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SHORT COMMUNICATION

A Highly Selective and Sensitive Probe for Cu^{2+} Based on Rhodamine-Pyridazine Conjugate and its Application

Peigang Ding · Shao Li · Linchao Yang · Junye Cheng · Zhenji Wang · Yufen Zhao · Yong Ye

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Abstract A naked-eye fluorescent chemodosimeter based on rhodamine-pyridazine conjugate L was synthesized and characterized. L exhibited high selectivity and excellent sensitivity in both absorbance and fluorescence detection of Cu^{2+} in aqueous solution with a broad pH span (1–10). The detection limit of the probe was shown to be up to 0.336 ppm. A simple paper test-strip system for the rapid monitoring of Cu^{2+} was developed, indicating its convenient use in environmental samples. Furthermore, fluorescence imaging experiments of Cu^{2+} in living MGC803 cells demonstrated its value of practical applications in biological systems.

Keywords Rhodamine B · Fluorescence · Chemodosimeter · Cu^{2+}

Introduction

Nowadays, selective detection and sensitive quantification of transition metal ions has attracted wide-spread interest because of their importance in biological and environmental processes [1]. Among transition metal ions, copper is the third most adequate and an essential trace element in living cells and biological systems of plants and humans [2–4]. But an excess amount of copper in the body would cause gastrointestinal disturbance and may damage to the liver and kidneys [5]. Traditional methods such as atomic absorption spectroscopy and inductively coupled plasma mass spectroscopy which require large and costly instruments, highly trained personnel, and tedious maintenance, which make them hard to be used effectively in detection and imaging of Cu^{2+} in biological samples. As a simple, efficient and economic method, fluorescent bioimaging has provided a facile and less cell-damaging mean of visualizing analytes of environmental and biological interest. Therefore, several fluorescent sensors for Cu^{2+} have been synthesized and investigated [6–10]. However, in most cases which fluorescence changes can only be observed in non-aqueous solvent [11–14], there is still an intense demand for new efficient Cu^{2+} optical chemosensors, especially those which can be applied in aqueous solution with high selectivity and sensitivity.

Rhodamine dyes provide an ideal mode for the construction of the "off-on" type fluorescent chemosensors, owing to their excellent spectroscopic properties, such as long wavelength excitation and emission profiles, large extinction coefficient and high fluorescence quantum yield [15, 16]. Upon metal binding, its structure can undergo a change from the spirolactam to an open ring amide, resulting in a magenta-colored, highly fluorescent compound [16]. To date, lots of rhodamine-based chemosensors for Hg^{2+} [17], Cr^{3+} [18], Ag^{+} [19], Pb^{2+} [20] and Fe^{3+} [21] ions have been developed. There were also some rhodamine-based probes for Cu^{2+} had been reported [22–25], but they could only work in narrow

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P. Ding · S. Li · J. Cheng · Y. Zhao · Y. Ye (✉)
Phosphorus Chemical Engineering Research Center of Henan Province, The College of Chemistry and Molecular Engineering, Zhengzhou University, Zhengzhou, China
e-mail: yeyong03@tsinghua.org.cn

L. Yang · Z. Wang
School of Pharmaceutical Science, Zhengzhou University, Zhengzhou, China

Y. Zhao · Y. Ye
Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology (Ministry of Education), Department of Chemistry, Tsinghua University, Beijing, China

pH span. As known to all, industrial wastewater is always acidic or alkaline solution. There is still an intense demand for new efficient Cu^{2+} optical chemosensors that can work in broad pH span. Herein, we reported a new rhodamine derivative bearing 3-chloro-6-hydrazinylpyridazine as a fluorogenic and chromogenic sensor for Cu^{2+} with a broad pH span (1–10). Among the various metal ions, this probe exhibits remarkably enhanced absorbance intensity and shows significant off-on fluorescence intensity for Cu^{2+} among the various metal ions in aqueous solution with high selectivity and sensitivity. A simple paper test strip system for the rapid monitoring of Cu^{2+} was developed, indicating its convenient use in environmental samples. Moreover, fluorescence imaging experiments of Cu^{2+} ions in living MGC803 cells demonstrated its value of practical applications in biological systems.

Experiment

Apparatus Regents and Chemicals

Fluorescence spectra measurements were performed on the F-4500 FL Spectrophotometer, and the excitation and emission wavelength band passes were both set at 5.0 nm. Absorption spectra were measured on a UV-2102 double-beam UV/VIS spectrometer, Perkin Elmer precisely. NMR spectra were recorded on a BrukerDTX-400 spectrometer in $CDCl_3$, using TMS as internal standard. Mass spectral determination was carried on a HPLC Q-T of HR-MS.

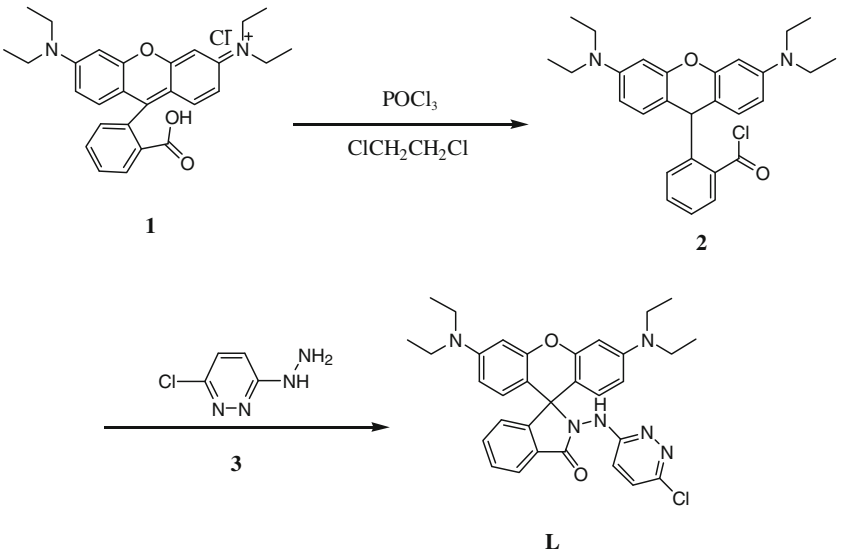
All the materials for synthesis were purchased from commercial suppliers and used without further purification. The solutions of metal ions were prepared from their nitrate salts, except for $FeCl_3$, $FeCl_2$, $CrCl_3$, $AlCl_3$ and $MnCl_2$. The metal ions were prepared as 10.00 mM in water solution.

Synthesis

Target compound L was synthesized on the basis of the route shown in Scheme 1. Compound 3-chloro-6-hydrazinylpyridazine 3 was synthesized from 3,6-dichloropyridazine and hydrazine according to reported method [26].

Synthesis of Compound L

To a solution of compound 1 (240 mg, 0.5 mmol) in dry 1, 2 − dichloroethane (8.0 mL) at room temperature, phosphorus oxychloride (240 mg, 1.5 mmoL) was added dropwise over a period of 5 min. After being refluxed for 4 h, the reaction mixture was cooled and concentrated under vacuum to give rhodamine B acid chloride [20]. This acid chloride was dissolved in dry acetonitrile (10 mL), and added dropwise to a solution of 3-chloro-6-hydrazinylpyridazine (86 mg, 0.6 mmol) in dry acetonitrile (6.0 mL) containing triethylamine (1.0 mL). After stirring for 4 h at room temperature, the mixture was concentrated under vacuum and the crude product was



Scheme 1 Synthetic route of target compound

purified by column chromatography using $CH_3COOCH_2CH_3$/PE (1:1, *v/v*) as eluent to give compound L as a pink solid in 49.3 % yield. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): 1.15(t, 12H, *J*=7 Hz), 3.33(q, 8H, *J*=7 Hz), 6.28(t, 4H, *J*=4.8 Hz), 6.46(m, 3H), 6.54(s, 1H), 6.88(d, 1H, *J*=9.2 Hz), 7.28(d, 1H, *J*= 7.6 Hz), 7.64(m, 2H), 8.01(d, 1H, *J*=7.6 Hz). ^{13}C NMR (100 MHz, CDCl3) δ (ppm): 12.50, 44.24, 66.79, 97.99, 104.75, 107.96, 116.11, 123.60, 124.59, 128.26, 128.54, 128.90, 130.03, 133.77, 149.08, 149.17, 149.62, 154.28, 160.03, 166.45. HR-MS: $C_{32}H_{33}ClN_6O_2$ $[M + H]^+$, calcd for 569.2426. Found: 569.2430 (Supporting Information, Figs. S1–S3).

Results and Analysis

The structure of compound L was characterized by ^{1}H NMR, ^{13}C NMR, and HRMS (shown in Fig. S1-3). The results were in good agreement with the structure shown in Scheme 1. Fluorescence and UV–vis studies were performed using a 10 μM solution of L in a CH_3CN/H_2O (6:4, *v/v*) solution with appropriate amounts of metal ions. The metal ions had chelated with the sensors sufficiently by shaking the solution for 30 min at 40 °C before measuring the absorption and fluorescence.

UV–Vis Spectral Responses of L

As shown in Fig. 1, UV–vis spectrum of compound L (10 μM) exhibited only very weak bands over 450 nm. Additing of 10 equiv. Cu^{2+} into solution immediately resulted in a significant enhancement of absorbance at about 554 nm, and simultaneously accompanying with the color changes from colorless to red. Under the identical condition, no obvious response could be observed upon the addition of other ions including Fe^{3+}, Cr^{3+}, Mg^{2+}, Ca^{2+}, Cd^{2+}, Al^{3+}, Pb^{2+}, Hg^{2+}, Ba^{2+}, Ni^{2+}, Fe^{2+}, Mn^{2+}, K^+, Li^+, Ag^+, Co^{2+} and Na^+ except for Zn^{2+} (Fig. 1), which caused a mild effect compared to Cu^{2+}. The results demonstrated that L was characteristic of high selectivity toward Cu^{2+} over other competitive metal ions.

To further investigate the interaction of Cu^{2+} and L, an ultraviolet titration experiment was carried out (Fig. 2). A linear increasing of absorption intensity of L could be observed accompanying with color changes from colorless to red along with the increasing concentrations of Cu^{2+} (Fig. 1, inset). To determine the stoichiometry of the copper–ligand complex, Job's method for absorbance measurement was applied [27]. (Fig. 3). The absorbance reached a maximum when the ratio being 0.67, indicating a 2:1 stoichiometry of the Cu^{2+} to L in the complex.

Fluorescence Spectral Responses of L

The selectivity of L for Cu^{2+} was further observed in the fluorescent spectra. As shown in Fig. 4, L exhibited a very weak fluorescence in the absence of metal ions. When 10 equiv. Cu^{2+} was introduced in a 10 uM solution of L in (6:4, *v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0), a remarkably enhancement of

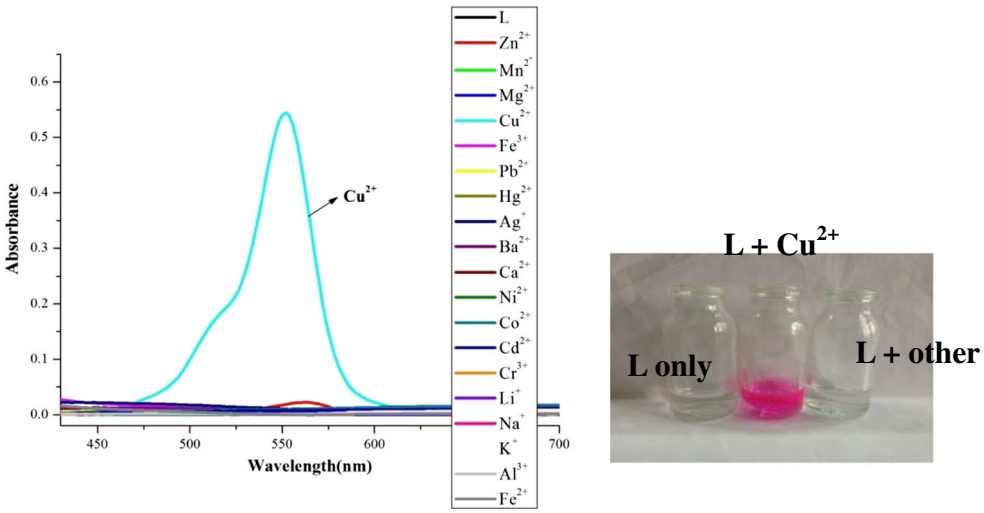


Fig. 1 Absorbance spectra of L (10 μM) in CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0) (6:4, *v/v*) with the presence of 10 equiv. of various species

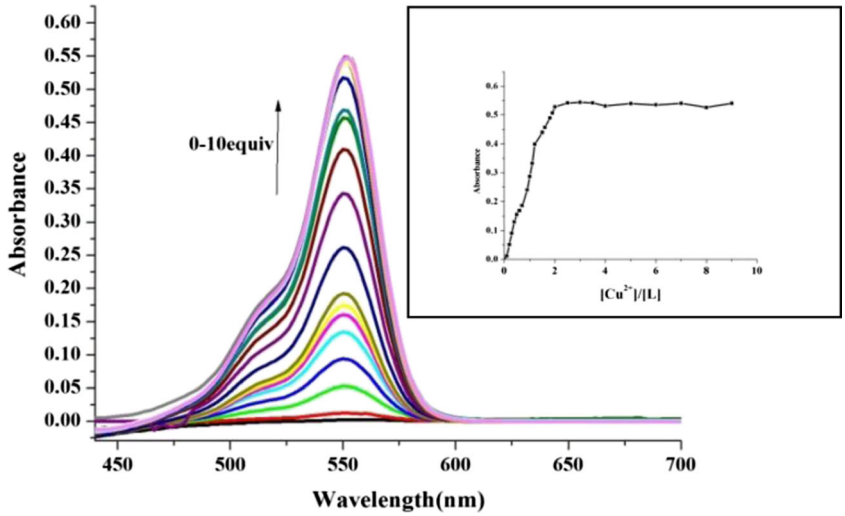


Fig. 2 Absorption spectra of L (10 μM) with gradual addition of various amounts of Cu^{2+} (from bottom 0–10 equiv.) in 6:4 (*v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0)

fluorescence spectra was observed. Competition experiments were carried out to explore the use of L as an ion-selective fluorescent probe for Cu^{2+}. L (1 mM) was treated with 10 equiv. Cu^{2+} in the presence of other metal ions (10 equiv.). As shown in Fig. 5, the competing metal ions showed very low interference with the detection of Cu^{2+}. Moreover, the competitive experiments also confirmed that the background metal ions (Fig. 5) showed very low interference with the detection of Cu^{2+} in water solution. The fluorescence response of L toward Cu^{2+} in the presence of various coexistent anions was also investigated, and it was gratifying to notice that all the tested anions had low interference (Fig. 6).

To further investigate the interaction of chemodosimeter L with Cu^{2+}, a fluorescence titration

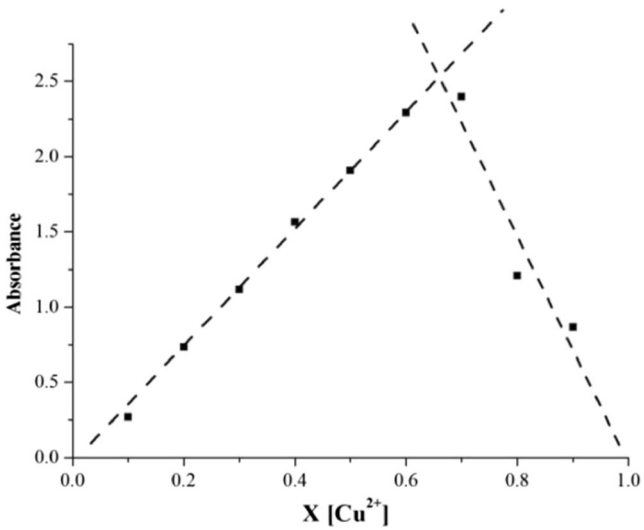


Fig. 3 Job's plot according to the method for continuous variations, indicating the stoichiometry for L-Cu^{2+} (the total concentration of L and Cu^{2+} is 100 μM). $X_{Cu^{2+}} = C_{Cu^{2+}}/C_L + C_{Cu^{2+}}$

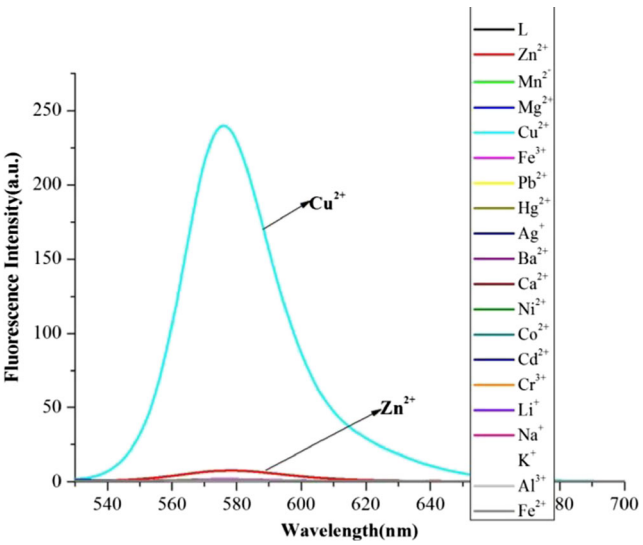


Fig. 4 Fluorescence spectra of L (10 μM) in the presence of 100 μM different metal ions in (6:4, *v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0). λ_{ex} = 520 nm, scan range 530–700 nm, slit width 5 nm

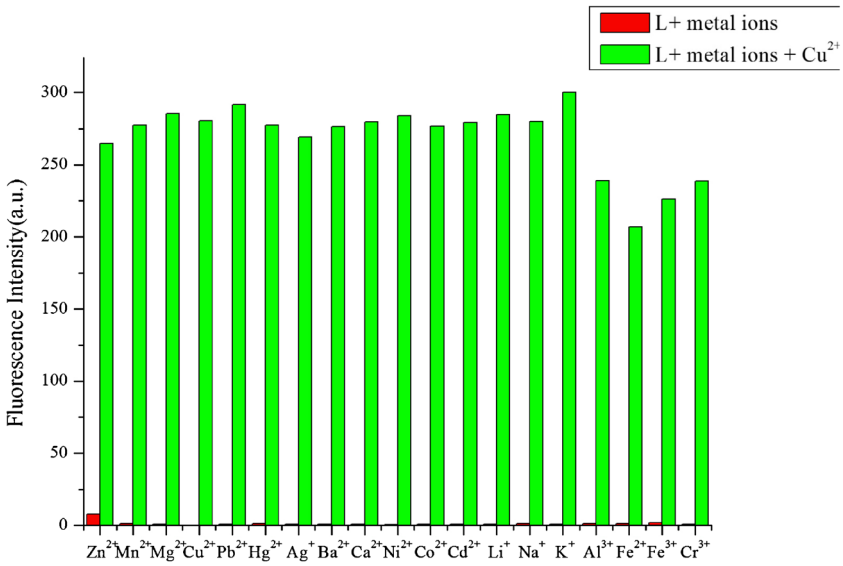


Fig. 5 Fluorescence responses of L to various cations in (6:4, v/v) CH_3CN /water solution (1 mM Tris–HCl, pH = 7.0). [L] = 10 μM, λex = 520 nm, λem = 577 nm

experiment was conducted. As shown in Fig. S4, the fluorescence intensity of L was enhanced with the increasing concentration of Cu^{2+}. The result indicated a 1:2 stoichiometry as the most possible for the binding mode of Cu^{2+} and L. Moreover, the time-dependence fluorescence of probe L was also evaluated in the presence of Cu^{2+} ions (Fig. S5). The kinetics of fluorescence enhancement at 577 nm by the probe L were recorded, and the results indicated that the recognizing event could complete in 70 min (T=25 °C) and 120 min (T=15 °C). These results also demonstrated that compound L was a selectivity and rapidly sensor for Cu^{2+} over various other metal ions.

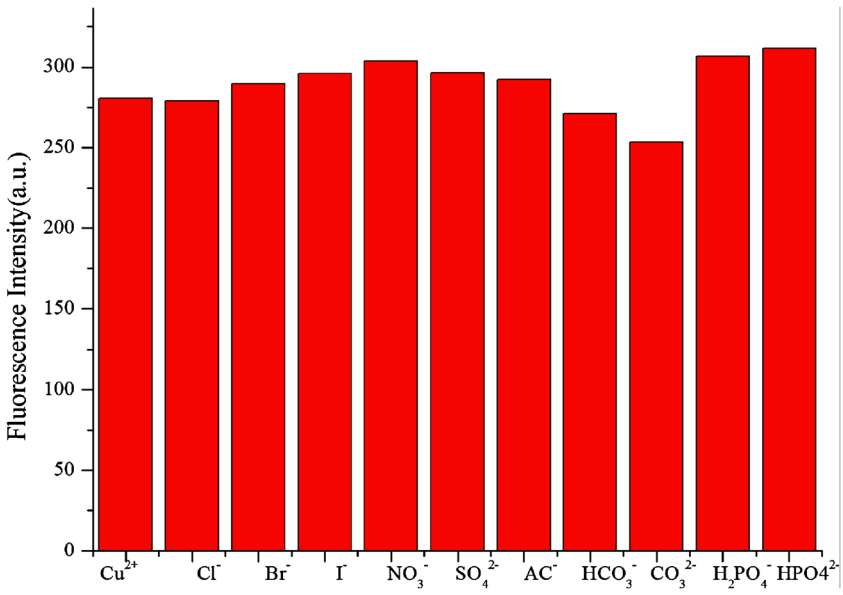


Fig. 6 Fluorescence responses of L to various anions in (6:4, *v/v*) CH_3CN /water solution (1 mM Tris–HCl, pH = 7.0). [L] = 10 μM, λ_{ex} = 520 nm, λ_{em} = 577 nm

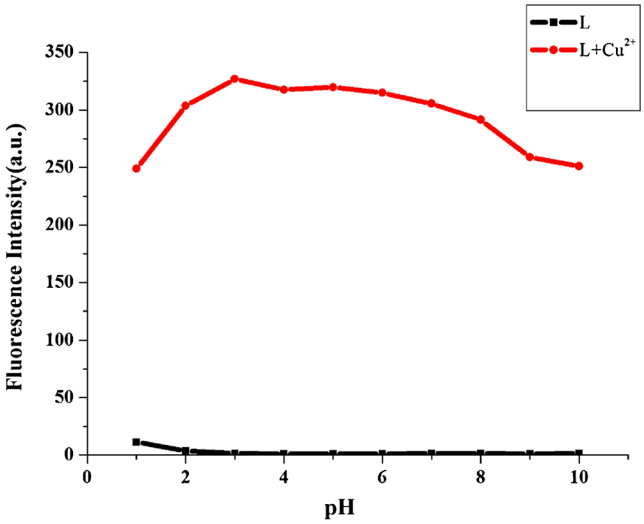


Fig. 7 Fluorescence intensity (577 nm) of free chemodosimeter L (10 μM) and in the presence of 10 equiv. Cu^{2+} in CH_3CN/Tris–HCl buffer (6:4, *v/v*) solutions with different pH conditions

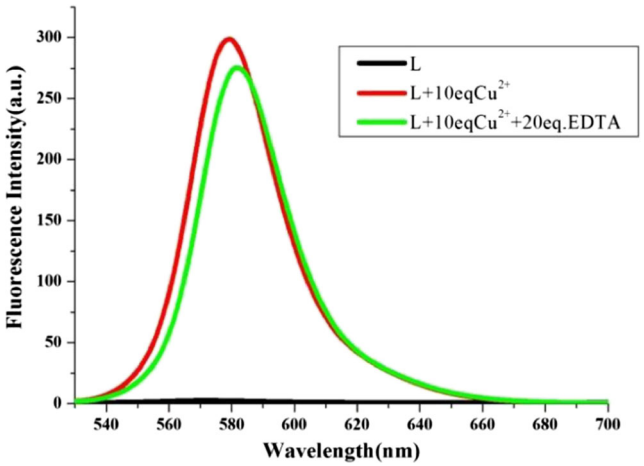


Fig. 9 Fluorescence intensity (at 577 nm) of L (10 μM) to Cu^{2+} in (6:4, *v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0). (1) Baseline: 10 μM L only; (2) red line: 10 μM L with 10 equiv. Cu^{2+}; (3) green line: 10 μM L with 10 equiv. Cu^{2+} and then addition of 20 equiv. EDTA

Effect of pH

To study the practical applicability, the fluorescence responses of sensor L in the absence and presence of Cu^{2+} in different pH values were evaluated. As shown in Fig. 7, the fluorescence titration curve of free chemodosimeter in CH_3CN/Tris–HCl buffer did not show obvious characteristic color of rhodamine between pH 1.0 and 10.0, suggesting that spirolactam tautomer of L was insensitive to the pH changes in this range. However, the addition of Cu^{2+} led to the fluorescence enhancement over a wide pH range (1.0–10.0), which was attributed to the opening of the rhodamine ring. Consequently, L might be used to detect Cu^{2+} in some environmental and physiological regions in a wide pH range.

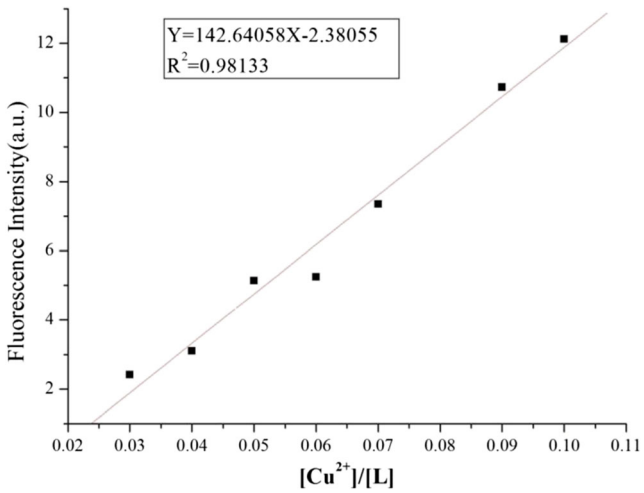


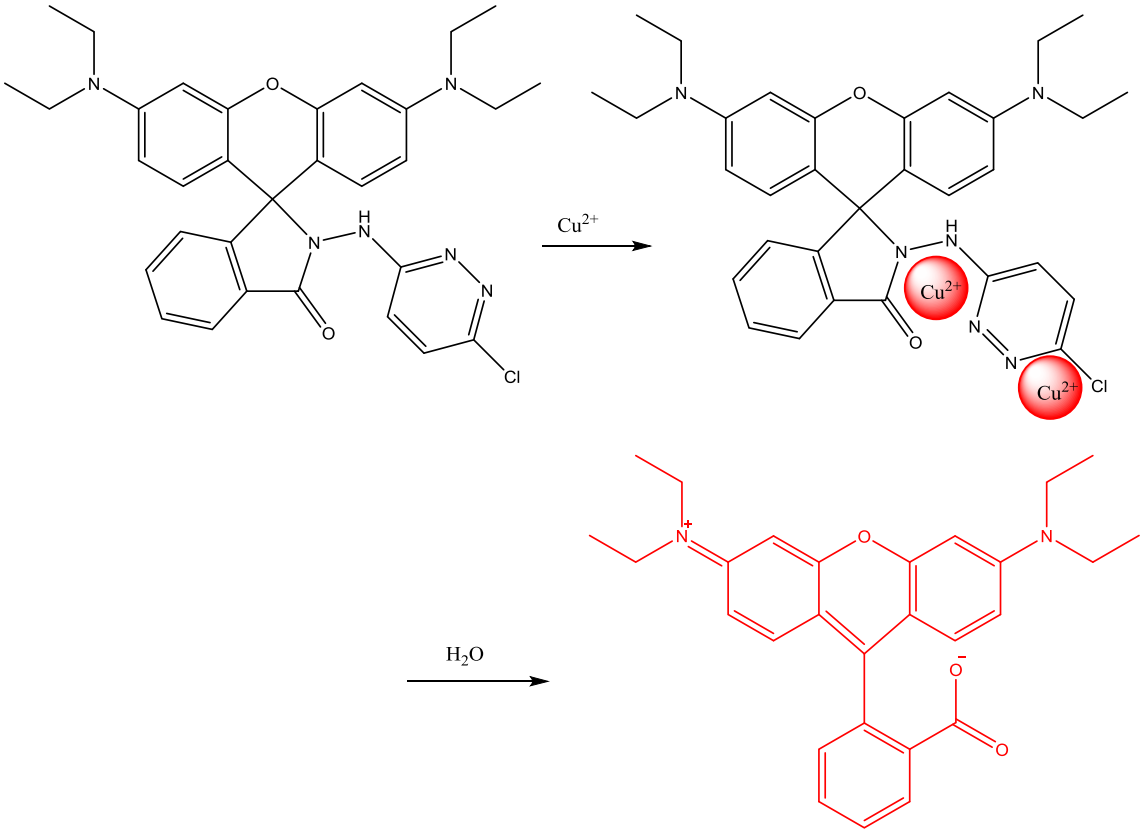
Fig. 8 The fluorescence intensity (at 577 nm) of compound L (10 μM) as a function of the Cu^{2+} concentration (0.1–1 μM) in water solution (λ_{ex} = 520 nm, slit width 5 nm)

The Detection of Cu^{2+}

Generally, one of the most important and useful applications of a fluorescent chemodosimeter is the detection of metal ions. Owning to its excellent spectroscopic properties, L is sensitive enough to detect relevant concentrations of Cu^{2+} in water samples. When L was employed at 10 μM in (6:4, *v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0), the fluorescent intensity of L was proportional to the concentration of Cu^{2+} added (Fig. 8). The detection limit was measured to be 0.336 ppm, establishing that L was capable of distinguishing safe and toxic levels of Cu^{2+} in drinking water according to the U.S. (MCL) standard [28] and China SA standard [29].

Mechanism

All of the above absorption spectra responses were irreversible, which was confirmed by the reversible titration using EDTA. When excess EDTA (2 equiv. of Cu^{2+}) was added to



Scheme 2 Possible sensing mechanism of L

the L + Cu^{2+} in (6:4, *v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0), the red color of the solution almost unchanged, indicating that the coordination of L with Cu^{2+} was chemically nonreversible. And the fluorescence emission changes of above solution had also confirmed it (Fig. 9). Thus, based on the 1:2 binding mode and the nonreversible behavior between L and Cu^{2+}, and according to our knowledge [24, 25, 30–32], we broached a conceivable mechanism of Cu^{2+} complex with L (Scheme 2). A directly evidence was obtained by comparing the HR-MS of L (Fig. S3) and L + Cu^{2+} (Fig. 10) in (6:4, *v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0). An unique peak at m/z 443.2 corresponding to [rhodamine B + H]$^+$ was clearly observed when 2 equiv. of Cu^{2+} was added to L, whereas L without Cu^{2+} exhibited peaks only at m/z 569.2 which corresponded to [L + H]$^+$(Fig. S3).

Applications

Many fluorescent sensors for Cu^{2+} detection could only be performed in organic solution, which would limit their applications under special circumstances such as on-site detection in situ. To demonstrate the practical application of our sensor, we prepared the test papers of sensor L. It was easily prepared by immersing a filter paper into the solution of L in DCM (1 mM) and then drying in air. Next, to different Cu^{2+} concentration solutions, these strips were immersed for 5 s and taken out of the solution. As shown in Fig. S6, the color of the test paper changed from colorless to red and deepened gradually with the increasing of Cu^{2+} concentration. These paper-made test kits might be used as a simple tool for detecting Cu^{2+} in environmental samples.

To further assess the potential applications of the probe as a Cu^{2+} probe in living cells, fluorescent imaging inside MGC-803 cells was monitored by fluorescence microscopy. As shown in Fig. 11b, very weak fluorescence of L inside the living MGC-803 cells was observed. After washing with water twice, 10 μM of Cu^{2+} were then supplemented to the cells. After incubated at 37 °C for 30 min later, a significant increase in

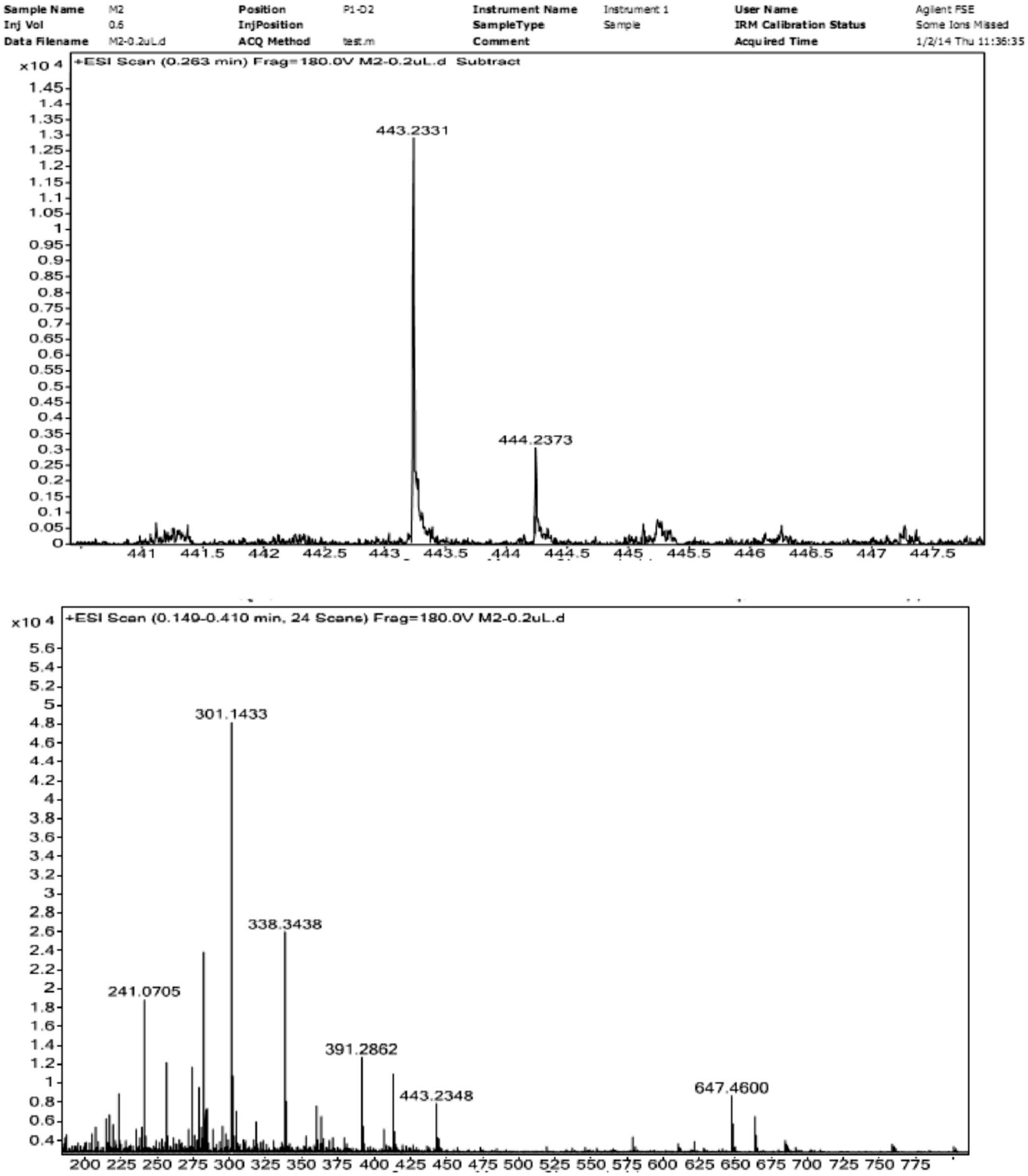


Fig. 10 HR-MS of L in the presence of 2 equiv. Cu^{2+} in (6:4, *v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0)

the fluorescence from the intracellular area was observed (Fig. 11d). A bright field transmission image of cells with Cu^{2+} and L confirmed that the cells were viable throughout the imaging experiments (Fig. 11a

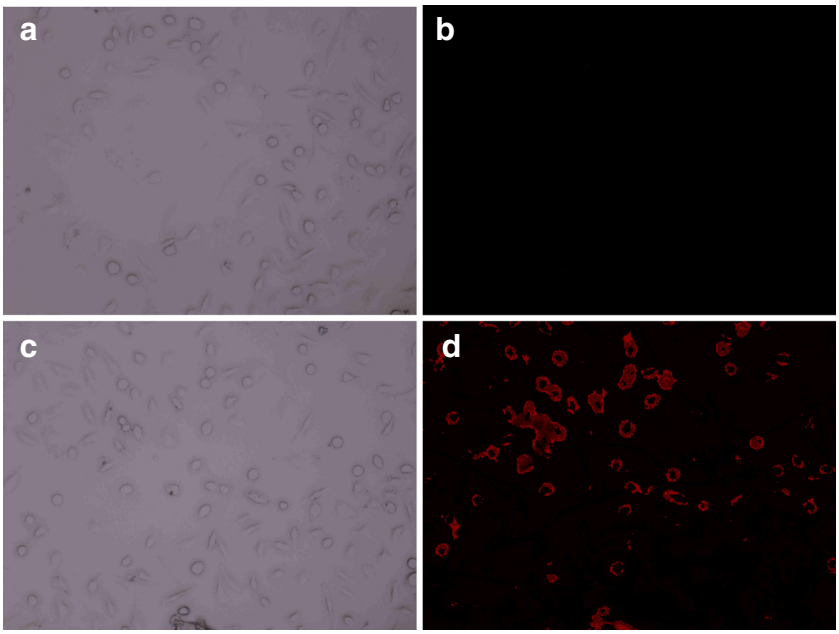


Fig. 11 Fluorescence images of Cu^{2+} in MGC-803 cells with 5 μM solution of L in H_2O for 30 min at 37 °C, bright-field transmission images (**a**, **c**) and fluorescence images(**b**, **d**) of MGC-803 cells incubated with 0, 10 μM of Cu^{2+} for 30 min, respectively

and c). These results indicated that L might be useful for detecting Cu^{2+} in biological samples.

Conclusion

In summary, an efficient rhodamine-based fluorescent chemodosimeter L was synthesized. Chemodosimeter L exhibited selectivity and sensitivity in (6:4, *v/v*) CH_3CN/water solution (1 mM Tris–HCl, pH = 7.0) with dramatic enhanced fluorescence intensities. The significant changes in the fluorescence color could be used for naked-eye detection. L might be used to detect Cu^{2+} in some environmental regions in a wide pH range with a detection limit up to 0.336 ppm. Moreover, it was applied for imaging in MGC803 cells to confirm that it could be used as a fluorescent sensor for monitoring Cu^{2+} in living cells.

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