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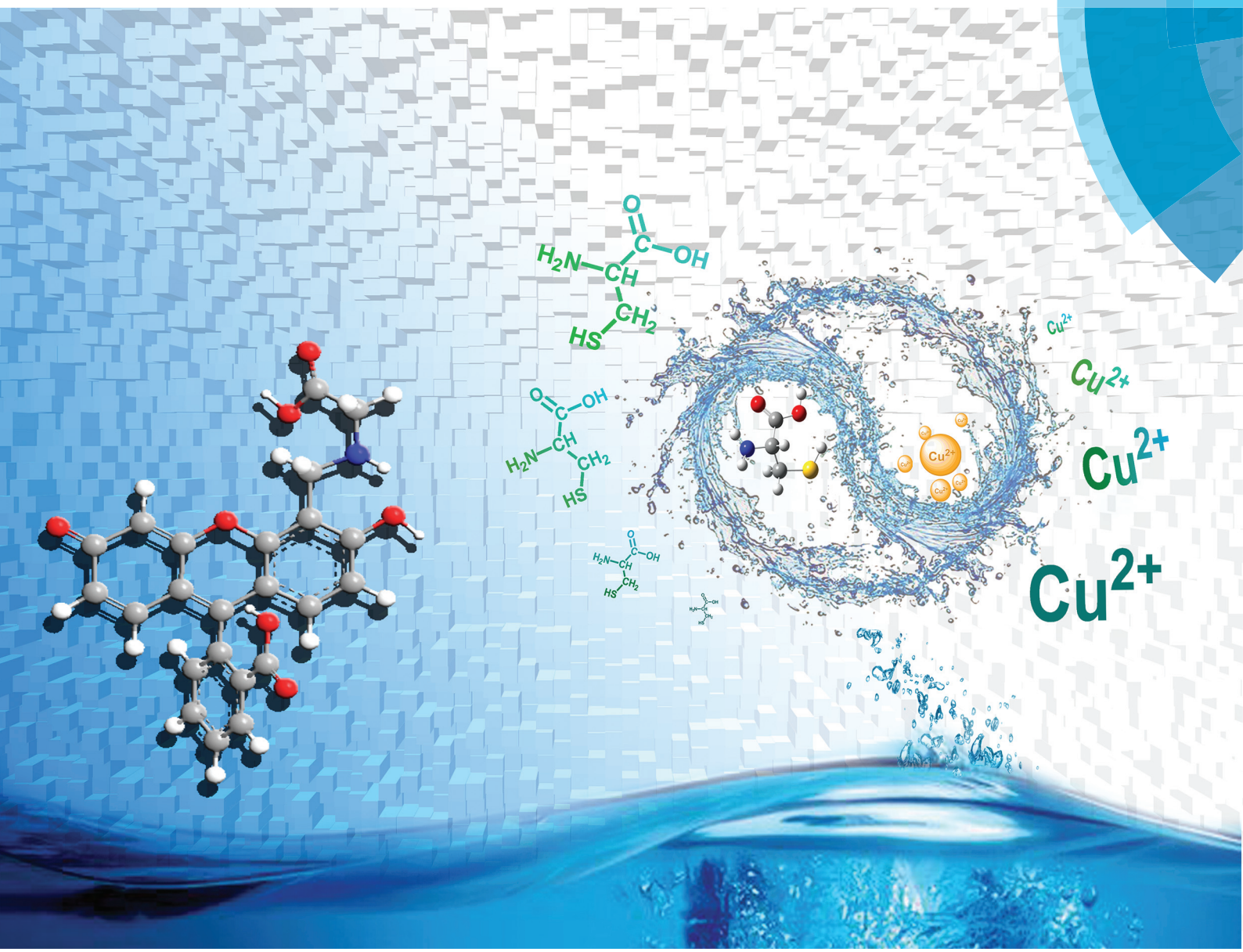

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A fluorescein-based chemosensor for relay fluorescence recognition of Cu(II) ions and biothiols in water and its applications to a molecular logic gate and living cell imaging†

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Relay recognition of copper(II) ions and biothiols *via* a fluorescence "on–off–on" cascade was designed and realized as a new sequential combination of cations and small molecules. Probe **1** bearing a fluorescein skeleton was thus synthesized, which performed well in 100% HEPES buffer (pH = 7.0) solution, as a highly sensitive, selective fluorescence sensor for Cu^{2+}. The limit of detection (LOD, 0.017 ppm) was obtained, and this value is much lower than 1.3 ppm, allowed by US EPA. The 1 : 1 complex generated from fast sensing of Cu^{2+} when excited at 491 nm, showed good relay recognition for biothiols (*i.e.*, Cys, Hcy and GSH with low detection limits of 0.12 μM, 0.036 μM and 0.024 μM, respectively) *via* remarkable fluorescence enhancement. The origin of this relay process was disclosed through ESI-MS and corresponding density functional theory (DFT) computations. Notably, probe **1** can be utilized for the construction of a molecular logic gate with the IMPLICATION function by using the above fluorescence changes. Moreover, this relay recognition was also applied to HepG2 cell imaging successfully.

Introduction

Over the past few years, fluorescent probes have been applied in many fields of molecular recognition, such as detection of various guest species and bioimaging.[1,2] Recently, multi-analyte fluorescent probes have attracted great attention due to their excellent performance.[3,4] As a result, bifunctional probes[5–10] and relay probes[11–18] emerged fast, and remarkable work has been performed to implement these unique strategies. Notably, due to the importance of sequence sensing in some biological processes,[19–21] relay probes would be useful for their investigation.

Biothiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) are essential to life due to their important roles in many biological processes, like cellular antioxidant defense and the growth of tissues in a living system.[22,23] Their irregular levels would lead to neurotoxicity[24] and the related Alzheimer's disease,[25] and cardiovascular disease.[26] Due to the importance of early diagnosis, convenient and sensitive methods based on fluorescence detection have been developed, and thus highly selective probes for biothiols have appeared in recent years.[27–30] Among these probes, Cu^{2+} complexes have been used to sense biothiols through an indicator displacement assay (IDA).[29,31,32] Very recently, this approach has been successfully extended to transmembrane transport of relevant amino acids.[33] The realization of this strategy relies on fluorescence quenching of a probe by coordinating with Cu^{2+}, and fluorescence recovery by the recognition of biothiols that rapidly compete with the probe for Cu^{2+}. However, some previous work like that done by Chen was carried out under different conditions (pure MeCN for Cu^{2+}; MeCN/HEPES = 1 : 99 for biothiols).[32] Their relay fluorescence recognition by a single probe under the same conditions is still rare, and most of the known probes functioned well merely in aqueous solution with organic solvents such as MeCN/H_2O = 1 : 1,[34] EtOH/HEPES = 9 : 1[35] and DMSO/HEPES = 5 : 95[36] and under the excitation of UV light (λ_{ex} = 305 nm or 355 nm) that would cause damage upon intracellular processes.[34,36] Thus, further advancement to work at pH within the physiological value in neat water is a significant challenge.

As a continuation of our investigation to bifunctional[37,38] and relay[39–43] fluorescent probes, herein we chose Cu^{2+} ions

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†Electronic supplementary information (ESI) available: Spectral data, copies of $^1H/^{13}C$ NMR, ESI-MS and other materials. See DOI: 10.1039/c7ob00525c

and biothiols as a new combination to study their relay recognition using a single probe (**1**). This probe was based on fluorescein, and therefore demonstrated a strong fluorescence character when excited by visible light (491 nm). Cu^{2+} was found to be the only metal ion that could quench the fluorescence of **1** by coordination to its amino acid moiety in 100% aqueous solution at pH 7.0. The recovery of fluorescence could be realized by the addition of biothiols. This on–off–on fluorescence mode[44,45] can be used for the sequential detection of Cu^{2+} and biothiols, and can be further extended for bioimaging.

Results and discussion

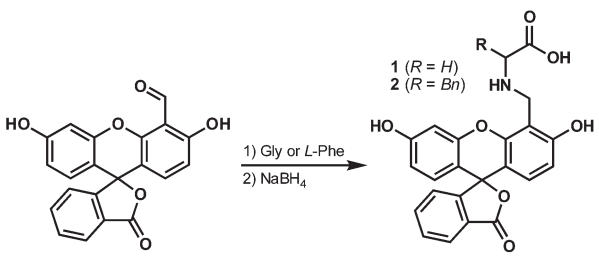
Design and syntheses of probes

As shown in Scheme 1, the designed probes **1** and **2** were synthesized from monoaldehyde-functionalized fluorescein[46] and commercially available α-amino acids. Their structures were confirmed by nuclear magnetic resonance and mass spectrometry (Fig. S1–S9, ESI†). Fluorescein has excellent spectroscopic properties[47–49] due to its strong fluorescence at about 550 nm when excited by a long absorption wavelength. According to our previous work,[38,50] an α-amino acid moiety in **1** may improve the probe's solubility in water and its response to some metal ions. The **1**-metal ensemble generated from the first recognition would in turn function as a new chemosensor to recover fluorescence through the displacement of metal ions induced by biothiols.

Fluorescence performance of probe 1 during tandem sensing of Cu^{2+} and biothiols

First, the relationship between the fluorescence intensity of this probe and the pH effect was investigated in detail (Fig. S10, ESI†). In general, fluorescein derivatives show two forms under different pH conditions:[51,52] one is a nonfluorescent "closed" spirolactone and the other is a "ring-opening" quinoid form with strong fluorescence. As demonstrated in Fig. S10,† fluorescence intensity of **1** increased significantly between pH 6.0 to 10.0, which suggested that its quinoid form was the main species. Considering the potential application, the following experiments were performed with a pH 7.0 HEPES buffer (100%).

Next, the fluorescence properties of this probe were investigated in detail. Probe **1** shows strong fluorescein emission (525 nm, Fig. 1a) upon excitation by visible light at 491 nm.



Scheme 1 Syntheses of probes **1** and **2**.

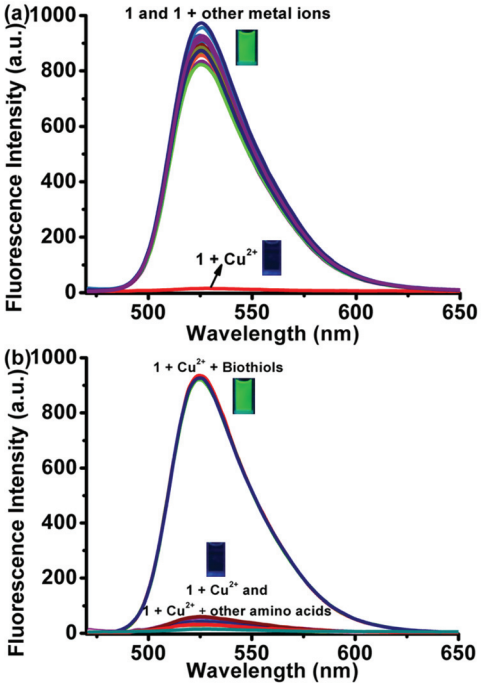


Fig. 1 (a) Fluorogenic performance of probe **1** (10.0 μM) with different cations (10.0 μM) in a pH 7.0 HEPES buffer (λ_{ex} = 491 nm). (b) Fluorescence variations of probe **1** + Cu^{2+} (10.0 μM) upon the addition of amino acids (50.0 μM).

Subsequently, metal ions such as K^+, Li^+, Ag^+, Na^+, Mg^{2+}, Fe^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Mn^{2+}, Cr^{3+}, Fe^{3+}, Al^{3+}, Ga^{3+} and Cu^{2+} were added into the solution of **1**, and only Cu^{2+} ions quenched the fluorescence severely. Subsequent studies indicated that the fluorescence intensity of the **1** + Cu^{2+} system was affected by pH (Fig. S10†). Its intensity remained at a minimum value when pH varied within the scope of 5.0–11.0. This observation means that there is no influence on the detection of Cu^{2+} ions at pH 7.0. Considering the strong affinity of the mercapto group with copper, Arg, Ser, Phe, Val, Pro, Tyr, Gln, Asn, Gly, Trp, Glu, Ile, Asp, His, Ala, Thr, Lys, Met, and Leu, and biothiols were chosen to study relay recognition of this *in situ* generated system by fluorescence spectra (Fig. 1b). To our delight, the fluorescence intensity was almost recovered merely after the addition of Cys, Hcy and GSH. Moreover, bright jade green fluorescence of probe **1** turned almost nonfluorescent upon the addition of Cu^{2+} (Fig. 1a, insert), and fluorescence recovery was also observed by the naked eye with the inclusion of biothiols (Fig. 1b, insert). Thus, our proposed tandem sensing of Cu(II) and biothiols was realized *via* a fluorescence "on–off–on" switch in 100% aqueous solution.

Relay fluorescence titrations of 1 with Cu^{2+} and biothiols

Titrations of Cu^{2+} were first carried out (Fig. 2a). With addition of Cu^{2+} to HEPES buffer of **1**, its fluorescence intensity at 525 nm decreased clearly, down to 1.8% in the presence of 1.0 equiv. of Cu^{2+} and fluorescence quantum yield[53] (78.1%) also

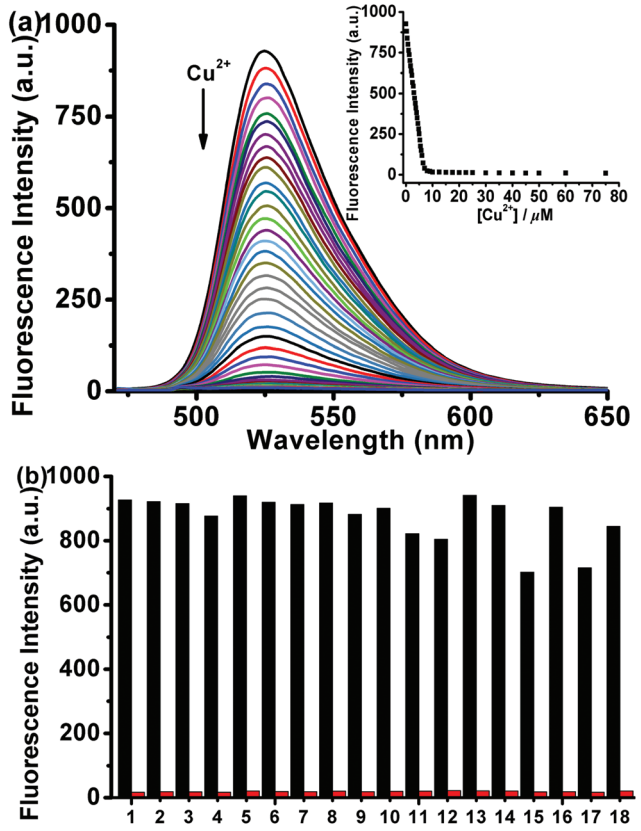


Fig. 2 (a) Fluorescent responses of **1** (10.0 μM) with variable Cu^{2+} in 100% HEPES (λ_{ex} = 491 nm). [Cu^{2+}] = 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00, 4.25, 4.50, 4.75, 5.00, 5.25, 5.50, 5.75, 6.00, 6.25, 6.50, 6.75, 7.00, 7.25, 7.50, 7.75, 8.00, 8.50, 9.00, 9.50, 10.00, 12.50, 15.00, 17.50, 20.00, 22.50, 25.00, 30.00, 35.00, 40.00, 45.00, 50.00, 60.00, 75.00 μM. Inset: Plot of the intensity of **1** as a function of [Cu^{2+}] at 525 nm. (b) The selectivity of **1** (λ_{em} = 525 nm). Black bars: Fluorescence intensity of **1** with other cations (Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, Ag^{+}, Fe^{3+}, Cr^{3+}, Al^{3+}, and Ga^{3+}). Red bars: Fluorescence intensity after inclusion of Cu^{2+}.

fell to 2.0%. As shown in Fig. 2a (inset), the increase of Cu^{2+} concentration did not result in additional fluorescence quenching of probe **1**, suggesting their equal stoichiometry. The Job's plot supported the 1 : 1 ratio as well (Fig. S11†). Based upon this stoichiometry, K_a (1.60×10^5 M^{-1}) of the formed **1**·Cu^{2+} was thus obtained by using emission variations of 525 nm with a Benesi–Hildebrand plot (Fig. S12†).[54] In addition, the peak at 481.0944 for [**1** + Cu(II) − H]$^+$ in the TOF mass spectrum confirmed the 1 : 1 binding model between **1** and Cu^{2+} (Fig. S13, ESI†). This fluorescence quenching could be attributed to a ligand-to-metal charge transfer (LMCT)[55] process between **1** and Cu^{2+} through effective coordination with an α-amino acid moiety. To confirm binding sites, control substrate **2** and monoaldehyde-functionalized fluorescein were evaluated (Fig. S14 and S15, ESI†). As shown in Fig. S14,† the high selectivity to Cu(II) under the same conditions was still observed for **2**. But monoaldehyde-functionalized fluorescein itself did not show significant selectivity for Cu^{2+}. These results indicated that the α-amino acid moiety of probe **1** was the main binding site, and the optimized structure of the corresponding complexes and the LMCT behavior were disclosed through DFT computations (Fig. 4, S28 and S29†). The LOD[56] (0.017 ppm) of Cu^{2+} was obtained (Fig. S16†), and this value is lower than 1.3 ppm, allowed by US Environmental Protection Agency. And the short response time (6–7 seconds) also suggested that **1** has high sensitivity for Cu^{2+} (Fig. S17, ESI†). In addition, the influence of other tested metal ions (60 μM) on the interaction of **1** with Cu^{2+} was also studied by competitive experiments (Fig. 2b). All of them have no apparent disturbance, which means that coexistent cations almost had no influence on probe **1**, and it could act as an on–off fluorescent sensor for the detection of Cu^{2+} in 100% HEPES buffer solution (pH = 7.0).

The *in situ* generated system (**1** + Cu^{2+}, 10.0 μM + 10.0 μM) was then utilized for the titration of biothiols. When Cys, Hcy or GSH was added to the HEPES buffer solution (pH = 7.0), an obvious turn-on response at 525 nm band could be detected (Fig. 3a, S18 and S19†). The fluorescence enhancement factor was up to 55-fold for these biothiols. The fluorescence

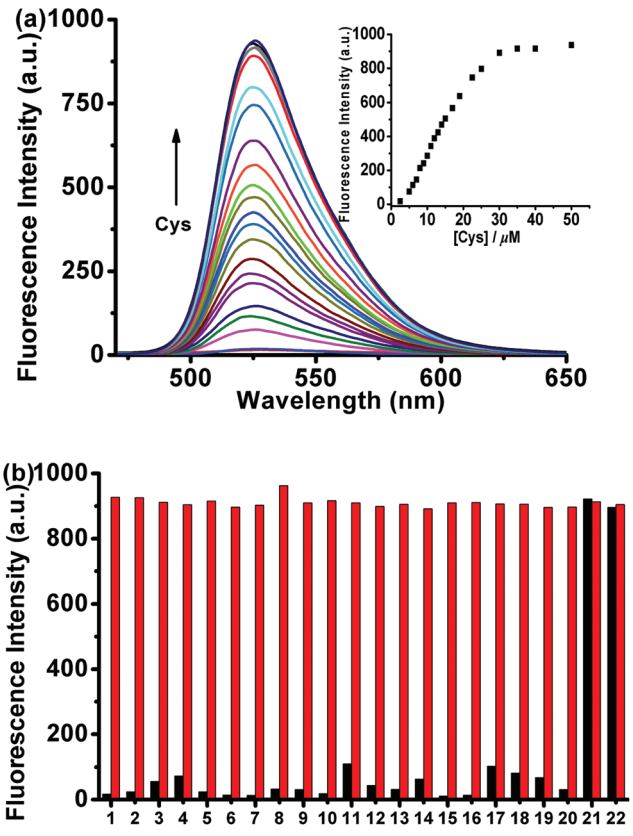


Fig. 3 (a) Fluorescence responses of **1** + Cu^{2+} with variable Cys in 100% HEPES (λ_{ex} = 491 nm). [Cys] = 0, 2.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0, 15.0, 16.0, 17.0, 19.0, 22.5, 25.0, 30.0, 35.0, 40.0, 50.0 μM. Inset: Plot of **1**·Cu^{2+} intensity *versus* [Cys]. (b) The selectivity of **1** + Cu^{2+} (λ_{em} = 525 nm). Black bars: Fluorescence intensity of **1** + Cu^{2+} with other amino acids (Arg, Ser, Phe, Val, Pro, Tyr, Gln, Asn, Gly, Trp, Glu, Ile, Asp, His, Ala, Thr, Lys, Met, Leu, GSH, Hcy). Red bars: The intensity after inclusion of Cys.

quantum yield (Φ) increased from 2.0% to 77.2% (Cys), 78.0% (Hcy) or 77.5% (GSH). The fluorescence recovery could be attributed to the regeneration of the initial probe **1** by the IDA approach, which had already been confirmed by ESI mass spectra. As shown in Fig. S20–S22 (ESI†), probe **1** is indeed the main species in the solution after the addition of biothiols to the **1** + Cu^{2+} system. This evidence was fully consistent with the above fluorescence changes. The corresponding LOD was determined as 0.12 μM, 0.036 μM and 0.024 μM for Cys, Hcy and GSH, respectively (Fig. S23–S25, ESI†). These values are lower than those reported previously,[32,35,36] and also lower than total concentrations in healthy plasma (200–300 μM for Cys and 12 μM for Hcy). The sensitivity of this probe would thus result in its potential applications. To validate the selectivity, these biothiols were added to the **1** + Cu^{2+} system with other amino acids in HEPES buffer (Fig. 3b, S26 and S27, ESI†). Pleasingly, all of competitive amino acids (125.0 μM) did not exert significant influence on the relay recognition of Cys, Hcy and GSH. So, the [**1** + Cu^{2+}] system would also work as a fluorescence turn-on sensor for these amino acids bearing the mercapto group in 100% HEPES buffer solution (pH = 7.0).

Computational studies

In order to deduce the inherent relationship of probe structures with the respective fluorescence response, DFT computations were performed by the Gaussian 09 program,[57] and the LANL2DZ effective core was used for Cu^{2+}.[58] As shown in Fig. S28,† there is a xanthene skeleton in the dominant configuration for **1**, which can lead to a delocalized system. As shown in Fig. 4 (left), most of the electron densities in these two molecular orbitals of **1** spread on the whole xanthene skeleton, which indicates that probe **1** would bear strong fluorescence. The optimized structure of the **1**·Cu^{2+} complex (Fig. S29, ESI†) reveals that the Cu^{2+} coordinates with –OH and $-CO_2H$ groups of probe **1**, in addition to some solvent molecule or counteranion. Notably, electron density on the HOMO of **1**·Cu^{2+} is still distributed at the xanthene moiety (Fig. 4, right) while that of its LUMO is almost concentrated on Cu^{2+}, thus leading to a LMCT process.[59] The origin of fluorescence quenching during the recognition of Cu^{2+} is thus attributed to a strong LMCT by an effective coordination between **1** and this metal ion.

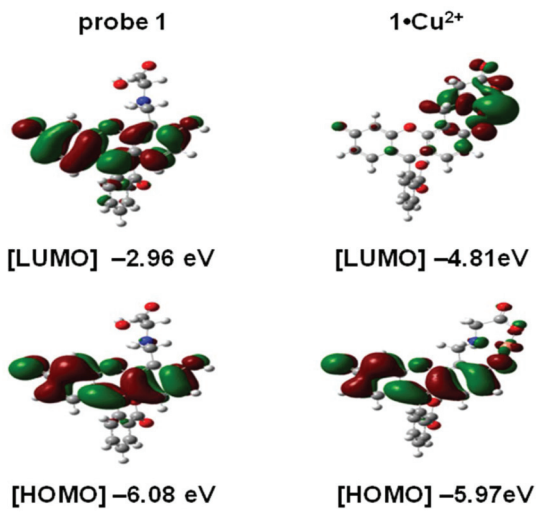


Fig. 4 Molecular orbital plots and energy levels.

Applications in molecular logic gates

With the development of sequential logic devices at the molecular level, inputted chemical information could be outputted as fluorescence signals.[3,12,17,60–65] As mentioned above, the fluorescence performance of **1**–Cu^{2+}-biothiols has been investigated systemically (Fig. 5), and herein the IMPLICATION logic gate could be constructed by using **1** as a logic device while Cu^{2+} and biothiols (Cys was selected as an example) as inputs and emission band (525 nm) as output (Table 1). Without any inputs (entry 1), the characteristic emission of probe **1** appeared, thus leading to output "1". When only Cu^{2+} was inputted, output "0" was obtained (entry 2) because the emission at 525 nm disappeared. With Cys or both inputs, the fluorescence emission was recovered again, and the output is "1" (entries 3 and 4). These results show that the molecular logic gate with the IMPLICATION function was constructed in this relay recognition system.

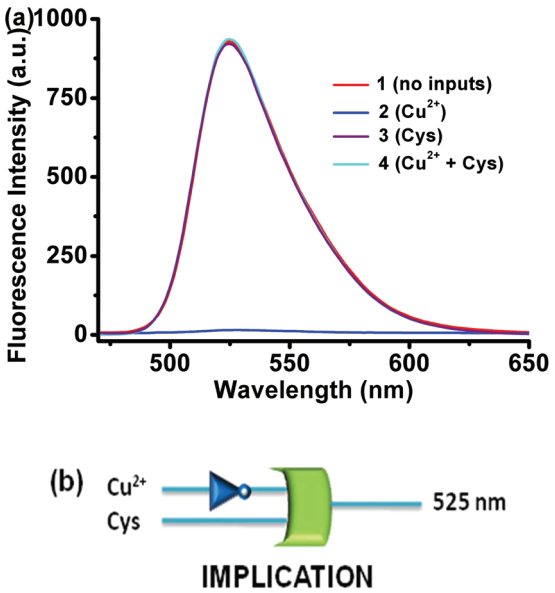


Fig. 5 Implication logic gate: inputs (Cu^{2+}, Cys); output (fluorescence intensity of 525 nm). (a) Fluorescent responses with none; Cu^{2+}; Cys; Cu^{2+} + Cys, respectively. (b) Logic circuit.

Table 1 Truth table

Entry	Cu^{2+} input **1**	Cys input **2**	525 nm output
1	0	0	1
2	1	0	0
3	0	1	1
4	1	1	1

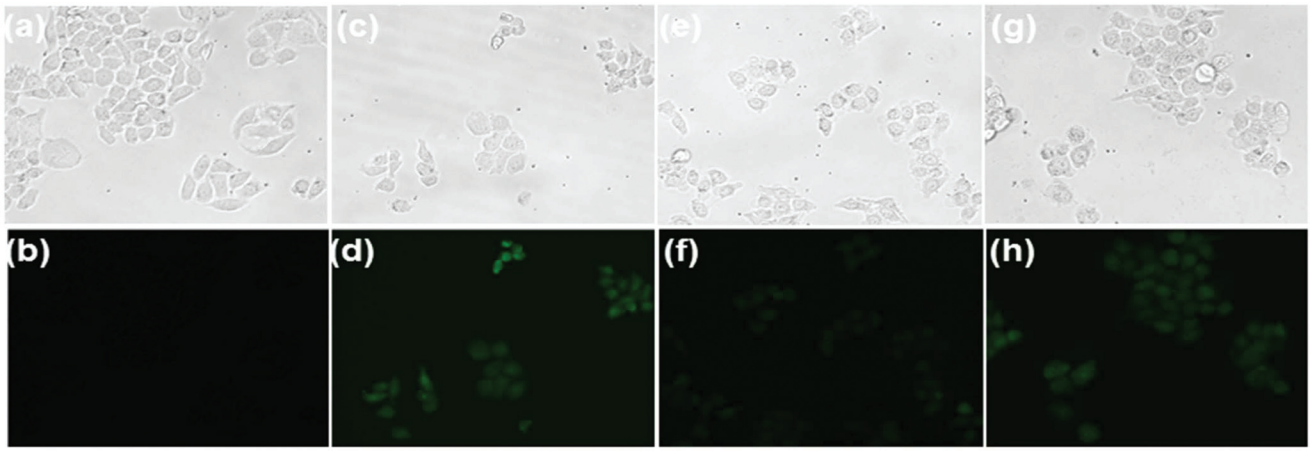
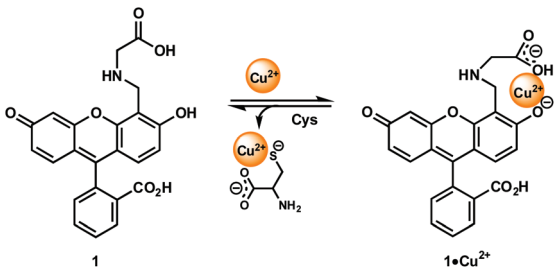


Fig. 6 Images of HepG2 cells: (a) bright field and (b) fluorescence images of HepG2 cells; (c) bright field and (d) fluorescence images of HepG2 cells incubated with **1** (50.0 μM) for 3 h; (e) bright field and (f) fluorescence images of HepG2 cells incubated with **1** (50.0 μM) for 3 h, and then incubated with Cu^{2+} (20 equiv.) for 15 min; (g) bright field and (h) fluorescence images of HepG2 cells incubated with **1** (50.0 μM) for 3 h, and then incubated with Cu^{2+} (20 equiv.) for 15 min, and finally incubated with GSH (100.0 μM) for 15 min.



Scheme 2 Mechanism of relay recognition.

Relay bioimaging in living cells

The application for bioimaging in living cells was investigated. One-hour incubation of HepG2 cells in PBS buffer provided nonfluorescence (Fig. 6b). However, green fluorescence within these cells could be seen (Fig. 6d), when HepG2 cells were treated with the probe (50.0 μM) for 3 h. After incubating with Cu^{2+} (20 equiv.) for 15 min, the fluorescence was quenched to some extent (Fig. 6f), due to the influence of the biothiols in cells. Upon further treatment of HepG2 cells with external GSH (100.0 μM) for 15 minutes, green fluorescence was recovered (Fig. 6h). In a control experiment, *N*-ethylmaleimide (NEM)[66] was added to HepG2 cells before they were treated with **1** and Cu^{2+}. As shown in Fig. S30 (ESI†), almost nonfluorescence was observed, indicating that the relay recognition of Cu^{2+} and biothiols by this probe also functions in living cells due to its good permeability.

Conclusions

The probe derived from fluorescein (**1**) shows high selectivity, sensitivity for Cu^{2+} ions by the quick formation of a 1 : 1 complex *via* fluorescence quenching in pure HEPES buffer. Then (**1**–Cu^{2+}) ensemble generated *in situ* exhibited interesting relay sensing to biothiols through the fluorescence recovery by effective displacement (Scheme 2). To this end, a new relay combination featuring in cations to small molecules compared to our previous work,[39–43] has been achieved *via* remarkable "on–off–on" fluorescence changes. The present investigation enriches our continuing studies toward unnatural amino acid-derived probes.[38,50]

Experimental

Details of chemicals and materials, spectra measurement, live cell imaging, and synthesis and characterization of monoaldehyde-functionalized fluorescein can be found in the ESI.†

Synthesis and characterization of probe 1

To a stirred solution of glycine (75 mg, 1.0 mmol) and NaOH (80 mg, 2.0 mmol) in MeOH/H_2O (v/v = 10 : 1, 10 mL) a solution of the above monoaldehyde-functionalized fluorescein (360 mg, 1.0 mmol) in MeOH (5 mL) was added dropwise at room temperature. The resulting mixture was stirred for 30 min and then allowed to warm gradually to 65 °C and further stirred for 8 h. The whole system was then cooled in an ice bath, followed by the addition of $NaBH_4$ (49 mg, 1.3 mmol) portionwise within 30 min. The resulting mixture was stirred for 4 h, and the pH of the solution was then adjusted to 5.0–6.0 by using glacial acetic acid (0.75 mL). After further stirring for 8 h, the reaction mixture was evaporated to give a viscous residue that precipitated upon the treatment with EtOH (10 mL). The filter cake was washed with EtOH (10 mL) and Et_2O (10 mL) successively, and was then collected and recrystallized in MeOH (7 mL) to afford probe **1** (334 mg, 80%). Mp: 250 °C (dec.). ^1H NMR (MeOH-d_4, 400 MHz) δ = 8.07–8.03 (m, 1H), 7.69–7.63 (m, 2H), 7.23–7.19 (m, 1H), 6.97

(d, J = 9.2 Hz, 1H), 6.91 (d, J = 8.8 Hz, 1H), 6.91 (d, J = 2.0 Hz, 1H), 6.65 (dd, J = 8.8, 2.4 Hz, 1H), 6.61 (d, J = 9.2 Hz, 1H), 4.52 (s, 2H), 3.60 (s, 2H) ppm; there are no signals of OH, –NH– and –COOH. ^{13}C NMR (MeOH-d_4, 150 MHz) δ = 172.6, 171.1, 156.04, 155.97, 135.4, 133.2, 132.6 (2C), 131.3 (2C), 130.8, 128.8, 128.3, 120.0, 117.3, 113.6, 113.3, 106.4 (2C), 104.0 (2C), 50.0, 41.6 ppm. ESI-MS: m/z 420.2 $[M + H]^+$.

Synthesis and characterization of probe 2

The procedure was similar to that of **1**, and the yield is 51% (262 mg). Mp: 230 °C (dec.). Two diastereoisomers exist in **2**, and the data for one of them is given below: ^{1}H NMR (MeOH-d_4, 400 MHz) δ = 8.02 (d, J = 7.6 Hz, 1H), 7.79 (t, J = 7.6 Hz, 1H), 7.71 (t, J = 7.6 Hz, 1H), 7.35 (d, J = 4.4 Hz, 2H), 7.32 (d, J = 4.4 Hz, 2H), 7.27 (dd, J = 9.2, 4.4 Hz, 1H), 7.20 (t, J = 8.8 Hz, 1H), 6.81 (s, 1H), 6.67 (dd, J = 8.8, 2.8 Hz, 1H), 6.60 (s, 1H), 6.58 (brs, 2H), 4.49 (d, J = 12.8 Hz, 1H), 4.44 (d, J = 12.8 Hz, 1H), 3.97 (dd, J = 8.8, 5.2 Hz, 1H), 3.42 (dd, J = 9.6, 5.2 Hz, 1H), 3.13 (dd, J = 8.4, 5.2 Hz, 1H) ppm; there are no signals of OH, –NH– and –COOH. ^{13}C NMR (MeOH-d_4, 150 MHz) δ = 173.3, 173.0, 157.0, 156.4, 137.4, 137.2, 132.6, 132.0, 131.9, 131.8, 130.6, 130.3 (2C), 130.1 (2C), 129.6, 129.2, 128.5, 121.8, 119.3, 119.0, 113.7 (2C), 106.2, 106.1, 104.1 (2C), 64.3, 42.4, 37.9 ppm. ESI-MS: m/z 510.3 $[M + H]^+$.

Acknowledgements

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