A highly selective fluorescent probe for the sensing of Cu\(^{2+}\) based on the hydrolysis of a quinoline-2-carboxylate and its application in cell imaging

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Abstract
A highly selective OFF–ON fluorescent probe is developed for the sensing of Cu\(^{2+}\) based on the hydrolysis of a quinoline-2-carboxylate moiety. The probe is weakly fluorescent due to esterification of the phenolic group. Upon treatment with 1 equiv. of Cu\(^{2+}\), the probe exhibits strong fluorescence at 570 nm. The probe also exhibits high selectivity for Cu\(^{2+}\) over other cations with a low detection limit of 0.2 \(\mu\)M, which is sensitive enough to meet the standard of the World Health Organization for Cu\(^{2+}\) in drinking water (30 \(\mu\)M). Moreover, the probe shows a very low cell cytotoxicity, and imaging experiments demonstrate that the probe can be used for the sensing of Cu\(^{2+}\) in living cells.

Keywords
cell imaging, Cu\(^{2+}\) sensing, fluorescent probe, quinoline-2-carboxylate

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Introduction
As the third-most abundant transition cation in the human body, copper ions play a vital role in physiological and pathological processes, such as signal transduction, cell respiration, cell proliferation, and apoptosis.\(^1\)–\(^5\) An abnormal elevation of the copper ion concentration will induce a series of neurodegenerative diseases, including Alzheimer’s disease,\(^6\) Parkinson’s disease,\(^7\) Wilson’s disease,\(^8\) and Menkes disease.\(^9\) It has been reported that intracellular copper ions mainly exist in the lower oxidative state (Cu\(^{+}\) form) under physiological environments.\(^10\) Furthermore, high levels of copper ions accumulating in lysosomes in the higher oxidative state (Cu\(^{2+}\) form) cause serious damage to living cells.\(^11\)–\(^14\) Therefore, it is desirable to design new methods and tools for the sensing of intracellular copper ions.

A variety of sensing methods and tools have been designed for the detection of copper ions, including atomic absorption spectrometry,\(^15\) atomic emission spectrometry,\(^16\) voltammetry,\(^17\) and inductively coupled plasma atomic emission spectrometry.\(^18\) However, these sensing methods still suffer from many weaknesses such as complicated sample preparation, time-consuming procedures, and expensive instruments, and are unsuitable for real-time analysis. In recent years, fluorescence imaging methods have attracted more and more attention due to their advantages over other methods such as easy operation, minimal sample consumption, real-time analysis, and nondestructive bioimaging. The development of an OFF–ON fluorescent probe for Cu\(^{2+}\) is a challenging task due to its paramagnetic nature.\(^19\)–\(^21\) To solve this problem, the development of reaction-based fluorescence probes is a desirable solution. Although many fluorescent probes for the sensing of Cu\(^{2+}\) are based on hydrolysis of a picolinate group,\(^22\)–\(^26\) hydrolysis of a hydrazide group,\(^27\)–\(^32\) and click chemistry,\(^33\),\(^34\) there is still great demand to design new fluorescent probes for copper-ion-sensing with new recognition sites or sensing mechanisms.

Herein, we have synthesized new fluorescent probe I bearing a quinoline-2-carboxylate moiety for the detection of Cu\(^{2+}\), which showed high selectivity and sensitivity for Cu\(^{2+}\) over other competing metal ions. The attachment of a
quinoline-2-carboxylate moiety to the phenolate group of highly fluorescent compound 2 inhibits intramolecular charge transfer (ICT) from the dicyanomethylene to the phenolate moiety. After Cu^{2+}-induced release of quinoline-2-carboxylate from probe 1, the ICT effect is recovered along with a dramatic enhancement in the fluorescence intensity. As shown in Scheme 1, probe 1 was obtained in high yield by the esterification of compound 2 with quinoline-2-carboxylic acid. The product was fully characterized by 1H NMR, 13C NMR, and high-resolution mass spectrometry (HRMS) (see Figures S1–S5, Supporting Information). Further studies revealed that probe 1 exhibits high selectivity and sensitivity for Cu^{2+} over other cations and anions with a low detection limit of 0.2 μM. The fast response (15 min) of the probe to Cu^{2+} and low cell cytotoxicity indicate that probe 1 is suitable for the sensing of Cu^{2+} in living cells.

Results and discussion

Initially, we investigated the selectivity of probe 1 toward various metal ions by UV-Vis and fluorescence spectroscopy. The absorption spectrum of probe 1 (10 μM) exhibited a strong band at 397 nm in DMSO–Tris buffer (1:1, v/v, 20 mM, pH 7.4). Next, a variety of metal ions including Li^{+}, Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Ag^{+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Pb^{2+}, Hg^{2+}, and Cu^{2+} were systematically introduced to the above-mentioned solution of probe 1. Only on treatment with Cu^{2+} did the absorbance at 397 nm bathochromically shift to 420 nm, with a slight decrease in the fluorescence intensity, while the addition of other metal ions did not induce any absorption change (Figure 1(a)). Due to the inhibition of ICT, probe 1 exhibited a very weak fluorescence. The addition of Cu^{2+} induced a strong emission band at 570 nm at 420 nm, with a slight decrease in the fluorescence intensity, while the addition of other metal ions did not induce any absorption change (Figure 1(a)). Due to the inhibition of ICT, probe 1 exhibited a very weak fluorescence. The addition of Cu^{2+} induced a strong emission band at 570 nm, while the addition of other metal ions induced negligible changes in the fluorescence intensity (Figure 1(b)). Meanwhile, the color of the solution turned from pale yellow to yellow after the addition of Cu^{2+} (inset, Figure 1(a)), and an enhanced orange fluorescence of the solution was observed under UV lamp irradiation (inset, Figure 1(b)). We also tested the selectivity of probe 1 toward various anions including F\(^{-}\), Cl\(^{-}\), Br\(^{-}\), I\(^{-}\), AcO\(^{-}\), H\(_2\)PO\(^{-}\), NO\(^{-}\), HSO\(^{-}\), and ClO\(^{-}\). The results indicated that anions did not induce any fluorescence change of probe 1 (Figure S6(a)).

To investigate the sensing ability of probe 1 for Cu^{2+}, titration experiments were also conducted with UV-Vis and fluorescence spectroscopy. As shown in Figure 2, upon addition of Cu^{2+} from 0 to 1.0 equiv., the absorption peak at 397 nm decreased with concomitant formation of a new peak at 420 nm, together with a clear isosbestic point at 412 nm. Upon excitation at 470 nm, the fluorescence intensity at 570 nm increased gradually upon the addition of Cu^{2+}, and reached a plateau after the addition of 1 equiv. of Cu^{2+} with a 10.2-fold fluorescence enhancement (Figure 3(a)). A linear relationship (R\(^2\)=0.985) was found between the fluorescence intensity at 570 nm and [Cu^{2+}] in the range of 0–6.0 μM (inset, Figures 3(b) and S7), suggesting that probe 1 is capable of sensing Cu^{2+} both qualitatively and quantitatively. The detection limit...
of probe 1 for Cu$^{2+}$ was measured to be 0.2 μM, which is sensitive enough to meet the standard of the World Health Organization (WHO) for Cu$^{2+}$ in drinking water (30 μM).35

For the practical sensing of Cu$^{2+}$, the coexistence of other metal ions and anions should not interfere with the detection of Cu$^{2+}$. The sensing properties of probe 1 for Cu$^{2+}$ in the presence of other metal ions were investigated (Figures 4 and S6(b)). It was found that the sensing ability of probe 1 to Cu$^{2+}$ was not affected significantly by the presence of other co-existing metal ions or anions (5 equiv.), which indicated that probe 1 is a promising for the sensing of Cu$^{2+}$ in living cells.

We also investigated the time-dependent responses of probe 1 to different concentrations of Cu$^{2+}$ (Figure 5). It was found that the fluorescence output of probe 1 became steady after 15 min following addition of Cu$^{2+}$. Furthermore, the sensing ability of probe 1 for Cu$^{2+}$ in the presence of other metal ions were investigated (Figures 4 and S6(b)). It was found that the sensing ability of probe 1 to Cu$^{2+}$ was not affected significantly by the presence of other co-existing metal ions or anions (5 equiv.), which indicated that probe 1 is a promising for the sensing of Cu$^{2+}$ in living cells.

To further identify the sensing mechanism of probe 1, high-performance liquid chromatography (HPLC) analysis was utilized to detect the hydrolysis process. Initially, probe 1 displayed a single peak with a retention time at 9.6 min (Figure 7(c)) while compound 2 and quinoline-2-carboxylic acid produced a single peak with a retention time at 6.3 and 2.3 min, respectively, (Figure 7(b) and (d)). Upon addition of Cu(ClO$_4$)$_2$ to the solution of probe 1, the peak at 9.6 min weakened while 6.3 and 2.3 min appeared (Figure 7(a)). This result confirmed that the fluorescence enhancement of probe 1 in the presence of Cu$^{2+}$ is ascribed to the release of molecule 2 from probe 1.
1 and Cu²⁺ (Scheme 2). The positions of the oxygen and nitrogen atoms in the quinoline-2-carboxylate moiety are suitable for the five-membered ring coordination of Cu²⁺. Furthermore, this coordination assists the attack of water on the carbonyl group; thus, a highly fluorescent molecule 2 is released due to the hydrolysis of probe 1.

Encouraged by the above experimental results, a further application of probe 1 for the sensing of Cu²⁺ in living cells was conducted. Initially, we evaluated the cytotoxicity of probe 1 at various concentrations using MTT assays (Figure S9). Living HeLa cells were incubated with different concentrations of probe 1 (0–50 μM) for 24 h at 37 °C, with the results suggesting that probe 1 has very low cytotoxicity to HeLa cells, even at high concentration.

The reaction time of probe 1 for Cu²⁺ was determined to be about 15 min, which suggested that probe 1 is suitable for the real-time detection of Cu²⁺ in living cells. When HeLa cells were incubated with probe 1 (10 μM) at 37 °C for 15 min, very weak fluorescence was observed (Figure 8). After 30 min of incubation of Cu(ClO₄)₂ (10 μM), a strong fluorescence was observed, suggesting that probe 1 has good cell membrane permeability and can be used to detect Cu²⁺ in living cells.

**Conclusion**

In summary, we have designed a highly selective fluorescent probe 1 for the sensing of Cu²⁺ based on the hydrolysis of a quinoline-2-carboxylate moiety catalyzed by Cu²⁺. The reaction of probe 1 with 1 equiv. of Cu²⁺ is complete in a short time (15 min) with a low detection limit of 0.2 μM. The sensing of probe 1 for Cu²⁺ ions is not interfered with by the presence of other metal ions. Furthermore, MTT assays suggest that probe 1 has a very low cytotoxicity toward living cells and can be utilized for the sensing of Cu²⁺ in living cells.

**Experimental section**

**Materials and instruments.** ¹H NMR and ¹³C NMR spectra were recorded on a Bruker Avance III 400 MHz Spectrometer.
Hg(ClO$_4$)$_2$·6H$_2$O, AgNO$_3$, Ni(NO$_3$)$_2$·6H$_2$O, Zn(NO$_3$)$_2$·6H$_2$O, and CaCl$_2$ were stored in a vacuum desiccator.

**Synthesis of (E)-4-(2-(3-(dicyanomethylene)-5,5-dimethylcyclohex-1-en-1-yl)vinyl)phenyl quinoline-2-carboxylate (1).**

To a mixture of compound 2 (209 mg, 0.72 mmol), quinoline-2-carboxylic acid (140 mg, 0.81 mmol), HOBt (113 mg, 0.84 mmol), and EDC HCl (210 mg, 1.1 mmol) in dry CH$_2$Cl$_2$ (5 mL) was added Et$_3$N (0.3 mL, 2.1 mmol) dropwise, and then the solution was stirred at room temperature overnight under an N$_2$ atmosphere. H$_2$O (20 mL) was added to the reaction mixture, and the aqeous phase was extracted with CH$_2$Cl$_2$ (2 × 20 mL). The combined organic phases were washed with brine and dried over anhydrous Na$_2$SO$_4$. After removal of the solvent, the residue was purified by column chromatography on silica gel (PE/EtOAc = 5:1) to give probe 1 as a yellow solid (247 mg, yield: 77%). m.p.: 234–235 °C. $^1$H NMR (400 MHz, CDCl$_3$): δ 8.43-8.33 (m, 3H), 7.97 (d, $J$ = 8.1 Hz, 1H), 7.89-7.86 (m, 1H), 7.76-7.72 (m, 1H), 7.63 (d, $J$ = 8.6 Hz, 2H), 7.39 (d, $J$ = 8.6 Hz, 2H), 7.12 (d, $J$ = 16.1 Hz, 1H), 7.01 (d, $J$ = 16.1 Hz, 1H), 6.89 (s, 1H), 2.64 (s, 2H), 2.51 (s, 2H), 1.12 (s, 6H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): δ 169.1, 164.0, 153.5, 152.1, 147.8, 147.1, 137.5, 135.8, 133.7, 130.9, 130.6, 129.6, 129.5, 129.1, 128.7, 127.6, 123.8, 122.6, 121.4, 113.4, 112.6, 79.1, 43.0, 39.3, 32.1, 28.0. HRMS (ESI): m/z [M + H$^+$] calcd for C$_{29}$H$_{24}$N$_3$O$_2$: 446.1863; found: 446.1858.

**Spectroscopic measurements.** The absorption and fluorescence spectral experiments were conducted with DMSO–H$_2$O solution (1:1, v:v, 20 mM Tris, pH 7.4). Stock solutions of cations (10.0 mM, used as chloride or perchlorate or nitrate salts) including LiCl, NaCl, KCl, MgCl$_2$, Cd(ClO$_4$)$_2$, Co(ClO$_4$)$_2$, Cu(ClO$_4$)$_2$, Fe(ClO$_4$)$_2$, Pb(NO$_3$)$_2$, Zn(NO$_3$)$_2$, Hg(ClO$_4$)$_2$, AgNO$_3$, Ni(NO$_3$)$_2$, Zn(NO$_3$)$_2$, and CaCl$_2$ were prepared in double distilled water (DI-H$_2$O). Stock solution of probe 1 (1.0 mM) was prepared in DMSO, and the stock solution was diluted in DMSO–H$_2$O solution (1:1, v:v, 20 mM Tris, pH 7.4) to 10 μM for absorption and fluorescence spectral experiments. In the fluorescence spectral experiments, the excitation wavelength was set at 470 nm, and the excitation and emission slit widths were set at 5 and 10 nm, respectively.

**HPLC measurement.** A Shimadzu LC-20A HPLC system equipped with a C18 column (Inertsil ODS-SP, 5 mm,
150 mm × 4.6 mm) was used. Eluent is 60% CH₃CN (0–15 min). The flow rate was 1.0 mL/min, and the eluents were detected at 254 nm. Injection volume is 10 mL.

**Cell imaging.** Probe 1 (1.0 mM) was prepared in DMSO solution. HeLa cells plated on coverslips were washed with phosphate-buffered saline (PBS), followed by incubating with 10 μM of the probe solution in DMSO for 15 min at 37 °C, and then washed with PBS three times. After incubating with 10 μM of Cu(ClO₄)₂ solution in PBS for 30 min at 37 °C, the cells were washed with PBS three times again.

**Cytotoxicity assay.** The cellular toxicity of probe 1 was performed using a Cell Counting Kit-8 (CCK-8). HeLa cells were seeded into 96-well plates at a density of 4000/well, cultured at 37 °C with 5% CO₂ for 24 h, and then different concentrations of probe 1 (5, 10, 20, 50 μM) were added to the wells. Subsequently, 10 μL of CCK-8 was added to each well followed by incubation for an additional 4 h at 37 °C under 5% CO₂. The absorbance of each well was measured on a micro-plate reader (Tecan, Austria) at a detection wavelength of 450 nm. The following formula was used to calculate the inhibition of cell growth: cell viability (%) = (mean of absorbance value of treatment group/mean of absorbance value of control) × 100%.

**Declaration of conflicting interests**

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**Supplemental material**

Supplemental material for this article is available online.

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**Figure 8.** Confocal microscopy images of HeLa cell staining with probe 1 (10 μM) (a–c) followed by the incubation of Cu²⁺ (10 μM) (d–f). (a) and (d): bright field images; (b) and (e): fluorescence images collected in the range of 500–550 nm; (c): overlay of (a) and (b); and (f): overlay of (d) and (e).
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