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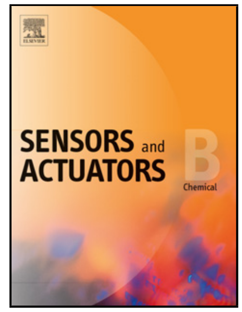

Title: Highly sensitive and fast responsive ratiometric fluorescent probe for Cu^{2+} based on a naphthalimide-rhodamine dyad and its application in living cell imaging

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Highly sensitive and fast responsive ratiometric fluorescent probe for Cu^{2+} based on a naphthalimide-rhodamine dyad and its application in living cell imaging

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Highlights

1. The detection limit of a new FRET probe for Cu^{2+} was 18.6 nM.
2. The probe exhibited high selectivity and sensitivity detection of in aqueous solution with a wide pH span (4.0-10.0).
3. The significant changes in color could be used for naked-eye detection
4. The response time to Cu^{2+} is less than 1 min.
5. The fluorescence imaging experiment demonstrated its value of practical application.

Abstract: Based on fluorescent resonance energy transfer (FRET), we developed a ratiometric fluorescent probe **1** for sensing Cu^{2+}. As expected, probe **1** exhibited high selectivity and excellent sensitivity in both absorbance and fluorescence detection of Cu^{2+} in aqueous solution. A significant color change from pale yellow to pink could be observed, enabling naked-eye detection of Cu^{2+}. Furthermore, fluorescence imaging experiment of Cu^{2+} in living MGC-803 cells demonstrated its value of practical applications in biological systems.

Keywords: Fluorescence, Ratiometric, FRET, Copper, Cell imaging, naphthalimide-rhodamine

1. Introduction

The determination of metal ions has received tremendous attention in the last two decades, because the metal ions play a significant role in the environment, biology and chemistry [1-3]. As the third-most abundant transition metal in human body and many living organisms, copper plays vital roles [4-5]. However, excess copper in the neuronal cytoplasm can lead many diseases, such as Parkinson's disease, Alzheimer's disease and Wilson disease [6-7]. Therefore, the detection of copper ion content in the physiological environment is very important.

Czarnik et al reported the rhodamine-B derivative and its ring-open reaction [8]. After then, many Cu^{2+} probes based on fluorescence intensity were synthesized [4, 9-16]. Although turn-on probes are more sensitive, a major limitation is that variations in the sample environment (pH, polarity, temperature, and so forth) might influence the fluorescence intensity measurements, due to the lack of background signal [17]. The ratiometric chemosensors can provide built-in correction for environmental factors by measuring the changes of emission intensities ratio at two different wavelengths followed by calculation of their intensity ratio [18-20]. From this point of view, fluorescence resonance energy transfer (FRET) based sensors were the most valuable [21-24]. To the best of our knowledge, only a few FRET probes for detecting Cu^{2+} based on rhodamine have been reported [25-30].

Scheme 1 Three kinds of rhodamine-based FRET probe

According to the reported rhodamine-based FRET probe, we found they can be classified into three types (Scheme 1. Type A-C). Most of reported probe were Type A which the energy donor is linked to the interaction site of rhodamine [25-28]. This may induce cleavage of the FRET dyad and are not suitable for analytes. There are several Type B probes were reported. An energy donor is selectively incorporated into the 4/5-position carboxylic acid group through an appropriate linker to form a Type B FRET platform. However, 4/5-position mixture isomers of FRET dyads are very difficult to separate by standard column chromatography [30]. For a FRET system, a substantial spectral overlap which is necessary between the donor emission band and the acceptor absorption band is closely related to the energy transfer efficiency. The naphthalimide derivatives have broad absorption spectra (450-650 nm) [31-33], which covers a part of absorption of rhodamine (500-560 nm) and fulfills a favorable condition for FRET (Fig. S1). In this paper, we construct a type C probe for sensing Cu^{2+} using piperazine to link the energy donor. Compared with Type A and Type B, this probe has the following advantages: 1) The interaction site is far away from the energy donor. 2) The strategy is suitable for target analytes that otherwise could induce the cleavage of the FRET dyad. 3) This approach only yields a single FRET dyad. The FRET energy transfer efficiency (E) was calculated to be 82.1% as $E=1-F_{DA}/F_{D}$. Where, F_{DA} and F_{D} denote the donor fluorescence intensity with and without an acceptor, respectively (Fig. S2) [34-35]. The synthetic route of type C probe was shown in Scheme 2. Probe **1** exhibited excellent sensitivity and selectivity with a detection limit of 18.6 nM. It also has the shortest response time (< 1 min) for Cu^{2+} in (CH_3CN/H_2O = 1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40). Further, fluorescence imaging experiments of Cu^{2+} in living MGC-803 cells demonstrated its value of practical application in biological systems.

2. Experimental

2.1. Apparatus

Fluorescence spectra measurements were performed on a HITACHI F-7000 fluorescence spectrophotometer, and the excitation and emission wavelength band passes were both set at 10.0 nm. Absorption spectra were measured on a Lambda 35 UV/VIS spectrometer, Perkin Elmer precisely. NMR spectra were measured on a Bruker DTX-400 spectrometer in $CDCl_3$ with TMS as internal standard. Mass spectral determination was carried on a HPLC Q-Tof HR-MS spectrometer (Waters Micromass) by using methanol as mobile phase.

2.2. Materials

All chemicals and reagents were used as received from commercial sources without further purification. Solvents for chemical synthesis and analysis were purified according to standard procedures. Double distilled water was used throughout the experiment. Chloride salts of metal ions (Li^{+}, K^{+}, Na^{+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Cr^{3+}) and the nitrate salt of Pb^{2+} and Ag^{+} were used to evaluate the metal ion binding properties by synthesized compounds. The metal ions were prepared as 10.00 mmol/L in water solution.

2.3. Synthesis

Part 1 and **part 2** was synthesized by reported methods (the detailed synthetic routes were shown in Supporting information) [29, 36].

To a solution of **part 2** (0.2 g, 0.43 mmol) and triethylamine (5 mL) in anhydrous CH_2Cl_2 (10 mL), **part 1** (0.15 g, 0.36 mmol) mixed with anhydrous CH_2Cl_2 (10 mL) were added dropwise at 0 °C for 30 min. After then the resulting mixture was stirred at room temperature for 4 h. After removed of solvent, the residue was purified by silica gel column chromatography (CH_3OH:CH_2Cl_2 = 1:10, v/v) to afford the pure product 0.13 g (42 %) of **1** as a yellow foamy solid. Mp: 122-125 °C. ^{1}H NMR ($CDCl_3$, 400 MHz, ppm): 1.18 (t, 6 H, J = 7.0 Hz), 3.25 (s, 4 H), 3.36 (q, 4 H, J = 6.8 Hz), 3.64-3.67 (m, 4 H), 3.70 (s, 4 H), 3.79 (s, 4 H), 5.5 (s, 2 H, NH_2), 6.31-6.34 (m, 1 H), 6.56 (d, 2H, J = 1.2 Hz), 6.66-6.69 (m, 4H), 7.09-7.11 (m, 1H), 7.26-7.32 (m, 3H), 7.47-7.49 (m, 2 H), 7.79 (t, 1 H, J = 7.8 Hz), 7.95-7.97 (m, 1 H), 8.26 (d, 1 H, J = 7.6 Hz), 8.64-8.66 (m, 1 H); ^{13}C NMR ($CDCl_3$, 100 MHz, ppm): 12.58, 44.39, 48.74, 65.66, 66.79, 97.96, 102.97, 108.32, 109.68, 112.09, 114.21, 121.88, 123.11, 123.79, 124.50, 124.62, 127.06, 128.04, 128.12, 128.37, 129.40, 129.44, 129.86, 131.57, 131.74, 132.68, 134.51, 148.35, 148.41, 149.00, 151.27, 151.85, 153.51, 153.60, 160.98, 166.28, 170.95; HR-MS: $C_{51}H_{47}N_7O_6$ $[M-H]^{-}$, calcd for 852.2546, found 852.2543.

Scheme 2. Synthetic route of **1**

3. Results and discussion

Fluorescence and UV-vis studies were performed using 10 μM solution of **1** in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) with 10 equiv. amounts of metal ions. Solutions were stayed for 10 minutes before measuring the absorption and fluorescence in order to make the metal ions chelate with the sensors sufficiently.

Compound **1** was pale yellow in aqueous solution due to the naphthalimide donor moiety and found very stable in the above-mentioned solution system for more than one week. Upon addition of Cu^{2+}, a significant color change from pale yellow to pink could be observed, enabling colorimetric and naked-eye detection of Cu^{2+}.

Based on our work in rhodamine-based Cu^{2+} probe [11], we know that organic solvents have great effect on the coordination reaction. When the coordination reaction was performed in acetonitrile–water solution, high F/F0 and A values were obtained, indicating that acetonitrile–water media is favorable for fluorescent measurement. According to the reported [11, 37], it was found that the fluorescence signal can reached maximum value at 30% aqueous acetonitrile for rhodamine-based Cu^{2+} probe. Taking into account its application in cell imaging, we choose 10% aqueous acetonitrile in Tris-HCl buffer for fluorescent assay.

3.1. UV-vis spectra

An important feature of **1** was its high selectivity toward the Cu^{2+} over the other competitive species. Changes of UV/vis spectra of **1** caused by Cu^{2+} and other metal ions including Li^{+}, K^{+}, Na^{+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Pb^{2+}, Ag^{+} and Cr^{3+}in CH_3CN/H_2O solution (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) were recorded in Fig. 1. All the competitive cations did not lead to any significant absorption changes in the visible region.

Fig. 1 UV-vis absorbance spectra of **1** (10 μM) in the absence and presence of 10 equiv. different metal ions in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution. Inset shows the photo **1** with different metal ions.

As shown in Fig. S14, an increase in the absorption intensity at 550 nm emerged soon after the addition of Cu^{2+}, which corresponded to the absorption of rhodamine. The absorption increased gradually after the addition of Cu^{2+} and a significant color change from pale yellow to pink could be observed easily by eyes, indicating that the addition of Cu^{2+} can promote the ring-opened reaction of the compound **1**.

To determine the stoichiometry of the Cu^{2+}-ligand complex, Job's method for absorbance measurement was applied. As shown in Fig. 2, the UV-vis titrations reached a maximum when the ratio of $[Cu^{2+}]/\{[Cu^{2+}]+[\mathbf{1}]\}$ was 0.5, indicating a 1:1 binding stoichiometry between Cu^{2+} and **1**.

Fig. 2 Job's plots of the complexation between **1** and Cu^{2+}. $[Cu^{2+}]+[\mathbf{1}]$ = 20 μM.

Fig. 3 Fluorescence spectra of **1** (10 μM) in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution with the presence of 10 equiv. of various species (λ_{ex} = 420 nm, slit = 10 nm).

3.2. Fluorescence spectral

The selectivity of **1** for Cu^{2+} was further observed in the fluorescent spectra. As shown in Fig. 3, **1** alone exhibited a strongly emission of the naphthalimide centered about 540 nm when excited at 420 nm. When 10 eq metal ions such as Li^{+}, K^{+}, Na^{+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Pb^{2+}, Ag^{+}, and Cr^{3+} were added, no obvious changes of fluorescence intense could be observed. However, upon addition of Cu^{2+} under the same condition, a new enhancement of the fluorescence emission at 570 nm appeared quickly. These changes could be ascribed to the Cu^{2+} induced opening of the spirocyclic ring of the rhodamine moiety that facilitated the acceptance of fluorescence resonance energy transfer from the naphthalimide donor.

Moreover, the competitive metal ions binding studies shown in Fig. 4 confirmed that background metal ions showed very low interference with the detection of Cu^{2+} in CH_3CN/H_2O solution (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40). These results also demonstrated that compound **1** was a selectivity sensor for Cu^{2+} over various other metal ions. Also, it was investigated that the fluorescence response of compound **1** toward $CuCl_2$ in the presence of various coexistent anions such as Br^{-}, NO_3^{-}, SO_4^{2-}, OAc^{-}, HCO_3^{-} and $H_2PO_4^{-}$. It is gratifying to note that all the tested anions have no interference with the fluorescence response of compound **1** toward Cu^{2+} (Fig. 5).

Fig. 4 Metal-ion selectivity of **1** in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution. The black bars represent the fluorescence emission ratio (F_{570}/F_{540}) of a solution of **1** (10 μM) and 10 equiv. of other metal ions. The red bars show the F_{570}/F_{540} ratio after the addition of 10 equiv. of Cu^{2+} to the solution containing **1** (10 μM) and different metal ions (100 μM) (λ_{ex} = 420 nm, slit = 10 nm).

Fig. 5 The ratiometric fluorescence responses (F_{570}/F_{540}) of **1** (10 μM) upon the addition of 100 μM $CuCl_2$ in the presence of 100 μM background anions in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution (λ_{ex} = 420 nm, slit = 10 nm).

In order to investigate the influence of the different acid concentration on the spectra of **1** and find a suitable pH span in which **1** can selectively detect Cu^{2+} efficiently, the acid titration experiments were performed. As shown in Fig. 6, the fluorescent titration curve of free sensor **1** in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer) solution did not show obvious changes of the fluorescence intensity ratio at (F_{570}/F_{540}) between pH 1.0 and 10.0, suggesting that spirolactam tautomer of **1** was insensitive to the pH changes in this range. However, the addition of Cu^{2+} led to the fluorescence intensity ratio enhancement over a comparatively wide pH range (4.0-10.0), which is attributed to opening of the rhodamine ring. Consequently, **1** might be used to detect Cu^{2+} in approximate physiological conditions.

Fig. 6 The ratiometric fluorescence responses (F_{570}/F_{540}) of free **1** (10 μM) and in the presence of 10 equiv. Cu^{2+} in CH_3CN/Tris-HCl (1/9, v/v, 1 mM) solution with different pH conditions (λ_{ex} = 420 nm, slit = 10 nm).

Moreover, a time course of the fluorescence response of **1** upon addition of Cu^{2+} was shown in Fig. 7. The kinetics of fluorescence enhancement at 570 nm by the new developed ratiometric fluorescent probe was recorded, and results indicated that the recognizing event is very quickly (< 1 min) and could complete in 5 min. That means compound 1 can be used as a ratiometric fluorescence probe for the fast detection of Cu^{2+}.

Fig. 7 Kinetics of the ratiometric fluorescence responses (F_{570}/F_{540}) of free **1** (10 μM) and in the presence of 10 equiv. Cu^{2+} in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution (λ_{ex} = 420 nm, slit = 10 nm).

We also have conducted the titration experiments with the addition of Cu^{2+} for probe **1** (Fig. S15). Upon addition gradual increase of Cu^{2+} to the solution of **1**, the emission at 570 nm was appeared and increased remarkably. The emission intensities ratio at 570 nm and 540 nm (F_{570}/F_{540}) exhibited a change from 0.7 to 2, which showed that the FRET process occurred. One of the most important and useful application for a fluorescence sensor is the detection limit of metal ions. The fluorescence intensity ratio was found to increase linearly with the Cu^{2+} concentration in the range of 0-16 μM (Fig. 8) and the detection limit of Cu^{2+} was measured to be 18.6 nM [38, 39], which were far lower than the WHO recommended value for Cu^{2+} (2.0 mg/L) and the U.S. Environment Protection Agency (EPA) set the maximum allowable level of copper (~20 μM) in drinking water [40, 41].

Fig. 8 Fluorescence intensity ratio changes (F_{570}/F_{540}) of **1** (10 μM) upon gradual addition of Cu^{2+} in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution (λ_{ex} = 420 nm, slit = 10 nm).

3.3. Mechanism

Further, it was of great interest to investigate the reversible binding nature of the sensor (Fig. 9). Upon addition of 10 equiv. EDTA to the solution of probe **1** (10 μM) with Cu^{2+} (10 equiv.), the fluorescence intensity at 570 nm was not quenched (blue line). These indicated that the coordination of probe **1** with Cu^{2+} is chemically irreversible.

Fig. 9 Fluorescence intensity of **1** (10 μM) to Cu^{2+} in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution (λ_{ex} = 420 nm, slit = 10 nm).

To further investigate the binding stoichiometry of **1** and Cu^{2+} ions, the form of **1**- Cu^{2+} complex was also confirmed by ESI-MS analysis (Fig. S16). When $CuCl_2$ was added to **1** in CH_3CN, the peak of $[\mathbf{1}+H]^+$ (854.58) was disappeared, in the same time, a new peak 840.65 was appeared, which was suggested the compound $[\mathbf{2}+H]^+$ (calcd = 840.34). Herein, according to our knowledge [29, 42-45], we broached a conceivable mechanism of Cu^{2+} complex with **1** (Scheme 3).

Scheme 3. Possible sensing mechanism of **1** with Cu^{2+}.

4. Bioimaging application of compound 1 in MGC-830 Cells [46, 47]

Bioimaging applications of compound **1** for monitoring of Cu^{2+} ions in living cells were then carried out. MGC-803 cells were used for this experiment. MGC-803 cells were incubated with **1** (10 μM) in RPMI1640 medium (containing 10% fetal bovine serum) for about 30 min at 37 °C showed a strong green intracellular fluorescence (Fig. 10b) and a weak red one (Fig. 10c). After then, 20 μM of Cu^{2+} were then supplemented to the cells, and all the reaction mixtures were incubated at 37 °C for another 30 min, a significant increase in the red fluorescence from the intracellular area was observed (Fig. 10f). A bright field transmission image of cells with Cu^{2+} and **1** confirmed that the cells were viable throughout the imaging experiments (Fig. 10a and d). These results clearly indicated that **1** might be used for ratiometric imaging of Cu^{2+} in biological samples.

Fig. 10 Bright field and fluorescence microscopic images of MGC-803 cells. MGC-803 cells incubated by compound **1** (10 μM) observed under bright field (a), green channel (b), red channel (c), then further incubation with Cu^{2+} (20 μM) for 30 min at 37 °C under bright field (d), green channel (e), red channel (f).

5. Conclusion

In summary, we reported a novel FRET based fluorescent probe **1** for the ratiometric detection of Cu^{2+} in aqueous solution. It exhibited high sensitivity and selectivity toward Cu^{2+} and could be conveniently detected even by naked eye. Probe **1** was suitable in a broad pH range 4-10 and allowed the ratiometric detection of intracellular Cu^{2+} levels in live MGC-803 cells. Moreover, probe **1** exhibited a low detection limit (18.6 nM) and a rapid response time (< 1 min) for Cu^{2+}.

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Biographies

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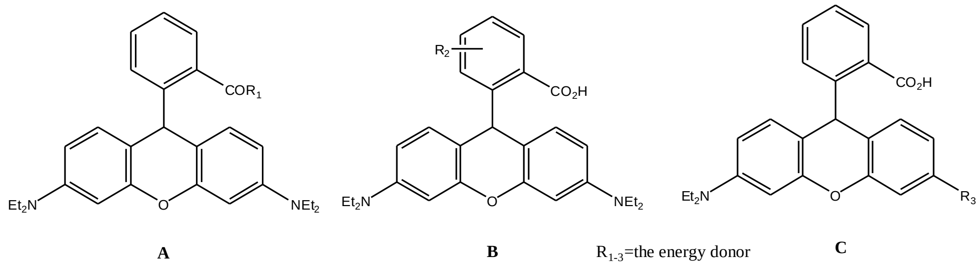
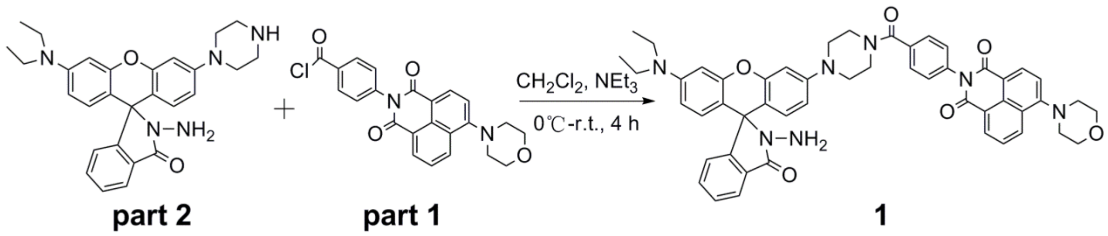


Scheme 1 Three kinds of rhodamine-based FRET probe



Scheme 2. Synthetic route of **1**

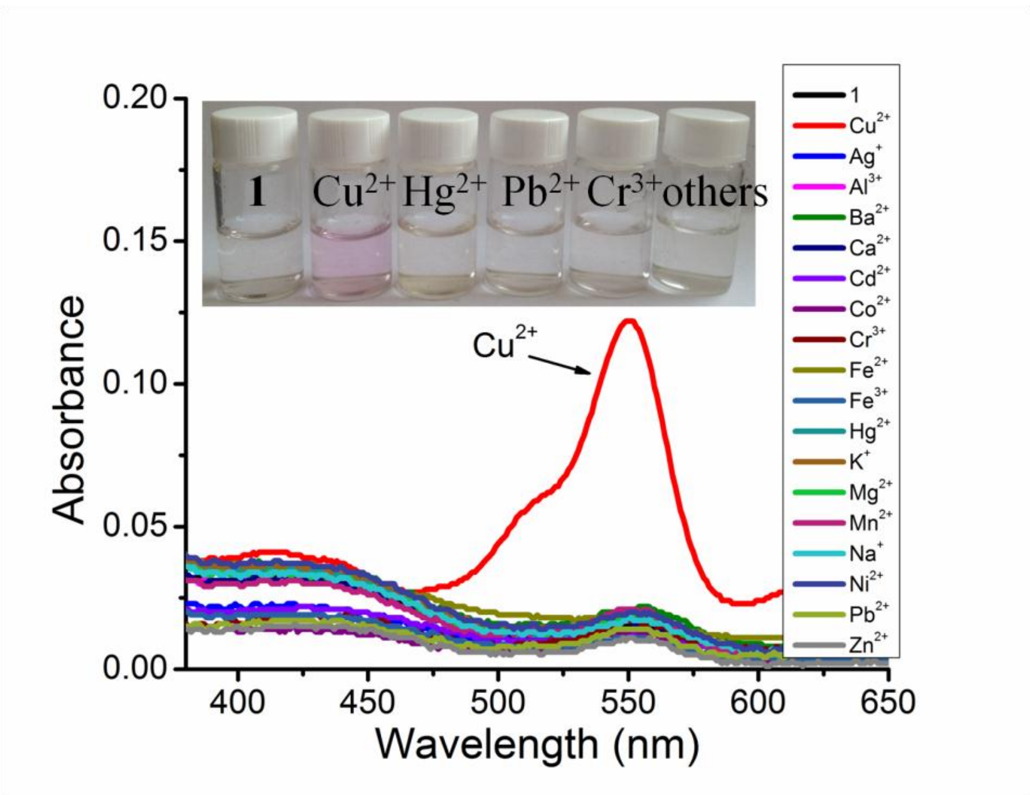


Fig. 1 UV-vis absorbance spectra of **1** (10 μM) in the absence and presence of 10 equiv. different metal ions in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution. Inset shows the photo **1** with different metal ions.

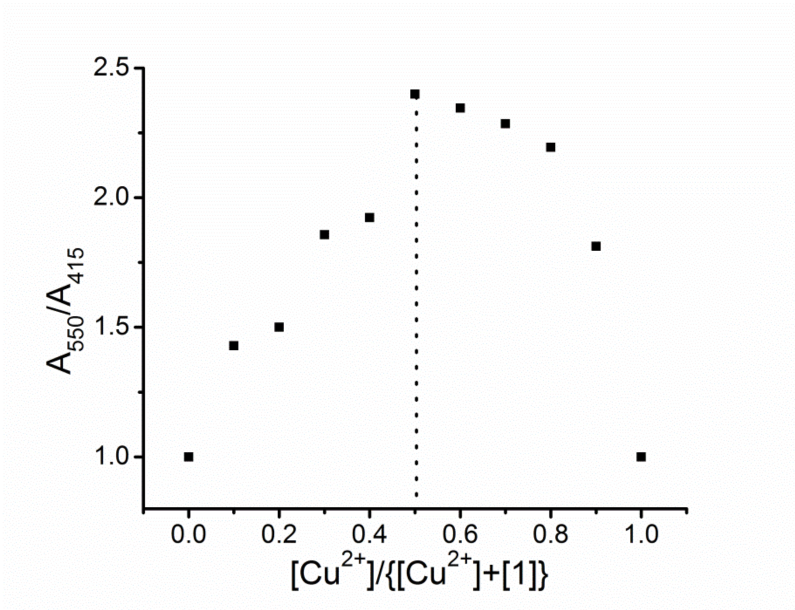


Fig. 2 Job's plots of the complexation between **1** and Cu^{2+}. $[Cu^{2+}]$+[**1**] = 20 μM.

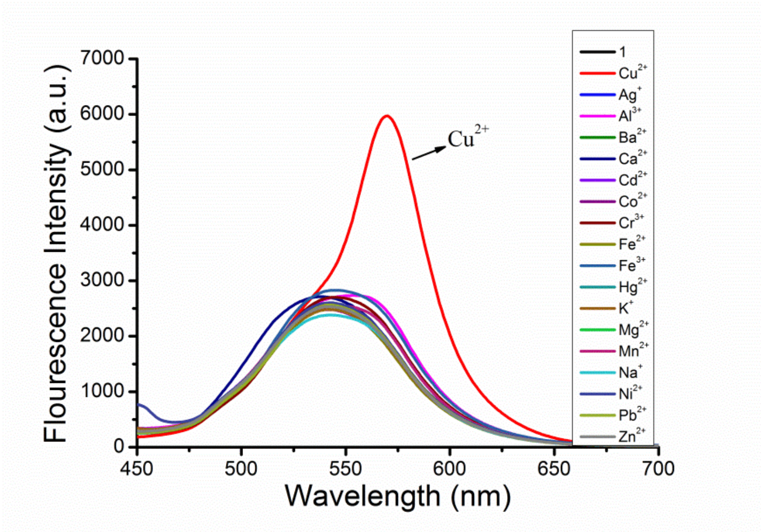


Fig. 3 Fluorescence spectra of **1** (10 μM) in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution with the presence of 10 equiv. of various species (λ_{ex} = 420 nm, slit = 10 nm).

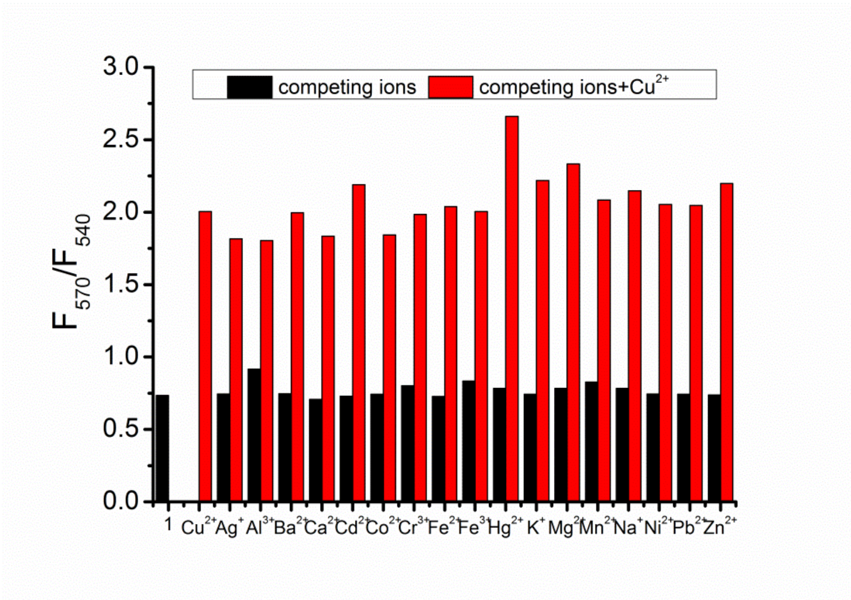


Fig. 4 Metal-ion selectivity of **1** in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution. The black bars represent the fluorescence emission ratio (F_{570}/F_{540}) of a solution of **1** (10 μM) and 10 equiv. of other metal ions. The red bars show the F_{570}/F_{540} ratio after the addition of 10 equiv. of Cu^{2+} to the solution containing **1** (10 μM) and different metal ions (100 μM) (λ_{ex} = 420 nm, slit = 10 nm).

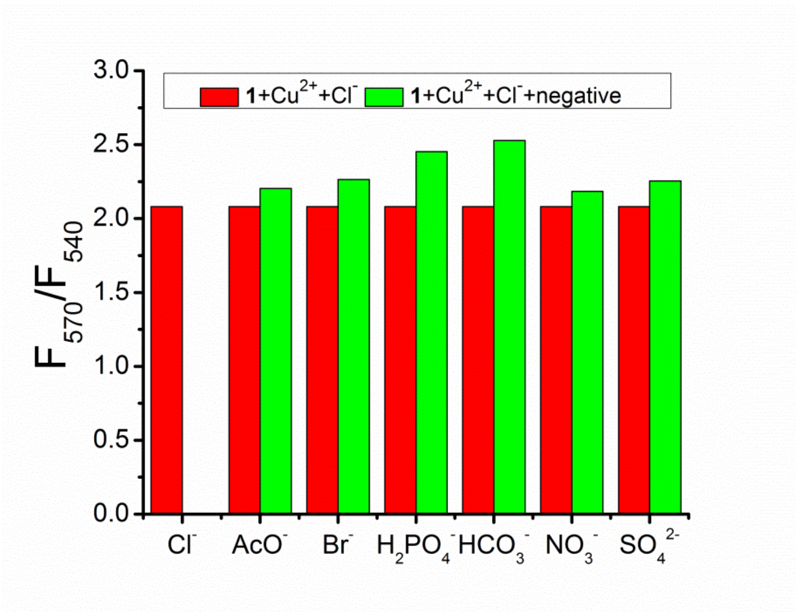


Fig. 5 The ratiometric fluorescence responses (F_{570}/F_{540}) of **1** (10 μM) upon the addition of 100 μM $CuCl_2$ in the presence of 100 μM background anions in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution (λ_{ex} = 420 nm, slit = 10 nm).

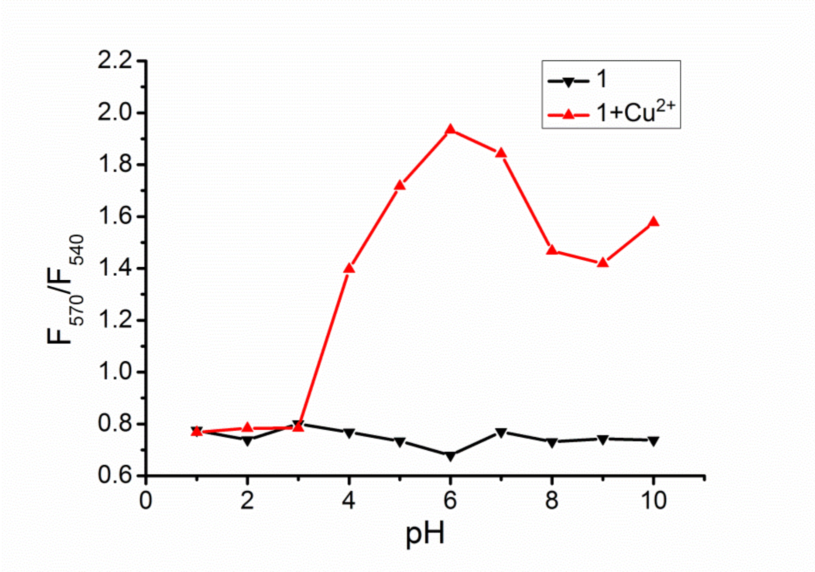


Fig. 6 The ratiometric fluorescence responses (F_{570}/F_{540}) of free **1** (10 μM) and in the presence of 10 equiv. Cu^{2+} in CH_3CN/Tris-HCl (1/9, v/v, 1 mM) solution with different pH conditions (λ_{ex} = 420 nm, slit = 10 nm).

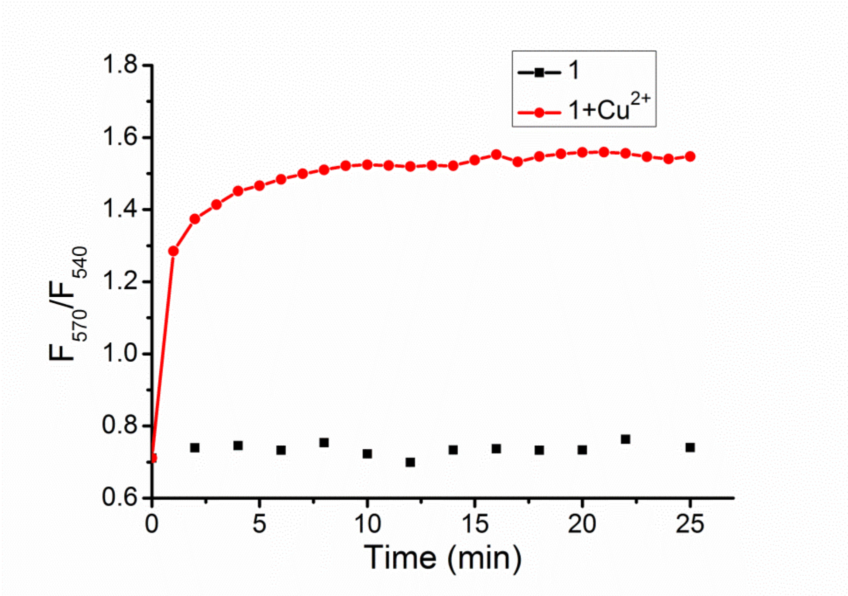


Fig. 7 Kinetics of the ratiometric fluorescence responses (F_{570}/F_{540}) of free **1** (10 μM) and in the presence of 10 equiv. Cu^{2+} in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution (λ_{ex} = 420 nm, slit = 10 nm).

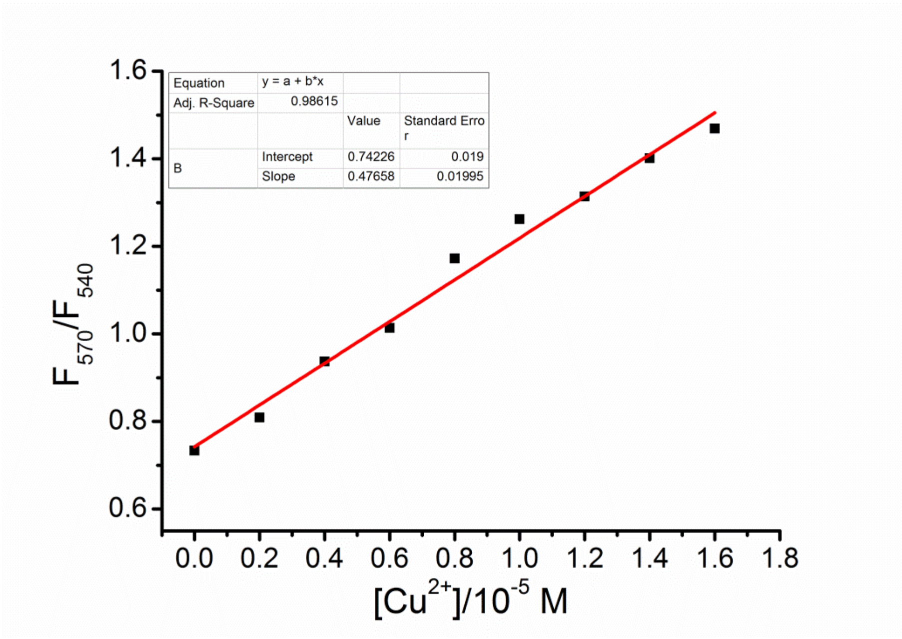


Fig. 8 Fluorescence intensity ratio changes (F_{570}/F_{540}) of **1** (10 μM) upon gradual addition of Cu^{2+} in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution (λ_{ex} = 420 nm, slit = 10 nm).

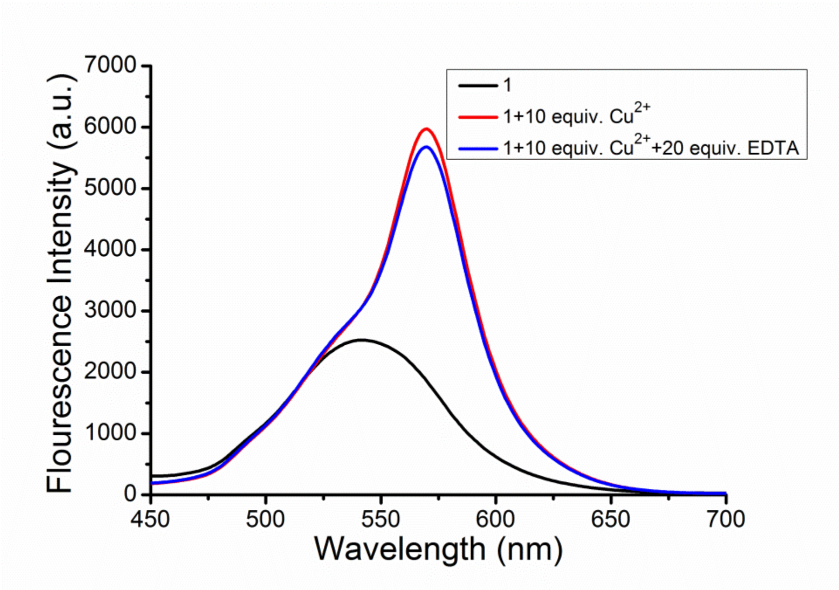
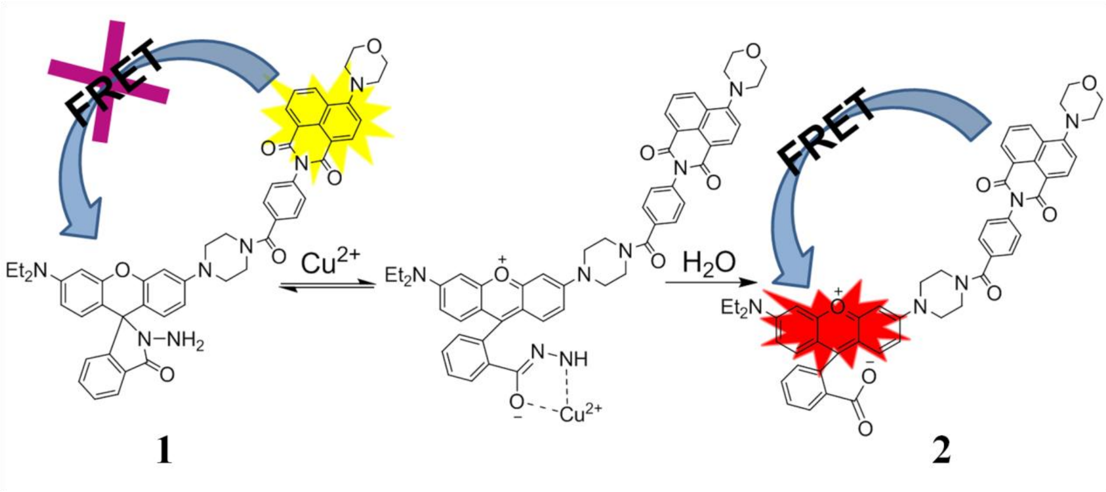


Fig. 9 Fluorescence intensity of **1** (10 μM) to Cu^{2+} in CH_3CN/H_2O (1/9, v/v, 1 mM Tris-HCl buffer, pH = 7.40) solution (λ_{ex} = 420 nm, slit = 10 nm).



Scheme 3. Possible sensing mechanism of **1** with Cu^{2+}.

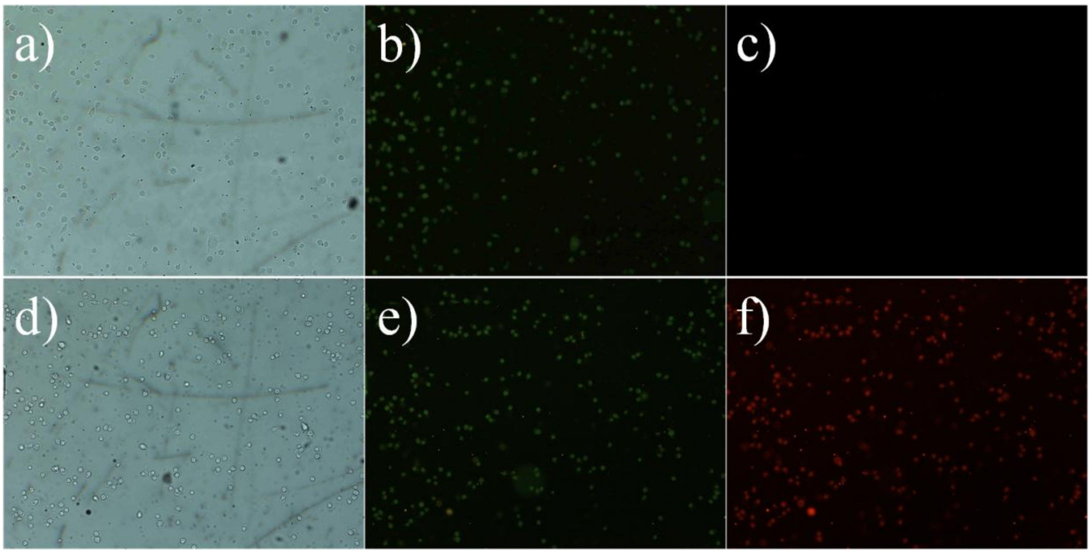


Fig. 10 Bright field and fluorescence microscopic images of MGC-803 cells. MGC-803 cells incubated by compound **1** (10 μM) observed under bright field (a), green channel (b), red channel (c), then further incubation with Cu^{2+} (20 μM) for 30 min at 37 °C under bright field (d), green channel (e), red channel (f).