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ORIGINAL ARTICLE

# Coumarin-Based Reversible Fluorescent Probe for Selective Detection of $Cu^{2+}$ in Living Cells

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## Abstract

Copper ion plays an important role in many biological processes in human body. $H_2S$ is considered as the third gasses transmitter after carbon monoxide and nitric oxide. Here a novel ICT-based fluorescent ON-OFF-ON probe for $Cu^{2+}$ and $H_2S$ detection was developed. Selectivity and sensitivity of probe was confirmed in aqueous Tris-HCl buffer (10 mM, pH 7.4, containing 90% acetonitrile). Probe DF-CU shows high selectivity over other analytes. The degree of fluorescence quenching is linearly associated with the concentration of $Cu^{2+}$ ($R^2 = 0.9919$). The limit of detection (LOD, calculated according to the 3σ/slope) for $Cu^{2+}$ was 6.4 μM. Probe can work in almost all pH. The probe shows a very fast response to $Cu^{2+}$ (within 10 s). Its response to copper ion could be reversed by $H_2S$. The complex of probe with $Cu^{2+}$ could be used for $H_2S$ detection. Furthermore, this ON-OFF-ON fluorescent probe successfully applied in the living cells for the detection of $Cu^{2+}$ and $H_2S$.

**Keywords** Fluorescent probe · $Cu^{2+}$ · $H_2S$ · MCF-7 cells · Fluorescence imaging

## Introduction

Copper ($Cu^{2+}$) is the third most abundant element found in the human body, along with zinc and iron. $Cu^{2+}$ is present low in concentration in different tissue of the body, and the highest quantity of $Cu^{2+}$ was found in the liver [1]. $Cu^{2+}$ plays an efficient role in the transportation of cofactor of many proteins. In general, the concentration of copper ions in the blood are in the range of 100–150 mg/dL (15.7–23.6 mM) [2]. The limit of copper ion in drinking water was set to be 1.3 ppm (~20 mM) by the U.S. Environmental Protection Agency (EPA) [3]. $Cu^{2+}$ has a significant role in cellular respiration, connective tissue establishment, bone growth, and response as a main catalytic cofactor for numerous metalloenzymes [4–11]. However, the exceeded amount of $Cu^{2+}$ in the body may generate many reactive oxygen species (ROS) that result in damage to tissue and cells in the body. According to the reported, we known the disorder of copper ions in cells can cause some neurodegenerative diseases such as Wilson diseases, Alzheimer's disease, and Menkes [12–16]. Recent research showed that $H_2S$ is very helpful in normal body function, such as a normality of inflammation and suppression of insulin signaling [17–19]. $H_2S$ is considered as the third gasses transmitter after carbon monoxide and nitric oxide [20, 21]. $H_2S$ has been reported as a type of never agents and dangerous gas for the biological system [22–24], excess amount of $H_2S$ in air cause headaches, coma and sometimes it results in death [25, 26]. Thinking of the importance of copper ion and $H_2S$, it is urgent to develop a good method for detection both copper ion and $H_2S$.

Many methods had been used to detect them, for example atomic absorptive spectrometry (AAS) [27], inductive coupled plasma mass spectroscopy (ICP-MS) [28], and cyclic

**Highlights**
1. A new selective coumarin-based probe for $Cu^{2+}$ detection was developed.
2. The probe showed a reverse response upon addition of $H_2S$.
3. The probes were successfully applied to image $Cu^{2+}$ and $H_2S$ in MCF-7 Cells.

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s10895-020-02585-0) contains supplementary material, which is available to authorized users.

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voltammetry (CV) [29]. These methods have some shortcoming, including the pretreatment of sample, long time required and expensive instrument needed, which limited them to be widely used. Different from above mentioned methods, fluorescent probe method gained wide attention because of its inexpensive, fast response, high selectivity, and sensitivity [30].

Our group has recently constructed some ratiometric probes for $Cu^{2+}$ or $H_2S$ [31–36]. But none of these probes could be used to detect both $Cu^{2+}$ and $H_2S$. Here, we developed a new ICT-based probe DF-Cu using 7-diethylaminocoumarin as a fluorophore and bis(pyridine-2-ylmethyl) amine as an active site for $Cu^{2+}$ binding. DF-Cu showed fluorescence quenching after the addition of $Cu^{2+}$ by forming a complex. The formed complex showed a fast response for the $HS^-$, and result in fluorescence turn-on. This kind of ON-OFF-ON fluorescent probe DF-Cu was successfully applied to image $Cu^{2+}$ and $HS^-$ in living cells.

## Experimental

### Apparatus

$^{13}C$ NMR and $^{1}H$ NMR spectra were gotten on a Bruker DTX-400 spectrometer in $CDCl_3$ and tetramethyl silane (TMS) as an internal reference. Absorptive spectra was conducted on a Lambda 35 UV/VIS spectrometer, PerkinElmer accurately. ESI-MS measurement was carried on a Q-Tof HR-MS spectrometer (Waters Micro mass) by using MeOH as a mobile phase. Fluorescent spectra dimensions were recorded on a HITACHI F-4600 fluorescent spectrophotometer. The excitation and emission slits were set at 2.5 nm. The excitation voltage was 700 V. MCF-7 cell fluorescence imaging was gotten by a LEICA TCS SP8 laser scanning confocal microscope.

### Materials

All used reagents including bis(pyridine-2-ylmethyl)amine, 7-(diethylamino) benzaldehyde, piperidine, acetic acid, ethanol, dichloromethane, 4-(dimethylamino)pyridine (DMAP), 1-(3-dimethylaminopropyl)- 3-ethylcarbodiimide hydrochloride (EDCI), methanol, diethyl malonate, NaOH, anhydrous sodium sulfate, were used directly after received from company. Double distilled water was used in all experiments. Common solvents were purified according to standard procedures. Probe stock solution (10 mM) was formed in $CH_3CN$. The solutions of amino acid (10 mM) and anions were gotten from corresponding salts including: NaCl, KCl, $MgCl_2$, $CuSO_4$, $CaCl_2$, $ZnCl_2$, $Na_2SO_4$, $MnCl_2$, $PbCl_2$, $CdCl_2$, $AgNO_3$, $BaCl_2$, $Fe_2SO_4$, $NiCl_2$ and $FeCl_3$.

### Cell Culture and Imaging

MCF-7 cell was cultivated in DMEM supplemented with 10% fetal bovine serum, in 5% $CO_2$, at 37 °C. For fluorescent imaging, 10 μM of **DF-Cu** was added to MCF-7 cell and nurtured at 37 °C for 30 min in 5% $CO_2$ incubator. Then, two groups of cells having probe DF-Cu and DF-Cu complex are treated with $Cu^{2+}$ and $HS^-$ solutions respectively and incubated for 1 h. At last, they were washed three times with PBS buffer.

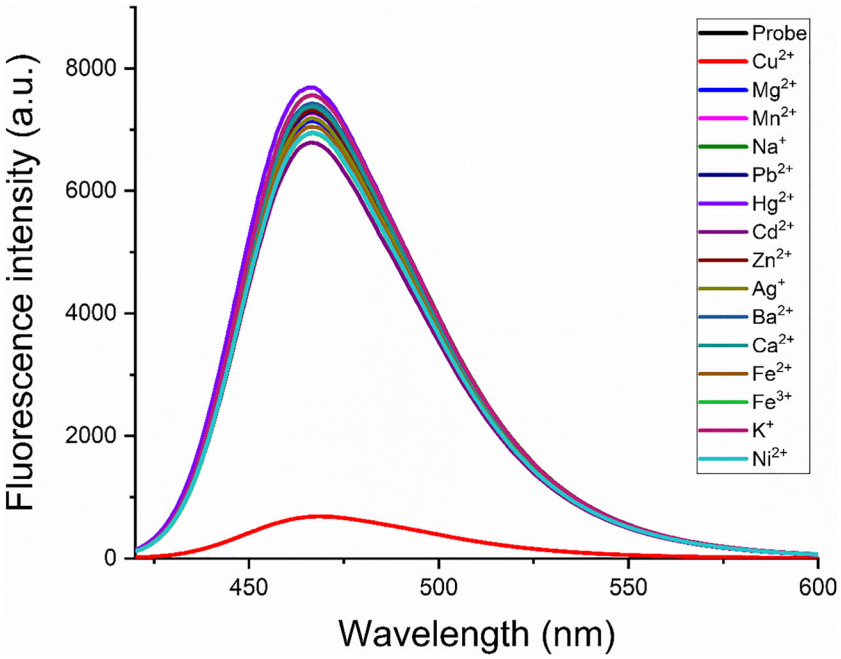



**Fig. 1** The fluorescence of **DF-Cu** (10 μM) and **Cu**$^{2+}$ (10 equiv.) or other various transition metal ions (10 equiv.) in Tris-HCl (10 mM, pH = 7.4, containing 90% acetonitrile) buffer. ($\lambda_{ex}$ = 420 nm, slit: 2.5 nm)

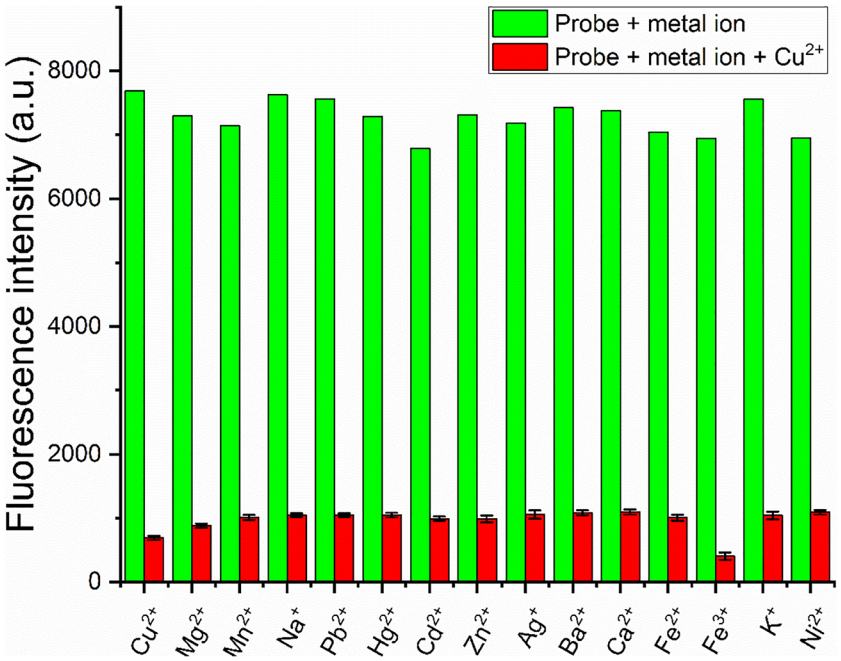



**Fig. 2** The fluorescence changes of **DF-Cu** (10 μM) at 465 nm on the addition of $Cu^{2+}$ (10 μM) in the presence of various ions (10 μM) in Tris-HCl buffer (10 mM, pH = 7.4, containing 90% acetonitrile). ($\lambda_{ex}$ = 420 nm, slit: 2.5 nm)

## Synthesis

Compound **D** was synthesized according to the previous reported paper [37].

Synthesis of **DF-Cu:** To the vigorously stirred solution of **D** (120 mg, 0.45 mmoL) in dichloromethane (10 mL) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (0.2 g, 1.0 mmoL), bis(pyridine-2-ylmethyl) amine (100 mg, 0.5 mmoL) and 4-(dimethylamino) pyridine (DMAP) (10 mg). The mixed solution was stirred overnight at room temperature after the reaction was confirmed as completion by TLC. The solution was washed with water and extracted with dichloromethane three times (30 mL × 3), dried over anhydrous $Na_2SO_4$. After the solvent was released by rotary evaporate, the crude **DF-Cu** was obtained and then purified by column chromatography (eluant: $CH_2Cl_2$/MeOH = 100 /1, *v*/v) to afford probe DF-Cu as a yellow solid (108 mg). $^1$H NMR (400 MHz, $CDCl_3$, ppm) δ: 1.22 (t, *J* = 7.4 Hz, 6H), 3.43 (q, *J* = 7.1 Hz, 4H), 4.70 (s, 2H), 4.89 (s, 2H), 6.5 (d, *J* = 6.3 Hz, 2H), 7.21–7.28 (m, 4H, PhH), 7.60–7.70 (m,

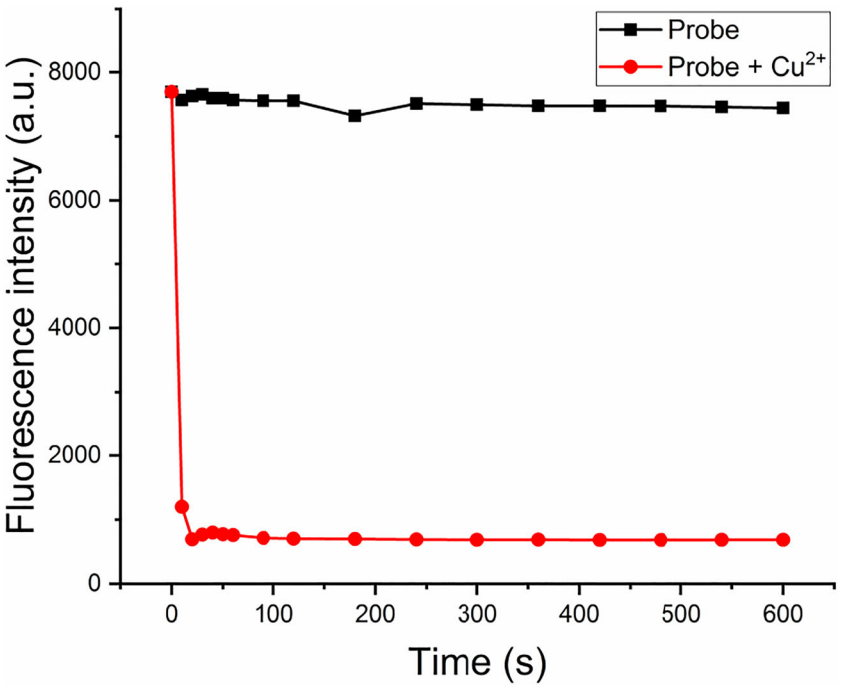



**Fig. 3** The fluorescence intensity of probe **DF-Cu** (10 μM) at 465 nm upon treatment with $Cu^{2+}$ (10 μM) with the progress of time in tris-HCl buffer (10 mM, pH = 7.4, containing 90% acetonitrile). ($\lambda_{ex}$ = 420 nm, slit: 2.5 nm)

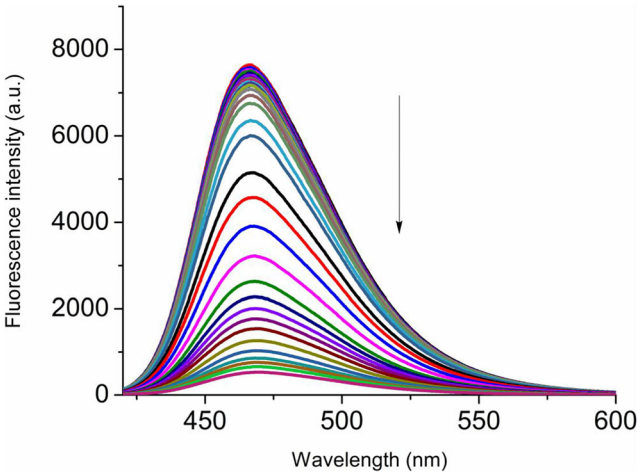



**Fig. 4** Fluorescence emission spectra of **DF-Cu** (10.0 μM) after $Cu^{2+}$ (0–10 equiv.) added in Tris-HCl buffer (10 mM, pH = 7.4, containing 90% acetonitrile). ($\lambda_{ex}$ = 420 nm, slit: 2.5 nm)

3H, PhH), 7.9 (s, 1H, PhH), 8.5 (s, 2H, PhH); $^{13}$C NMR (100 MHz, $CDCl_3$, ppm) δ: 167.72, 159.54, 157.09, 156.77, 156.32, 151.50, 149.63, 149.02, 144.11, 137.22, 136.70, 129.73, 122.51, 122.19, 121.95, 121.89, 116.73, 109.22, 107.68, 97.07, 54.24, 50.44, 44.91, 29.70, 12.42; HR-MS: *m/z* calcd for $[C_{26}H_{26}N_4O_3]^+$ 443.2083, Found: 443.2079.

## Results and Discussion

### Design and Synthesis of DF-Cu

We choose 7-diethylaminocoumarin as a fluorophore because of its good photostability, high fluorescence quantum yield and large stoke's shift. Because bis (pyridine-2-ylmethyl)

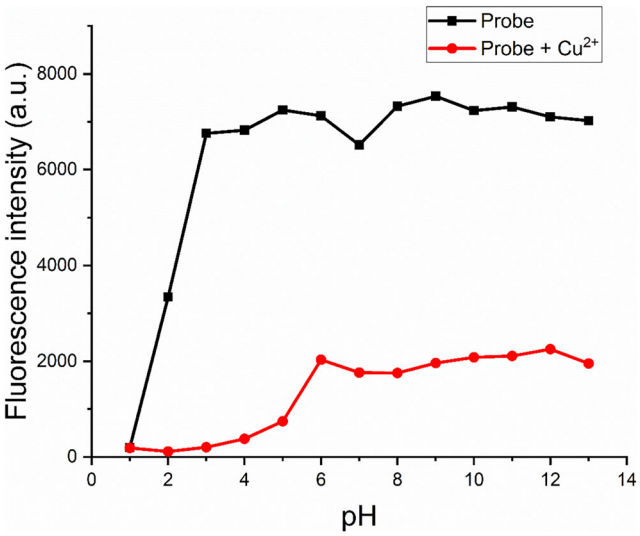



**Fig. 5** The fluorescence intensity of probe **DF-Cu** (10 μM) in different pH (1–13) as presence and absence of $Cu^{2+}$ (10 equiv.). ($\lambda_{ex}$ = 420 nm, slit: 2.5 nm)

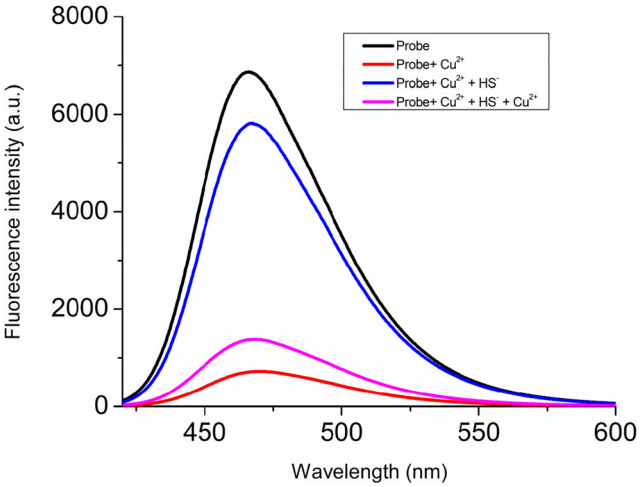


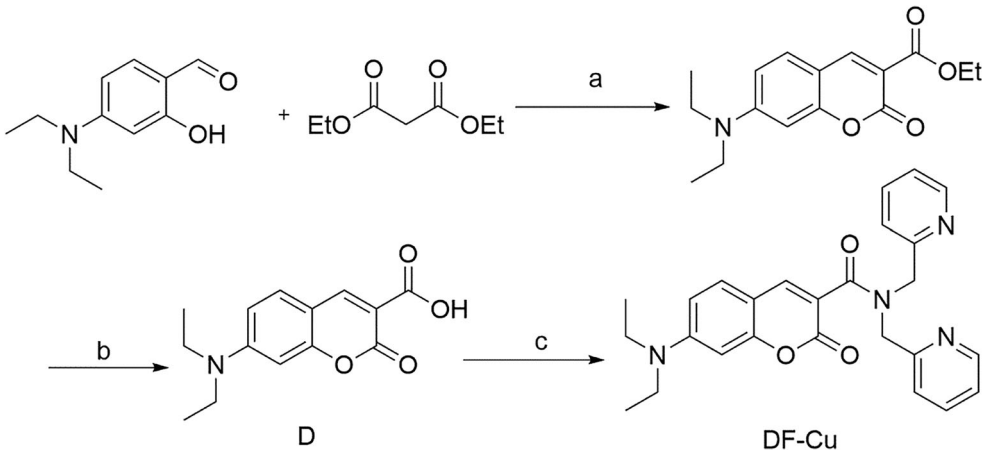

**Fig. 6** The fluorescent intensity of **DF-Cu** (10 μM), probe with **$Cu^{2+}$** (10 equiv.), and then **$H_2S$** added in Tris-HCl buffer (10 mM, pH = 7.4, containing 90% acetonitrile). $\lambda_{ex}$ = 420 nm, slit: 2.5 nm

amine is good ligand for $Cu^{2+}$, we choose it as a response group for $Cu^{2+}$, and hope it has a fast response to $Cu^{2+}$ over other metal ions and amino acids. By introducing response group into coumarin fluorophore, we synthesized a new probe DF-Cu in three steps with good yield. The probe DF-Cu was well characterized by $^1$H NMR, $^{13}$C NMR, and HR-MS (Fig. S1–S3**).**

### Spectroscopic Response of DF-Cu toward $Cu^{2+}$

To confirm the selectivity of probe DF-Cu (10 μM) toward $Cu^{2+}$, we conducted UV absorption and fluorescence titration in aqueous Tris-HCl buffer (10 mM, pH 7.4, containing 90% acetonitrile). The alone probe DF-Cu showed an absorption peak centered at 400 nm (Fig. S4, S5)**. After** 100 μM of $Cu^{2+}$ was added, the absorption peak shifted to 410 nm and there was no change as other metal ions added. It illustrated that the probe DF-Cu was selective for $Cu^{2+}$ detection by UV. From Fig. 1, it was observed and the fluorescence intensity of probe DF-Cu at 465 nm has not change when various common ions added, except $Cu^{2+}$. The fluorescence was quenched dramatically because the complex formation between probe DF-Cu and $Cu^{2+}$. It means the probe possesses a high fluorescent selectivity to $Cu^{2+}$.

Next, we performed competitive test (Fig. 2). The fluorescent intensity of DF-Cu was almost 7 times higher than that of the complex DF-Cu + $Cu^{2+}$. The fluorescence intensity ratio of DF-Cu / (DF-Cu + $Cu^{2+}$) is kept stable when other metal ions were added. The results showed the DF-Cu having good selectivity and specificity toward $Cu^{2+}$. Kinetic study of probe DF-Cu showed that the reaction between probe DF-Cu and $Cu^{2+}$ was finished within 10 s (Fig. 3**)**, which demonstrated the response speed of probe to $Cu^{2+}$ is very fast. The results showed that the probe DF-Cu is applicable for the fast and real-time detection of $Cu^{2+}$.




**Scheme 1** Synthetic route for the probe DF-Cu. Reagents and conditions: (**a**) 7-(diethylamino) benzaldehyde, piperidine, acetic acid, ethanol, 80 °C, 12 h; (**b**) 2 M NaOH 12 h, rt., acidified with 2 M hydrochloric acid; (**c**) bis(pyridine-2-ylmethyl) amine, dichloromethane, EDCI, DAMP, rt., overnight

## Fluorescent Titration of Probe DF-Cu with $Cu^{2+}$

Next, we performed fluorescent titration of probe DF-Cu. The fluorescence intensity of probe DF-Cu gradually decreased by the addition of a low concentration of $Cu^{2+}$ (Fig. 4**)**. The addition of $Cu^{2+}$ was continued until there was no further change in fluorescence intensity. The degree of fluorescence quenching is linearly associated with the concentration of $Cu^{2+}$ ($R^2 = 0.9919$) (Fig. S6**)**. The limit of detection (LOD, calculated according to the 3σ/slope) for $Cu^{2+}$ was 6.4 μM, which also represents a fairly high sensitivity for $Cu^{2+}$. Accordingly, Stern–Volmer plots (Fig. S7) illustrate that the emission of probe DF-Cu is quenched by $Cu^{2+}$ and the complexation constant is $7.52*10^3$ L/Mol.

## The Effect of pH on the Fluorescent Spectra

Next, we performed a pH experiment for probe DF-Cu. For monitoring pH response of probe DF-Cu, we prepared pH solution ranges from values 1 to 13. We studied the UV absorption and fluorescence emission of probe DF-Cu in different pH (1–13) as presence and absence of $Cu^{2+}$ (10 equiv.) (Figs. S8–11**)**. Probe DF-Cu can work well in pH 3–13 (Fig. 5**)**. It's worthy to note that probe can be used to detect co $Cu^{2+}$ in physiological pH environment.

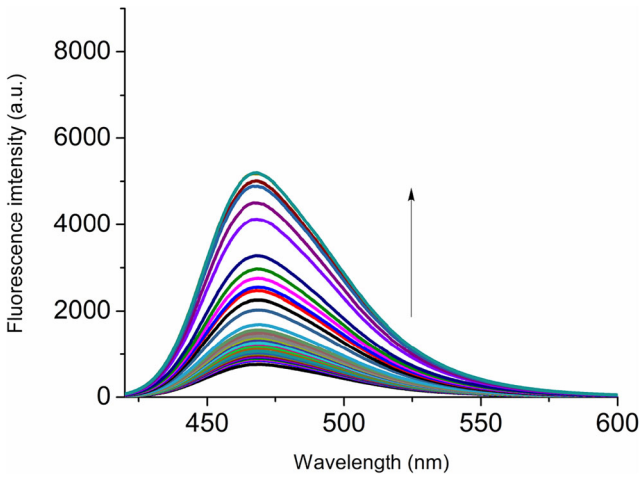


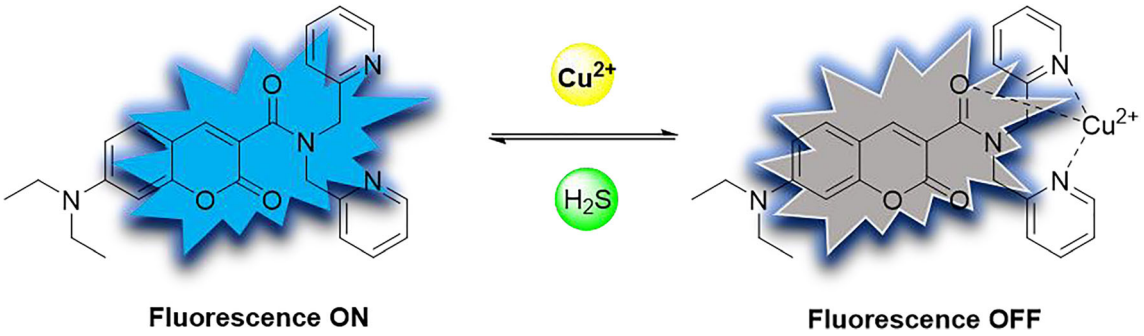

**Fig. 7** The fluorescence of **DF-Cu** + $Cu^{2+}$ complex (10 μM) upon treatment with $H_2S$ (0–12 equiv.) in Tris-HCl buffer (pH = 7.4, 10 mM, containing 90% acetonitrile, $\lambda_{ex}$ = 420 nm, slit: 2.5 nm)

## Fluorescent Performance of Probe DF-Cu Complex with $H_2S$

The addition of $Cu^{2+}$ to probe DF-Cu result in the fluorescence off. We speculated that is due to the complex probe+ $Cu^{2+}$ formed. Because $Cu^{2+}$ could react with $S^{2-}$ and form a stable compound CuS, we thought that complex DF-Cu + $Cu^{2+}$ may used for the sensitive detection of sulfide ions by the removal of $Cu^{2+}$ from complex and result in fluorescence recover. We checked this idea by making a sample solution of probe DF-Cu complex and then adding sulfide ion. As we expected, the fluorescence intensity restored and nearly same to the fluorescence intensity of probe DF-Cu (Fig. 6). Then, we added copper ions to this solution and found that the fluorescence off again, which mean the complex between probe DF-Cu and $Cu^{2+}$ formed again. **The results illustrated that the response of probe to $Cu^{2+}$ is reversible. The response mechanism was proposed in** Scheme 2**.** Scheme 1

## Fluorescent Titration of DF-Cu Complex for $H_2S$

Fluorescence titration of probe DF-Cu complex for $H_2S$ was done in situ. Firstly, the sample solution of probe DF-Cu was prepared. Then $Cu^{2+}$ solution was added to this sample solution to make probe DF-Cu complex. Last, we started the fluorescent titration of probe DF-Cu complex for sulfide ions. There was a gradual enhancement in fluorescence intensity of probe DF-Cu along with the concentration of $H_2S$ increasing (Fig. 7**)**. The fluorescence emission is linearly associated with the concentration of $H_2S$ ($R^2 = 0.9848$) (Fig. S12). The limit of detection for $H_2S$ was 134.2 μM. The UV-vis spectra of DF-Cu + $Cu^{2+}$ complex (10.0 μM) after $H_2S$ (0–10 equiv.)




**Scheme 2** Proposed mechanism of probe DF-Cu with $Cu^{2+}$ and $H_2S$

added shown in Fig. S13. Along with $H_2S$ added, a little blue-shift was observed in the UV-vis spectra of DF-Cu + $Cu^{2+}$ complex.

## Sensing Mechanism

To gain insight into the sensing mechanism, we continue to study the sensing process by MS. An intense peak at m/z 601.27 corresponding to $(C_{26}H_{26}CuN_4O_7S)^+$ was present in the MS spectrum (Fig. S14). The results demonstrated that $Cu^{2+}$ could form coordinated complexes with probe DF-Cu (Scheme 2).

## Cell Imaging

The cytotoxicity of probe DF-Cu in living MCF-7 cells was assessed by a conventional MTT assay. The experimental results demonstrated that almost 80% of the cells survived (Fig. 8). It means probe DF-Cu is low Cytotoxicity and could be applied for cell imaging in living cells.

Subsequently, probe DF-Cu was used to trace Copper ions in living MCF-7 cells (Fig. 9). The MCF-7 cells were incubated with 10 μM probe DF-Cu for 30 min at 37 °C in 5% $CO_2$. There was strong fluorescence in blue channel (Fig. 9b). Upon the addition of 100 μM $Cu^{2+}$ and incubated another 1 h, an obvious fluorescence decreasing was observed as expected (Fig. 9e). As the designed compound is an ON-OFF-ON probe, we also checked its complex for $H_2S$ imaging. When probe DF-Cu + $Cu^{2+}$ complex was treated with hydrogen sulfide (500 μM) and incubated for 1 h, a strong fluorescence in blue channel was observed (Fig. 9h). The result indicates that $Cu^{2+}$ and $H_2S$ can be detected by probe DF-Cu in a living cells.

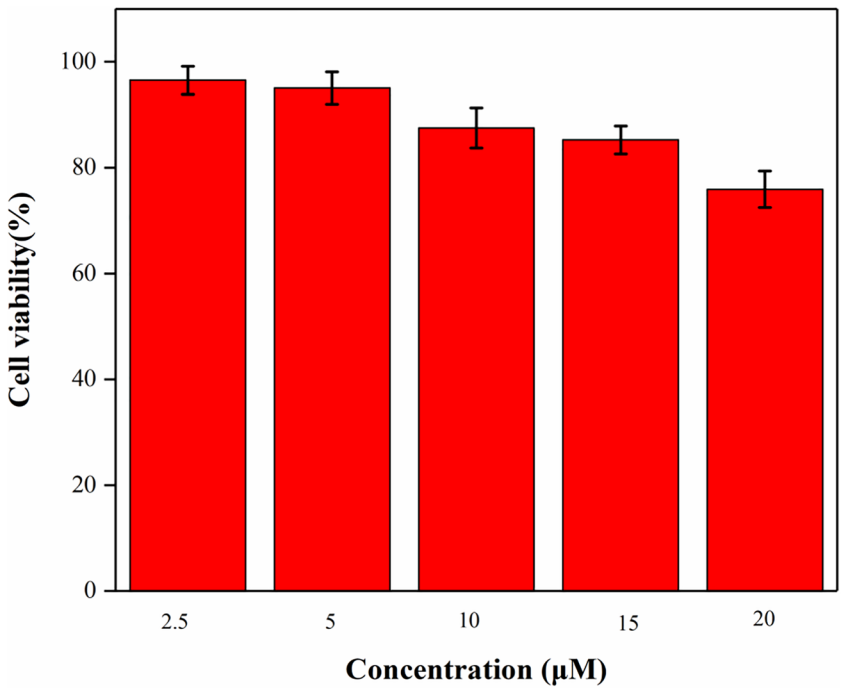



**Fig. 8** Cytotoxicity assay of probe DF-Cu at different concentration in MCF-7 cells

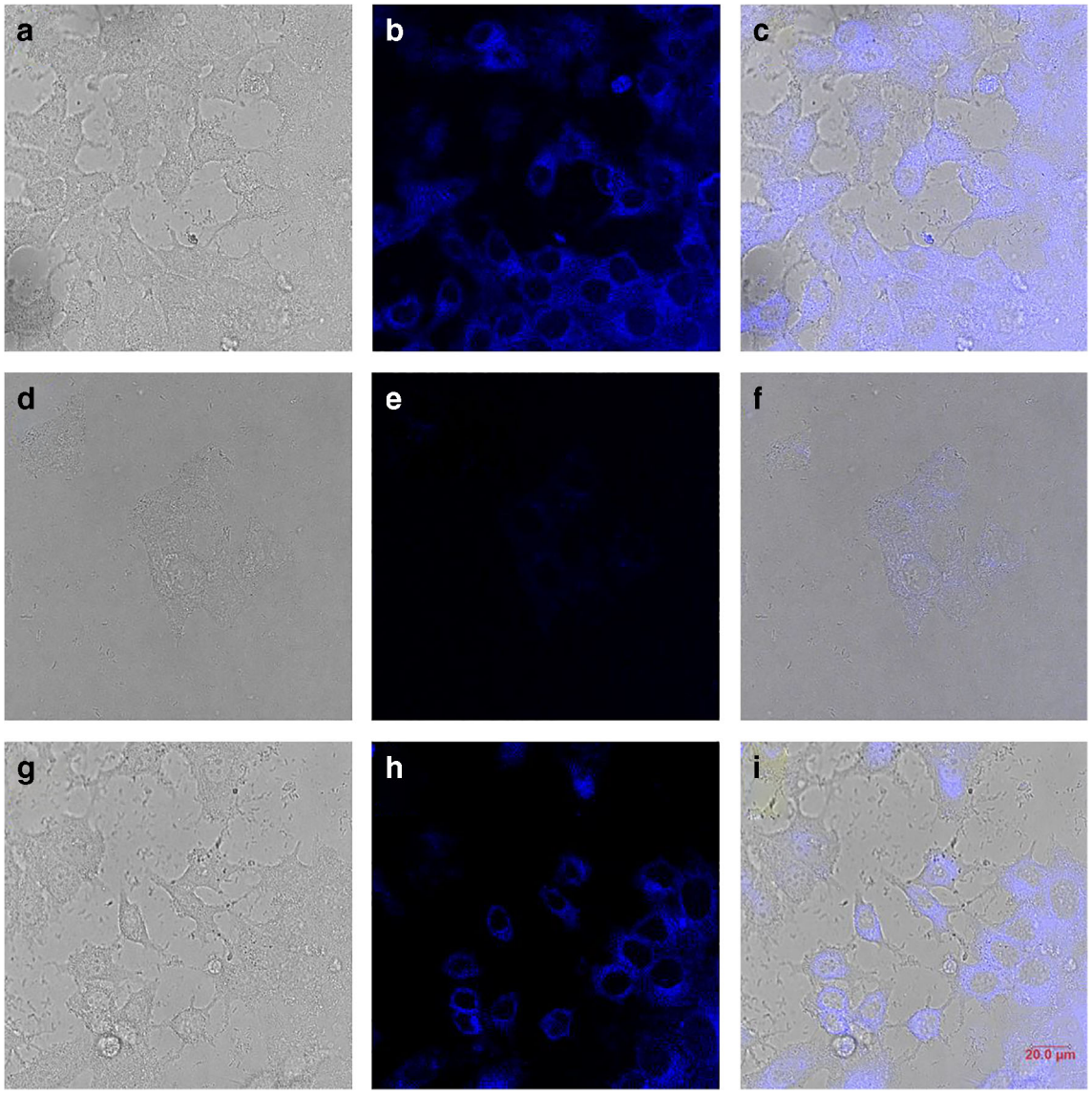



**Fig. 9** Confocal fluorescence images of $Cu^{2+}$ with **DF-Cu** in MCF-7 cells. (**a**, **b**) MCF-7 cells pretreated **DF-Cu** (10 μM) for 30 min. (**d**, **e**) MCF-7 cell was pretreated with **DF-Cu** (10 μM) for 30 min, and then mixed with $Cu^{2+}$ (100 μM). (**g**, **h**) MCF-7 cells were pretreated with **DF-Cu** + $Cu^{2+}$ complex (10 μM) for 30 min and then incubated with $HS^-$ (500 μM) for 1 h. (**c**, **f**) and (**i**) are merged images of a with **b**, **d** with **e**, and **h** with **i** (excitation: 400 nm emission: 430–510 nm)

## Conclusion

In summary, we have designed and synthesized a novel highly selective and sensitive ICT probe for $Cu^{2+}$ detection. When probe response to $Cu^{2+}$, the fluorescence turn-off and probe-$Cu^{2+}$ complex was formed. Upon addition of hydrogen sulfide to probe-$Cu^{2+}$ complex, the fluorescence could be recovered. This ON-OFF-ON system was repeatable. Furthermore, the probe DF-Cu showed a very fast response to $Cu^{2+}$ and the probe-$Cu^{2+}$ complex showed a fast response to hydrogen sulfide. Moreover, the probe DF-Cu could be used to detect $Cu^{2+}$ and hydrogen sulfide ion in MCF-7 cell. We believe that the probe DF-Cu has potential application for the quantitative analysis of in a cell.

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