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ORIGINAL PAPER

A New Pyrazoline-Based Fluorescent Probe for Cu^{2+} in Live Cells

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Abstract A new pyrazoline-based probe was synthesized and the structure was determined by using X-ray diffraction analysis. The probe responds to Cu^{2+} in aqueous medium in "turn-off" fluorescent manner with selectivity and sensitivity. Furthermore, the probe could be used for real-time tracking of Cu^{2+} in Hela cells.

Keywords Pyrazoline · Fluorescent probe · Copper ion · Living cell · Imaging

Introduction

Copper is an essential trace element in the human body and plays a pivotal role in a variety of many fundamental physiological processes in organisms [1–3]. However, Cu^{2+} is high toxic to biological systems when Cu^{2+} is at the high concentration exceeding cellular needs. It leads to severe neurodegenerative diseases such as Menkes disease, Wilson disease, and Alzheimer's disease [4–6]. Therefore, the highly selective, sensitive and rapid recognition of Cu^{2+} in living cells is demanded.

Based on the different sensing methods for detecting Cu^{2+}, fluorescent probes have several advantages over other methods due to their high sensitivity, selectivity and real-time monitoring [7–12]. In recent years, a number of studies on the design and synthesis of fluorescent probes for the detection of Cu^{2+} ions have been reported and a lot of probes have been used with some success in biological applications [13–16]. Several "turn-off" fluorescent probes for Cu^{2+} have also been reported to monitor Cu^{2+} in biological applications [17, 18], but some of them have shortcomings for practical application such as cross sensitivity towards other metal ions, longer response times, low water solubility, a narrow pH span, tricky procedures for synthesis and poor sensitivity [19–21]. In addition, some "turn-off" fluorescent probes can only be applied in non-aqueous solvents which limit their application in biological systems [22, 23]. Thus it is of significance to develop fluorescent probes that would allow accurate, sensitive, rapid recognition of Cu^{2+} in aqueous media.

The pyrazoline-based fluorescent probes have attracted considerable interest because of their blue light emission with high fluorescence quantum yield [24–27]. Consequently, several pyrazoline-based probes for metal ions, such as Zn^{2+} [28] have been studied. However, some of them were used with no success in biological applications. As an extension of our work on the development of fluorescent probe for monitoring metal ions [29–33], herein we report the design and synthesis of a new pyrazoline-based fluorescent probes **4** (Scheme 1) for Cu^{2+} recognition. Probe **4** is not only of

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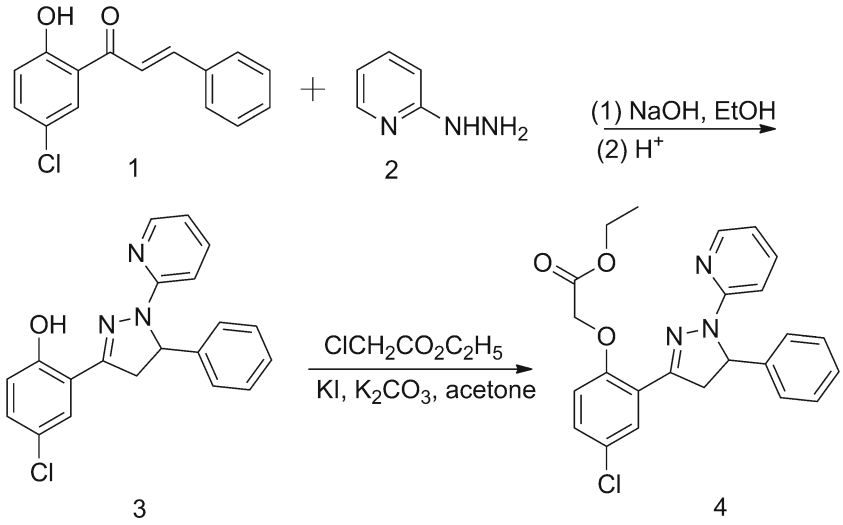
Scheme 1 Synthesis of compound **4**



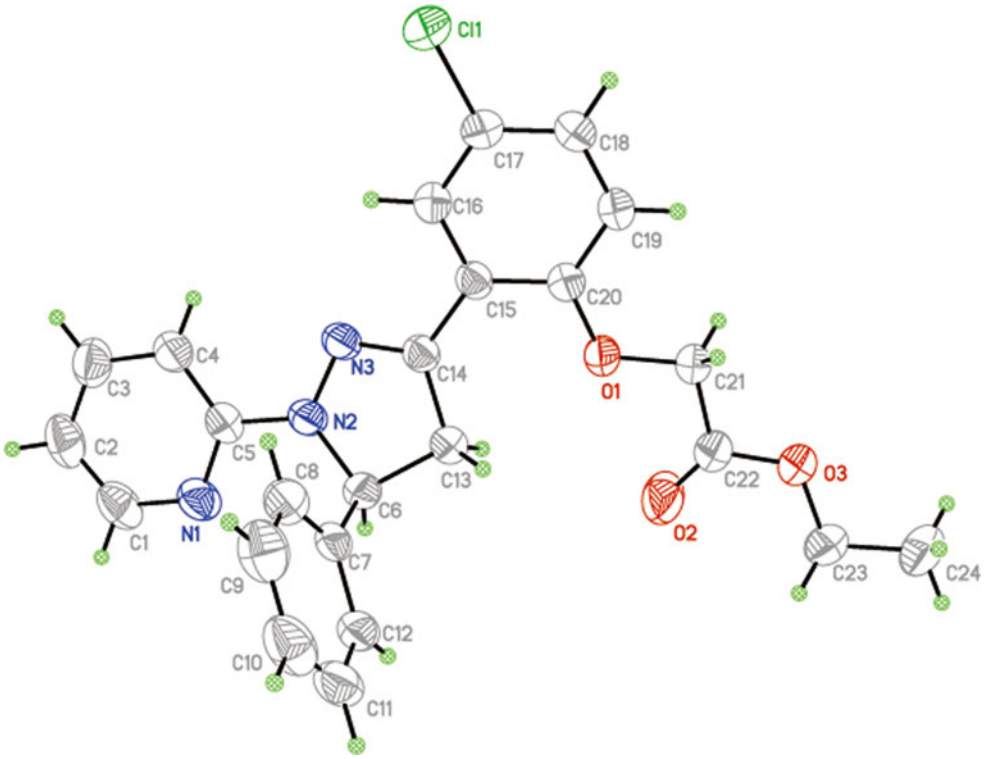
high sensitivity and selectivity in aqueous media but also can detect Cu^{2+} in living cells.

Experimental Details

Apparatus

Thin-layer chromatography (TLC) was conducted on silica gel 60 F_{254} plates (Merck KGaA). ^{1}H NMR spectra were recorded on a Bruker Avance 400 (400 MHz) spectrometer, using DMSO as solvent. ^{13}C NMR spectra were recorded on a Bruker Avance 300 (75 MHz) spectrometer, using $CDCl_3$ as solvent, and tetramethylsilane (TMS) as internal standard. Melting points were determined on an XD-4 digital micro melting point apparatus. IR spectra were recorded with an IR spectrophotometer VERTEX 70 FT-IR (Bruker Optics). HRMS spectra were recorded on a QTOF6510 spectrograph (Agilent). UV–vis spectra were recorded on a U-4100 (Hitachi). Fluorescent measurements were recorded on a Perkin–Elmer LS-55 luminescence spectrophotometer. All pH measurements were made with a Model PHS-3C pH meter (Shanghai, China) and operated at room temperature about 298 K.

Fig. 1 Crystal structure of **4**



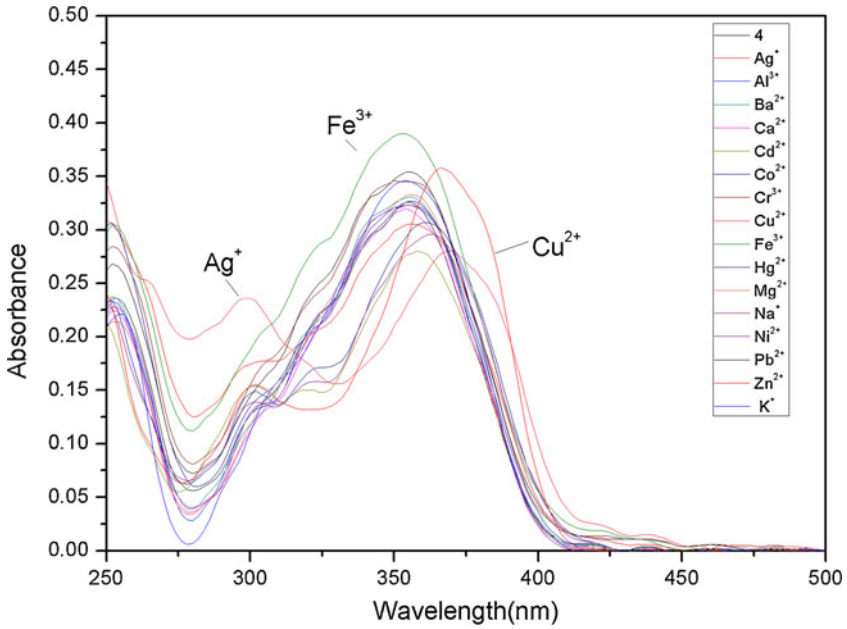


Fig. 2 Absorption spectra of **4** (20 μM) in buffered EtOH/HEPES solution (20 mM, pH= 7.2, 2:3, v/v) with 5 equiv. of metal ions: Al^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cr^{3+}, K^{+}, Mg^{2+}, Na^{+}, Ag^{+}, Hg^{2+}, Zn^{2+}, Cu^{2+} ions and blank

Reagents

Deionized water was used throughout the experiment. All the reagents were purchased from commercial suppliers and used without further purification. The salts used in stock aqueous solutions of metal ions were $NaNO_3$, $Fe(NO_3)_3 \cdot 9H_2O$, $AgNO_3$, KNO_3, $Co(NO_3)_2 \cdot 6H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, $Al(NO_3)_3 \cdot 9H_2O$, $Ba(NO_3)_2$, $Cr(NO_3)_3 \cdot 9H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$, $Pb(NO_3)_2$, $Cu(NO_3)_2 \cdot 3H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$ and $HgCl_2$.

Synthesis of Ethyl 2-(4-chloro-2-(5-phenyl-1-(pyridin-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)phenoxy)Acetate (**4**)

The synthetic route of the proposed compound **4** is shown in Scheme 1. Starting material pyrazoline (**3**) was prepared according to the literature [31]. A mixture pyrazoline (**3**) (0.350 g, 1 mmol), KI (0.168 g, 1 mmol), K_2CO_3 (0.331 g, 2.4 mmoL), ethyl chloroacetate (0.150 g, 1.2 mmol) and acetone (25 ml) was stirred at reflux for 2 h. After cooling, the mixture was filtered and the filtrate was evaporated to afford

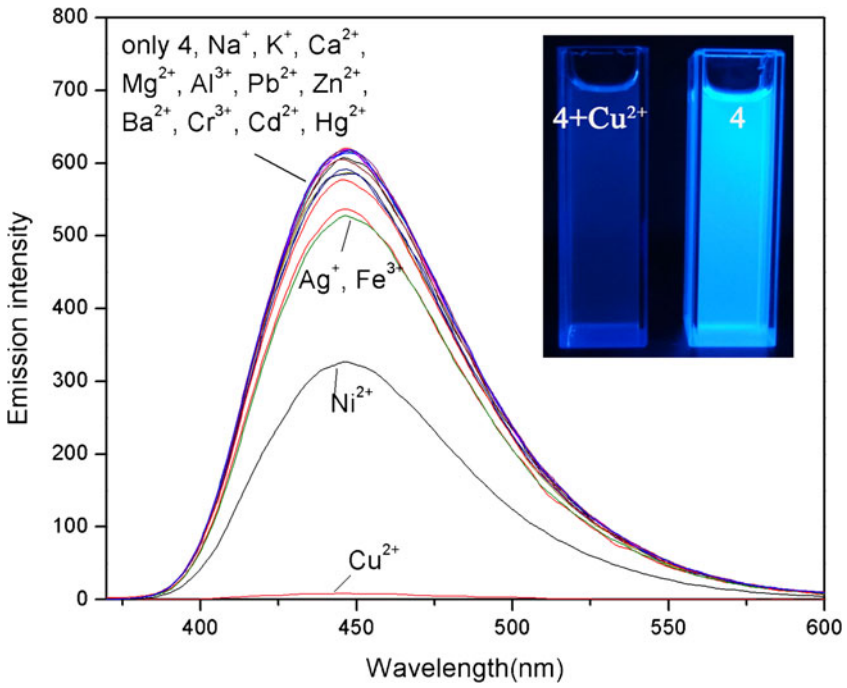


Fig. 3 Fluorescence emission spectra of **4** (10 μM) in buffered EtOH/HEPES (20 mM, pH= 7.2, 2:3, v/v) solution with 5 equiv. of metal ions: Al^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cr^{3+}, K^{+}, Mg^{2+}, Na^{+}, Ag^{+}, Hg^{2+}, Zn^{2+}, Cu^{2+} ions and blank. Excitation wavelength was 354 nm. Emission wavelength was 445 nm. Inset: the color change of color **4** (10 μM) in buffered EtOH/HEPES (20 mM, pH=7.2, 2:3, v/v) solution with 5 equiv. Cu^{2+}

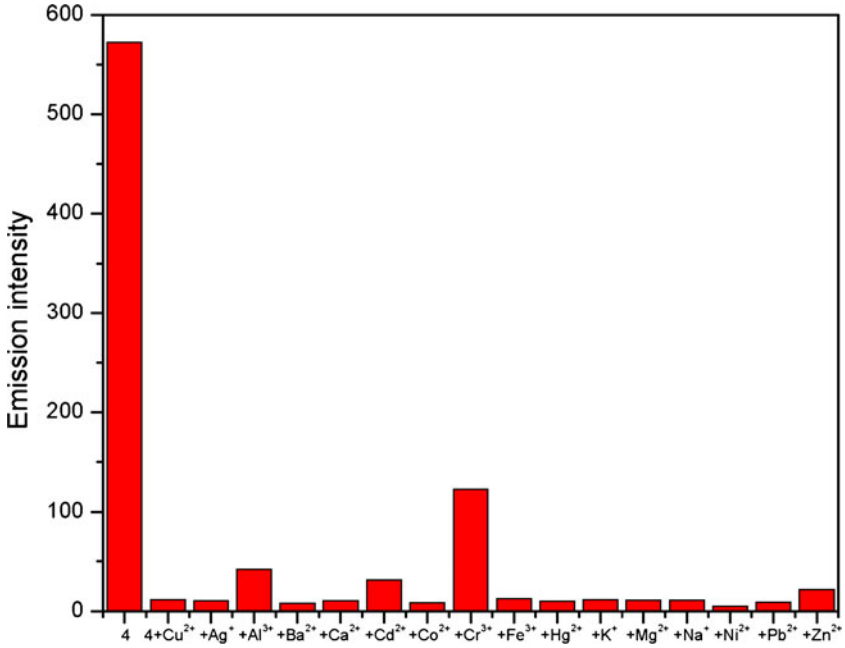


Fig. 4 Fluorescence emission spectra of **4** (10 μM) in buffered EtOH/HEPES (20 mM, pH= 7.2, 2:3, v/v) solution containing 5 equiv. of Cu^{2+} to the selected metal ions Al^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Ag^{+}, Hg^{2+}, Zn^{2+}, Ba^{2+}, Cd^{2+}, Cr^{3+} (5 equiv.) and Mg^{2+}, Ca^{2+}, K^{+}, Na^{+} (50 equiv.). Excitation wavelength was 354 nm. Emission wavelength was 445 nm

residue. The residue was crystallized from ethanol to afford **4** (0.227 g), Yield: 55.2 %; mp: 158–160 °C; IR (KBr, cm^{-1}): 3501.7, 2924.2, 1764.5, 1590.2, 1481.8, 1417.8, 1219.8, 1137.4, 1075.3, 765.4, 700.3; ^{1}H NMR (400 MHz, DMSO): δ 1.14 (t, 3H, *J*=7.04 Hz, CH_3), 3.34 (dd, 1H, *J*=5.28, 18.60 Hz, 4-H*trans*), 4.03 (dd, 1H, *J*=12.28, 18.60 Hz, 4-H*cis*), 4.12 (q, 2H, *J*=7.11 Hz, CO_2CH_2), 4.84 (s, 2H, OCH_2CO), 5.72 (dd, 1H, *J*=5.28, 12.28 Hz, 5-H of pyrazoline), 6.69 (t, 1H, *J*=6 Hz, pyridine-H), 7.09 (d, 1H, *J*=8.88 Hz, Ar-H), 7.17–7.22 (m, 3H, Ar-H), 7.2–7.30 (m, 2H, Ar-H), 7.39 (dd, 1H, *J*=2.8, 6 Hz, Ar-H), 7.46 (d, 1H, *J*=8.56 Hz, Ar-H), 7.60 (t, 1H, *J*=4.34 Hz, Ar-H), 7.95 (d, 1H, *J*=2.84 Hz, Ar-H), 7.97 (s, 1H, Ar-H), ^{13}C NMR (75 MHz, $CDCl_3$): 167.96, 155.17, 154.26, 147.86, 147.39, 143.19, 137.23, 129.60, 128.65, 128.61 (2C), 127.16, 127.00, 125.81 (2C), 123.99, 114.48, 113.70, 109.27, 77.43, 77.01, 76.58, 65.90, 62.25, 61.52, 45.54, 14.08; HRMS: calcd for $[M+H]^{+}$ $C_{24}H_{23}ClN_3O_3$: 436.1428; found: 436.1409.

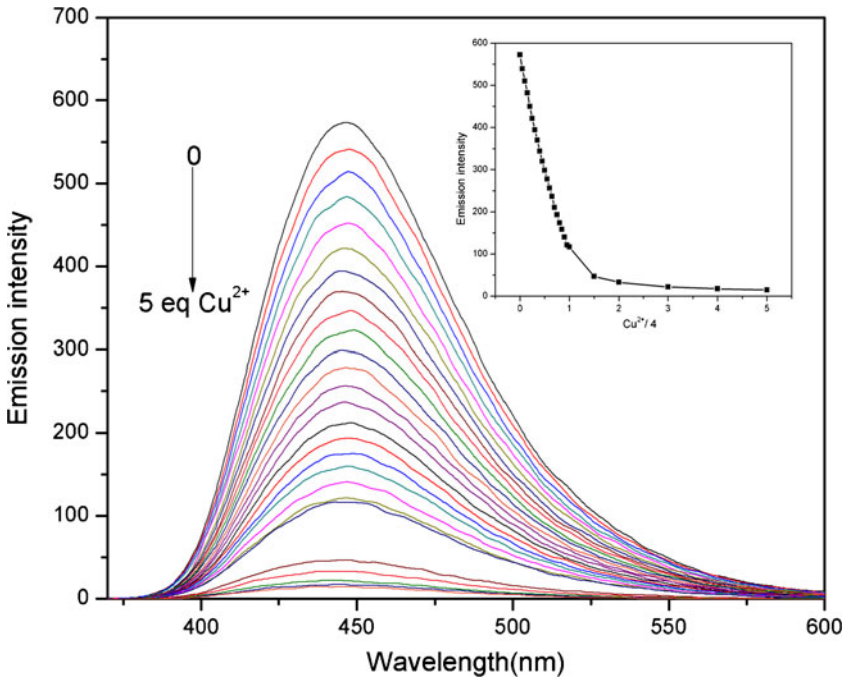


Fig. 5 Fluorescence emission spectra of **4** (10 μM) upon the addition of Cu^{2+} (0–5 equiv.) in buffered EtOH/HEPES (20 mM, pH=7.2, 2:3, v/v) solution. The *inset* shows the emission of **4** at 445 nm as a function of Cu^{2+} concentration

A single crystal of **4** was obtained from an ethanol solution and was characterized using X-ray crystallography (Fig. 1).

Cell Culture and Imaging

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10 % calf bovine serum (HyClone) at 37 °C in humidified air and 5 % CO_2. For fluorescence imaging, the cells ($5\times10^4 mL^{-1}$) were seeded into 24-well plates, and experiments to assay Cu^{2+} uptake were performed in the same media supplemented with 1 μM of $CuCl_2$ for 0.5 h. The cells were washed twice with PBS buffer before the staining experiments, and incubated with 1 μM of probe **4** for 1 h in the incubator. After washing twice with PBS, the cells were imaged under a Phase Contrast Microscope (Nikon, Japan).

Results and Discussion

Absorption Properties

As shown in Fig. 2, free probe **4** exhibits a maximum absorption wavelength at 354 nm in buffered EtOH/HEPES solution (20 mM, pH=7.2, 2:3, v/v). Upon addition of Cu^{2+} or Ag^{+}, a maximum absorption at 370 nm appeared. The addition of other metal ions such as Al^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cr^{3+}, K^{+}, Mg^{2+}, Na^{+}, Hg^{2+}, Zn^{2+} had little change to the UV–vis spectrum of **4**. In addition, during the UV–vis spectrum of **4** with different concentration, the absorption maximum shows at 354 nm and the ε is $2.7\times10^4 M^{-1}cm^{-1}$ (Fig. S1, Fig. S2).

Selectivity Studies

The fluorescence spectrum changes of probe **4** (10 μM) in buffered EtOH/HEPES solution (20 mM, pH=7 .2, 2:3, v/v) before and after addition of different metal ions were examined. As shown in Fig. 3, free probe **4** shows a strong fluorescence emission at 445 nm. Interestingly, addition of 5 equiv. of Cu^{2+} resulted in a remarkably quenching of fluorescence intensity. However, the addition of other metal ions such as Al^{3+}, Fe^{3+}, Co^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cr^{3+}, K^{+}, Mg^{2+}, Na^{+}, Ag^{+}, Hg^{2+}, Zn^{2+} with the same concentration does not trigger obvious fluorescence change of the probe, which well illustrates the selectivity of the probe to Cu^{2+}. The phenomenon was also justified by the change in color from stronger blue emission to almost dark in presence of Cu^{2+} under the irradiation at 365 nm (Fig. 3 inset).

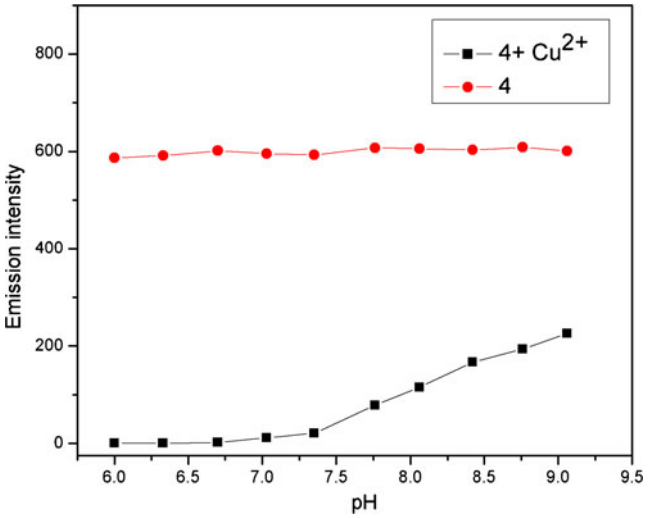


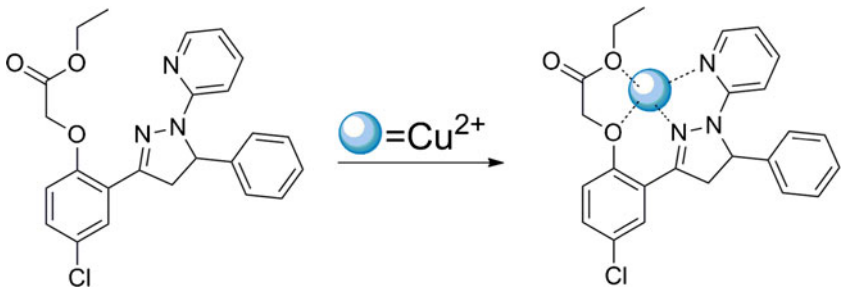
Fig. 6 The effect of pH (6.0–9.06) on the relative fluorescence intensity probe **4** with 5 equiv. Cu^{2+} in buffered EtOH/HEPES (20 mM, pH=7.2, 2:3, v/v) solution. Excitation wavelength was 354 nm. Emission wavelength was 445 nm

Interestingly, upon addition of different anions (1 equiv.) such as $CuSO_4$, $CuCl_2$, $Cu(NO_3)_2$ and $Cu(OAc)_2$ to a solution of probe **4** (10 μM), no significant change in the optical properties was observed (Fig. S3). So the copper salts with different anions all can quench the fluorescence intensity of probe **4**.

Tolerance Over Other Metal Ions

To check the practical ability of probe **4** as a Cu^{2+} selective fluorescent chemosensor, the competition experiments were carried out in the presence of 5 equiv. Cu^{2+} mixed with other competing metal ions. As shown in Fig. 4, only Cr^{3+} slightly disturbed the intensity of **4**-Cu (II) and the initial fluorescence intensity of **4**-Cu (II) did not changed significantly with other metal ions such as Al^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, K^{+}, Mg^{2+}, Na^{+}, Ag^{+}, Hg^{2+}, Zn^{2+}. This indicated

Scheme 2 The proposed binding mode



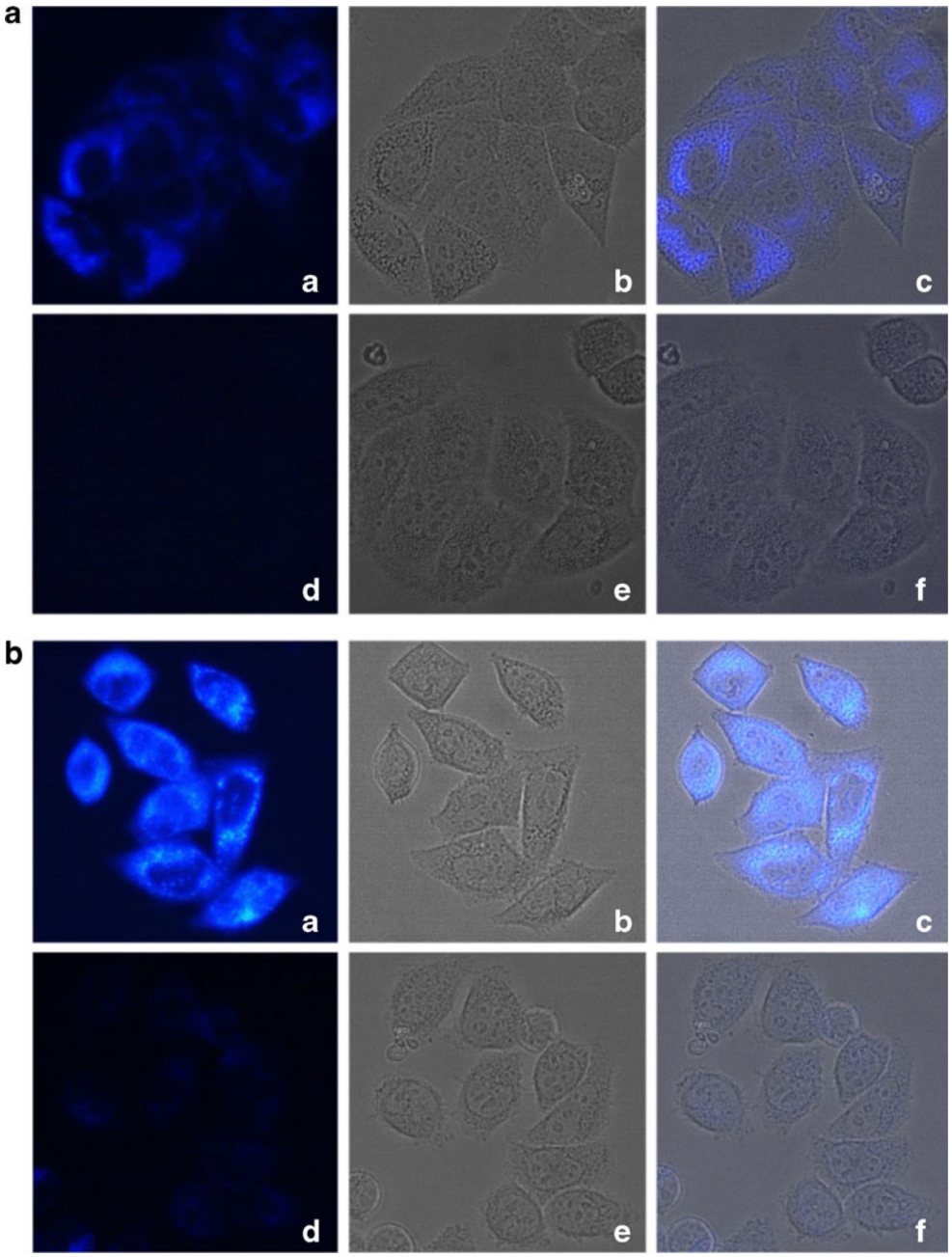


Fig. 7 Fluorescence microscope images of living Hela cells. **a** using 1 μM probe and 2 μM $Cu(NO_3)_2$; **b** using 5 μM probe and 10 μM $Cu(NO_3)_2$. **a** Cells incubated with probe **4** for 2 h at 37°C; **b** Bright-field view of panel (**a**); **c** Overlay image of (**a**) and (**b**). **d** Cells incubated with probe for 2 h at 37 °C and then incubated with Cu^{2+} added to the growth medium for 30 min at 37 °C. **e** Bright-field image of the live Hela cells shown in panel (**d**). **f** Overlay image of (**d**) and (**e**)

that probe **4** had a high selectivity for Cu^{2+} in the presence of other related species.

Cu^{2+}-titration

To further investigate the interaction of probe **4** and Cu^{2+}, a fluorescence titration experiment was carried out, as shown in Fig. 5. With increasing the Cu^{2+} concentration, the original strong fluorescence emission at 445 nm was gradually quenched and it was quenched almost completely after the addition of 1 equiv. Cu^{2+}. And when the Cu^{2+} concentration increased up to 5 equiv., the fluorescence emission did not cause significant changes.

The Proposed Reaction Mechanism

To investigate the probable complexation of the probe **4** with Cu^{2+}, the binding model of the probe **4** and Cu^{2+} was proposed as shown in Scheme 2. The probe is the most likely to chelate Cu^{2+} via its oxygen on the ether, oxygen on the carbonyl group, as well as nitrogen on the pyridine and pyrazoline. This binding mode was further supported by a Job's plot. As shown in Fig. S4, the

absorbance reached a inflection point when the ratio was 0.5, which indicated a 1:1 stoichiometry of the Cu^{2+} to **4** in the complex [16].

Kinetic Assay and Effect of pH

The spectra response of probe **4** in the absence and presence of Cu^{2+} in different pH values were firstly evaluated. As shown in Fig. 6, in the pH range of 6.0–9.06, obvious characteristic emission of **4** could be observed. However, the addition of Cu^{2+} led to the fluorescence quenching over a comparatively wide pH range (6.0–7.5). It means that probe **4** can serve as a good selective chemosensor for Cu^{2+} in near neutral and weak acidic medium, which is important in both environmental and biological analysis. Further, the time-dependence of fluorescence of probe **4** was also evaluated in the presence of Cu^{2+}. As shown in Fig. S5, Cu^{2+} induced fluorescence quenching can complete within 10 min after addition of Cu^{2+}. This suggests that probe **4** could be used for real-time tracking of Cu^{2+} in cells.

Linear Range and Limit Of Detection

Generally, as a highly selective and sensitive fluorescent probe, the detection limit is an important factor in practical application. As shown in Fig. S6, the limit of detection of probe **4** for Cu^{2+} is measured to be 3.74×10^{-7}M, and the correlation coefficient is 0.997 [34].

The binding stoichiometry of probe **4** with Cu^{2+} was calculated through the Benesi–Hildebrand equation [35]. As shown in Fig. S7, when the Cu^{2+} concentration ranged from 4 μM to 8.5 μM, the plot of $1/(I_0- I)$ against $1/[Cu^{2+}]$ shows a linear relationship, which demonstrates that probe **4** coordinated with Cu^{2+} in a 1:1 ratio, and the binding constant was calculated from the ratio of intercept/slope to be $1.49\times10^{4}M^{-1}$ with the correlation coefficient as high as 0.9991. Thus the fact suggests the high copper affinity of probe **4** as speculated.

Imaging of Intracellular Cu^{2+}

The ability of probes to sensitively and selectively detect analyte in living cells is significant for biological application. Considering that higher level of Cu^{2+} in tumors takes a possible key role in promoting angiogenesis, we carried out experiment using the probe on Hela cells.

From Fig. 7, we can clearly observe significant confocal imaging changes of probe **4** (1 and 5 μM) in the medium upon addition of Cu^{2+} (2 equiv.) for 2 h at 37 °C. Hela cells incubated with probe **4** initially display a strong fluorescent image, but the fluorescence image completely quenched in the presence of Cu^{2+}. Moreover, with increasing concentration of the probe **4** incubated, fluorescence intensity enhanced obviously in concentration-dependent manner and at the presence of Cu^{2+} fluorescence images of the cells revealed a remarkable quenching. These results demonstrate that probe **4** can be useful for monitoring Cu^{2+} in living cells.

Conclusion

In summary, we have developed a highly sensitive and selective probe for Cu^{2+} in aqueous solution. The change in color of solution from fluorescent blue to a non-fluorescent was readily distinguishable. Job's plot confirmed the presence of Cu^{2+} induced the formation of complex in the 1:1 binding mode. When 10 μM of probe **4** was used in buffered EtOH/HEPES (20 mM, pH=7.2, 2:3, v/v) solution, the detection limit was low to 0.4 μM. Meanwhile, the experimental results demonstrate that probe **4** could be used to detect Cu^{2+} in living cells.

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