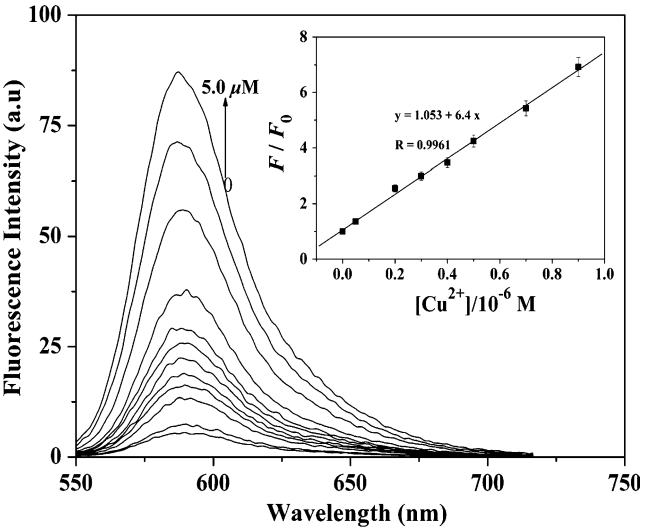


Fig. 1 Fluorescence response of 1 (2 μM) with various concentrations of Cu^{2+} in aqueous methanol (2:8, *v/v*) at pH 7.0. Inset: the fluorescence at 580 nm of 1 (2.0 μM) as a function of Cu^{2+} concentrations (0–0.9 μM)

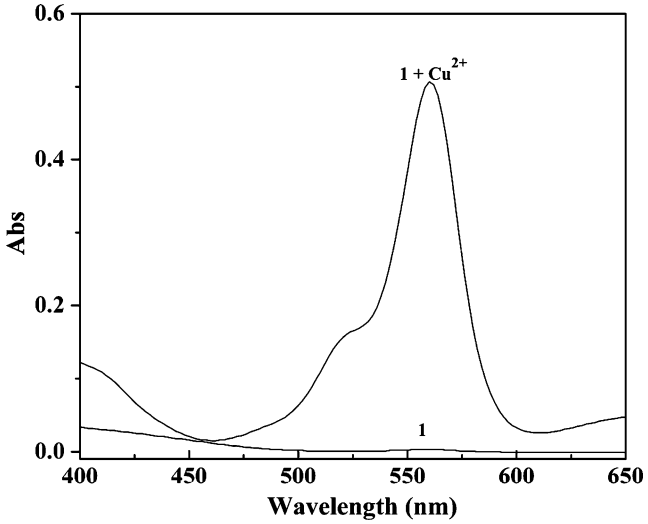


Fig. 2 The absorption spectra of 1 (10 μM) with Cu^{2+} (50 μM) in aqueous methanol (2:8, *v/v*) at pH 7.0

Fig. 3, a maximum absorbance at 556 nm was observed when the molecular fraction of 1 was close to 0.5, which established the 1:1 complex formation between 1 and Cu^{2+}. The stoichiometry was also supported (Fig. S1, Supporting Information) by the Benesi-Hildebrand method [32, 33]. As well as, the binding constant was further found to be 1.3×10^5 M^{-1}, which was determined also according to the Benesi-Hildebrand method [32, 33] with details in Cal. 1 (Supporting Information). Moreover, this binding mode was also confirmed by the presence of two distinct peaks with m/z 691.98 and 719.98, respectively, corresponding to $[1\text{-Cu-(OH)}]^+$ and $[1\text{-Cu-(CH}_3\text{CH}_2\text{OH)}]^+$ in the ESI–MS spectrum of a mixture of 1 and 1 equiv of Cu^{2+} (Fig. S2, Supporting Information), indicating that 1 actually coordinates with Cu^{2+} in a 1:1 stoichiometry.

On the basis of 1:1 stoichiometry, the linear response of the fluorescence intensity toward $[Cu^{2+}]$ was obtained in Cu^{2+} concentration range of 50–900 nM (inset of Fig. 1). And the limit of detection (LOD) was obtained of 7.0 nM, which was calculated based on 3 s/m (s is the standard deviation of the measured intensity of the blank solution and m is the slope of the plot in the inset of Fig. 1). The results demonstrated that the proposed probe can quantitatively determine Cu^{2+} at the ultra-trace level.

Effects of pH

The effects of pH were evaluated in the pH range from 4.0 to 10.0 (Fig. 4). No obvious absorbance of probe 1 at 556 nm was observed within pH 4.0–10.0, suggesting that it was insusceptible to the change of acid–base solution. However, in the presence of Cu^{2+}, a remarkable absorbance band at 556 nm was formed under different pH values in the pH range. It showed the pH value corresponding to the highest response was approximately 7.0, which revealed that the probe 1 for Cu^{2+} could work well in approximate physiological conditions with a very low background response. Therefore, further studies were carried out in aqueous methanol (2:8, *v/v*) at pH 7.0.

Selectivity and competition

The fluorescence responses of probe 1 to other respective metal ions (50 equiv) are investigated, illustrated in Fig. 5a. Addition of Na^+, Ag^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+} and Cr^{3+}, led to no obvious fluorescence

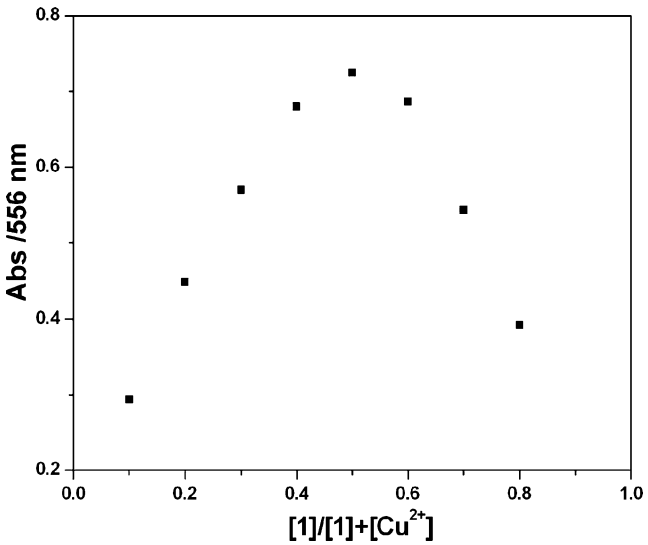


Fig. 3 Job's plot of 1 with Cu^{2+} according to the method of continuous variation. The total concentration of ([1] + $[Cu^{2+}]$) was 50 μM

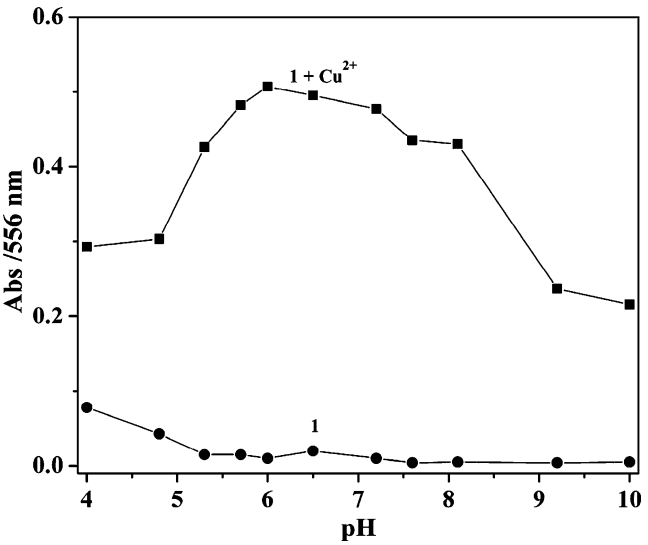


Fig. 4 pH-dependent absorbance of 1 (10 μM) (●) and 1 (10 μM) plus 50 μM Cu^{2+} (■) in HEPES buffers as a function of different pH values

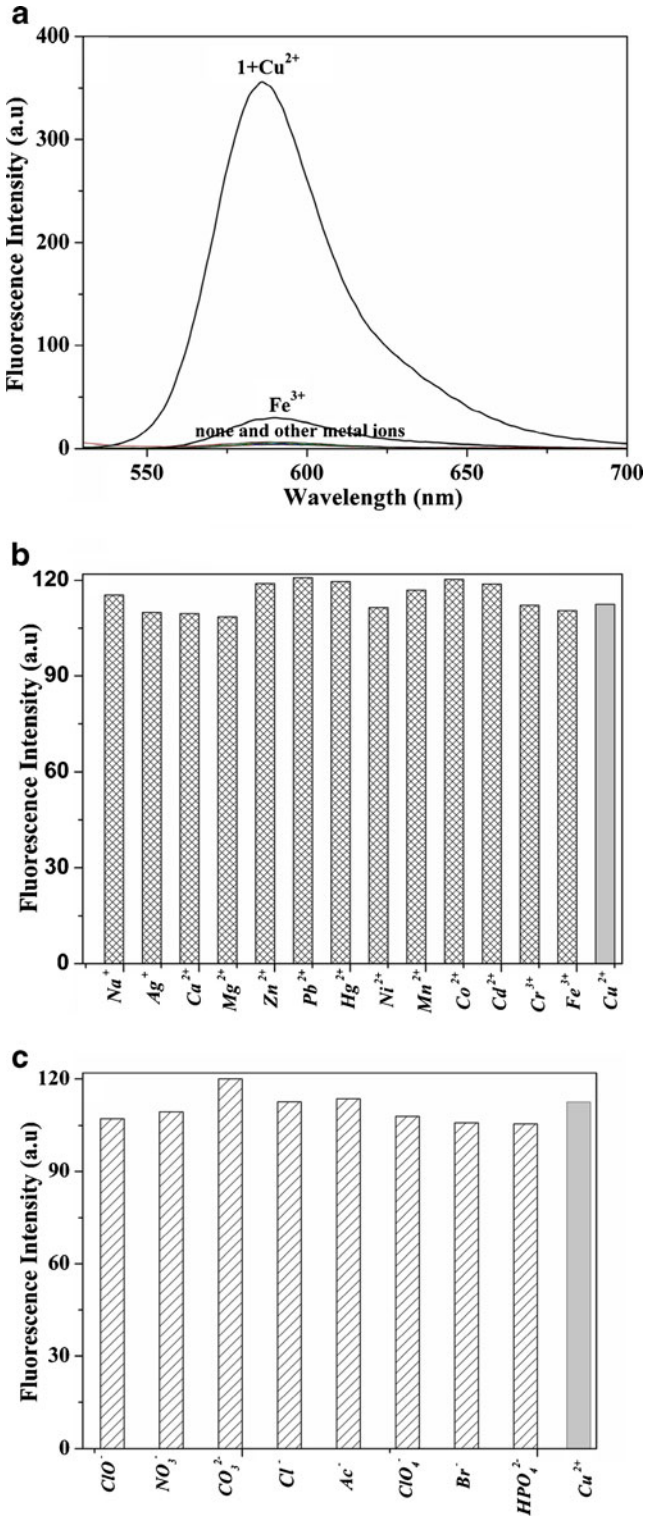


Fig. 5 (**a**) Fluorescent emission spectra of 1 (1.0 μM) to different metal ions (50 μM) in aqueous methanol (2:8, *v/v*) at pH 7.0; (**b**) fluorescence response of 1 (1.0 μM) to 10 μM of Cu^{2+} and to the mixture of 50 μM individual other metal ions with 10 μM of Cu^{2+}; (**c**) fluorescence response of 1 (1.0 μM) to 10 μM of Cu^{2+} and to the mixture of 50 μM individual anions with 10 μM of Cu^{2+}

enhancements, and only Fe^{3+} caused a less than 10-fold enhancement. In contrast, upon the addition of Cu^{2+} at the same concentration, the F/F_0 value was almost 100-fold increase, with a high quantum yield (Φ=0.60, see Cal. 2 in Supporting Information). This finding indicated that 1 could selectively recognize Cu^{2+} in aqueous condition.

One challenge for the probe is to obtain a specific detection system to Cu^{2+} over a wide range of potentially competing ions, since the system might show cross-sensitivity toward other metal ions or toward anions. Competition experiments were conducted in the presence of 10 equiv of Cu^{2+} mixed with 50 equiv of other metal ions mentioned above, respectively. No significant variation in fluorescence intensity was found by comparison with that the same amounts of Cu^{2+} solution without other metal ions, and the relative error was less than ±5% (Fig. 5b). Also, it was investigated that the fluorescence response of probe 1 toward Cu^{2+} in the presence of various coexistent anions such as ClO^-, NO_3^-, CO_3^{2-}, Cl^-, Ac^-, ClO_4^-, Br^- and HPO_4^{2-}. It is gratifying to note that all the tested anions have no interference (Fig. 5c). For the Cu^{2+} probe, neither cross-sensitivity to the other metal ions nor to the commonly present anions was observed, while an excellent selectivity toward Cu^{2+} was exhibited. Thus, the probe 1 was indicated a Cu^{2+}-specific fluorescent probe.

Reversibility and mechanism for the binding of 1 with Cu^{2+}

As is well known, the reversibility is an important property to obtain an excellent probe. Thus, the EDTA–adding experiments were conducted to examine the reversibility of the probe 1. As clearly shown in Fig. 6, absorbance

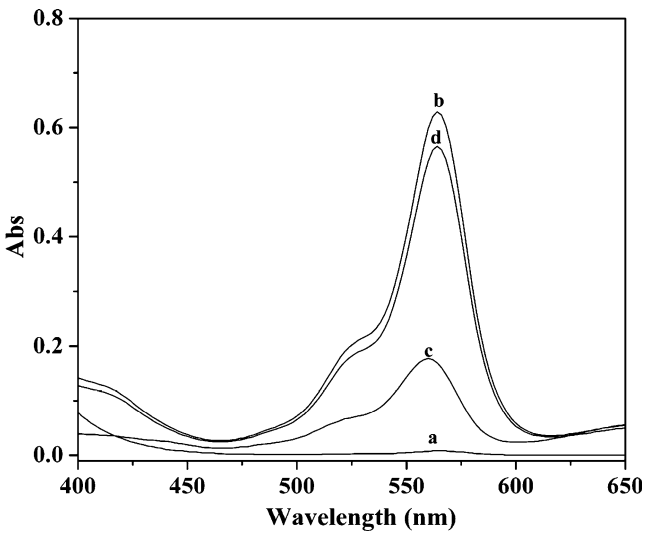
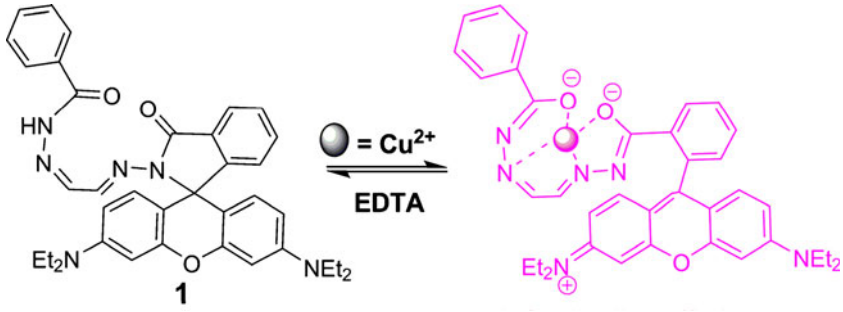


Fig. 6 Absorption spectra in aqueous methanol (2:8, *v/v*) at pH 7.0: (**a**) 1 (10 μM); (**b**) 1 (10 μM) with Cu^{2+} (0.1 mM); (**c**) 1 (10 μM) with Cu^{2+} (0.1 mM) and then addition of EDTA (50 μM); (**d**) 1 (10 μM) with Cu^{2+} (50 μM) and EDTA (50 μM) and then addition of 0.1 mM Cu^{2+}

Scheme 2 Proposed binding mode of Compound 1 with Cu^{2+}



decreased when EDTA was added to the solution containing 1 and Cu^{2+} (Fig. 6c). Besides, the color also changed from pink to colorless. When Cu^{2+} was added to the system again, the signals was almost completely reproduced (Fig. 6d) and the colorless solution turned to pink. These findings indicated that probe 1 can reversibly coordinate with Cu^{2+}.

Thus, the most likely binding sites for Cu^{2+} are the conjugated moieties including carbonyl O, imino N, benzoyl hydrazine N and O atoms (Scheme 2). It is very likely due to the chelation–induced ring opening of rhodamine spirolactam, rather than other possible reactions [26, 27].

Detection of Cu^{2+} in living cells

To further demonstrate the practical applicability of the probe in biological samples, fluorescence imaging experiments were conducted in living cells. The fluorescence images of HeLa cells were recorded before and after addition of Cu^{2+}, shown in Fig. 7. In the absence of Cu^{2+}, free 1 showed no detectable fluorescence signal in living cells (Fig. 7a). After incubation with Cu^{2+}, a bright fluorescence was observed in living cells (Fig. 7c). The results suggested that probe 1 can penetrate the cell membrane and can be applied for in vitro imaging of Cu^{2+} in living cells and potentially in vivo.

Method performance comparison

The performance of the proposed probe 1 toward Cu^{2+} was compared with the corresponding performance of some reported fluorescent probes for Cu^{2+} determination, as shown in Table 1. All the fluorescent methods present

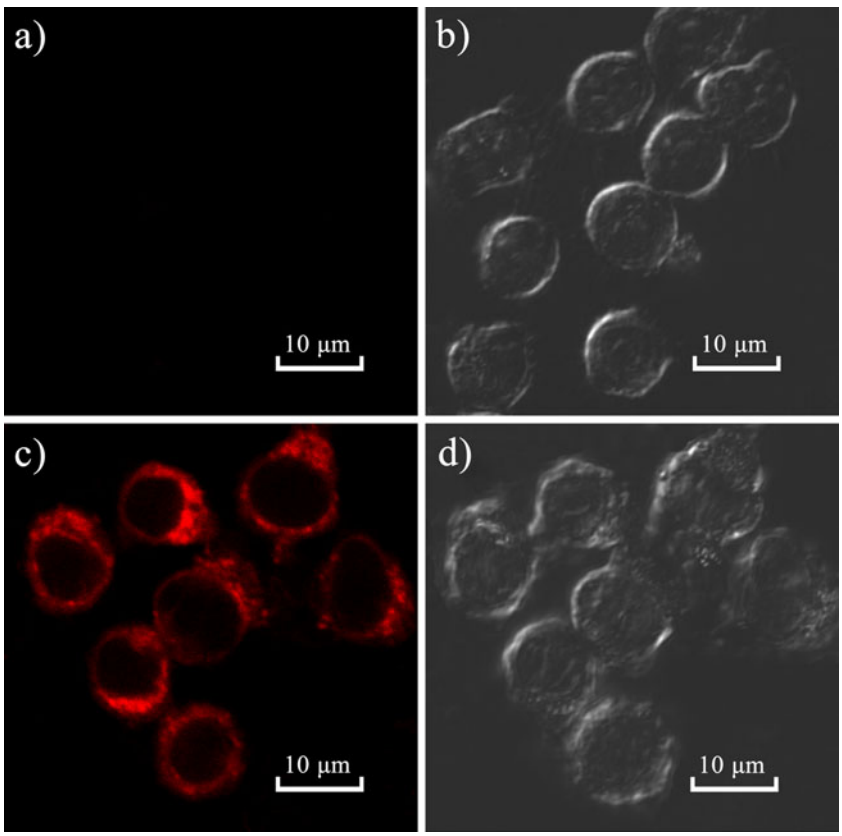


Fig. 7 Confocal fluorescence and brightfield images of HeLa cells. (**a**) Cells stained with 20 μM 1 for 30 min at 37 °C; (**b**) bright field image of cells shown in panel (**a**); (**c**) cells supplemented with 1 μM $CuCl_2$ in the growth media for 1 h at 37 °C and then incubated with 20 μM 1 for 30 min at 37 °C;. (**d**) brightfield image of cells shown in panel (**c**). (λ_{ex}=559 nm)

Table 1 Performance comparison of various fluorescent probes for Cu^{2+} ion

Fluorescence modes	Fluorescence reagents	Reproducibility	Linear range (nM)	LOD (nM)	Testing media	Applications	Remarks	Ref.
Quenching $\lambda_{ex/em}$ =388/460 nm	Luminol derivative	Reversible	0–500	0.27	pH 10.0 (alkaline buffer)	Mineral water samples	Big background fluorescence, long synthetic time	[4]
Quenching $\lambda_{ex/em}$=556/603 nm	Spiropyran derivative	Reversible	750–3600	150	Ethanol or pH 6.98 (0.1 M Tris-HCl)	NA	High yield (89%), big background fluorescence.	[[7]
Enhancement $\lambda_{ex/em}$=495/516 nm	Fluorescein hydrazide	Irreversible	100–1000	64	pH 7.2 (0.01 M Tris-HCl)	Human serum and cerebrospinal fluid	Low yield (70%), long analysis time	[[11]
Enhancement $\lambda_{ex/em}$=530/575 nm	Rhodamine derivative	NA	0–14000	10	Water-CH_3CN (1:1, *v/v*, pH 7.1, 50 mM HEPES)	Waste water samples	Low yield (60%), complicated purification	[22]
Enhancement $\lambda_{ex/em}$=500/552 nm	Rhodamine derivative	Reversible	800–10000	300	Water-ethanol (8:2, *v/v*, pH 7.1, Tris-HCl,)	River samples and HeLa cells	Low yield (23%), complicated purification	[23]
Enhancement $\lambda_{ex/em}$=510/580 nm	Rhodamine derivative	Reversible	50–900	3	Water-methanol (2:8, *v/v*, pH 7.0, 20 mM HEPES)	NA	High yield (80%), simple synthetic route, simple purification	[24]
Enhancement $\lambda_{ex/em}$=510/580 nm	Rhodamine derivative	Irreversible	4.5–160	10	Water-CH_3CN (3:7, *v/v*, pH 7.2, 50 mM HEPES)	HeLa cells	Low yield (68%), simple synthetic route	[25]
Enhancement λex/em=540/586 nm	Rhodamine derivative	NA	1–10	NA	Water-CH_3CN (9:1, *v/v*, pH 7.0, 10 mM Tris-HCl)	NA	Low yield (60%), dual-function chemosensor for Cu^{2+} and ClO^-	[28]
Enhancement $\lambda_{ex/em}$=495/552 nm	Rhodamine derivative	Reversible	NA	NA	Water-CH_3CN (1:1, *v/v*)	EJ cells	Low yield (55%)	[29]
Enhancement $\lambda_{ex/em}$=510/580 nm	Rhodamine derivative	Reversible	50–900	7	Water-methanol (2:8, *v/v*, pH 7.0, 20 mM HEPES)	HeLa cells	High yield (85%), low background fluorescence, simple synthetic route, simple purification	This work

good selectivity for Cu^{2+}. The fluorescence quenching methods using luminol derivative [4] and spiropyran derivative [7], respectively, exhibit wide linear range and good reversibility, even down to 0.27 nM LOD [4], but they both give big background fluorescence and aren't applicable for living cells. Most of the fluorescence enhancement methods possess wide quantitation span [11, 22–25], but some of them have more or less disadvantages, such as irreversibility [11, 25], complicated purification [22, 23], and long analysis time [11]. As for the two types of enhancement probes based on rhodamine derivative, dual-function detection for Cu^{2+} and ClO^- are realized [28] and reversible response in living cells are exhibited [29], however, low yields still restrict their further applications [28, 29]. Our newly developed fluorescence enhancement method presents a number of attractive analytical features such as high sensitivity, wide linear range, good reversibility and reproducibility, good selectivity, high yield and wide applicability. The fluorescence probe 1 based on rhodamine spirolactame derivative is easy to prepare with low cost and can be used for routine analysis of ultra-trace level Cu^{2+} in living cells.

Conclusions

In summary, we describe a new rhodamine-based probe 1, which shows a reversible, selective, and sensitive fluorescence enhancement response to Cu^{2+} via a 1:1 binding mode in aqueous solution. High selectivity toward Cu^{2+} is exhibited and little cross-sensitivity is observed to other commonly coexistent metal ions or anions. Furthermore, probe 1 for Cu^{2+} presents high sensitivity of nanomolar level detection. The molecular design might greatly contribute to the development of more efficient and useful probes based on rhodamine platform. The excellent biological value of probe 1 is demonstrated by the fluorescence imaging in HeLa cells. It is anticipated that the probe will significantly promote the studies on the effects of Cu^{2+} in biological systems.

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