A turn-on PET fluorescence sensor for imaging Cu^{2+} in living cells†

Guangjie He,[a] Xiuwen Zhao,[a] Xiaolin Zhang,[a] Hongjun Fan,*[b] Shuo Wu,[a] Huaqiang Li,[a] Cheng He[a] and Chunying Duan*[a]

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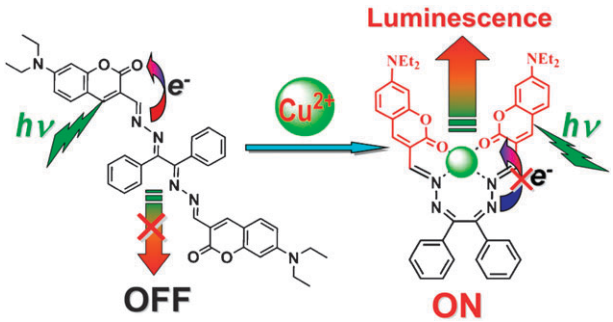
By the incorporation of coumarin fluorophores within the benzyldihydrazone moiety and by carefully adjusting the redox potential of the fluorophore and its excited state energy, one of the brightest Cu^{2+}-binding sensors in aqueous media was achieved and could be used for the detection of Cu^{2+} in living cells.

Copper is an essential trace element that acts as a cofactor for many fundamental biological processes in all currently known life forms, but alterations to its cellular homeostasis are connected with serious neurodegenerative diseases.[1,2] Highly sensitive imaging with copper-selective fluorescent sensors is therefore strongly demanded for visualizing labile copper with a sub-cellular resolution at the molecular level.[3,4] Since the divalent copper ion is a well-known efficient fluorescence quencher in most cases,[5] fluorescence enhancement for copper binding is fairly faint and therefore poses a significant design challenge.[6,7] Electronic decoupling combined with a rigid sensor architecture is provided, especially for typical quenchers, to achieve fluorescence enhancement.[8,9] The design of Cu^{2+}-selective PET sensors still requires careful adjustment of the redox potential of the fluorophore and its excited state energy fluorescence intensity,[10] because Cu^{2+} is also a redox-active species, besides the intrinsic quenching behavior of these paramagnetic heavy metal ions.

By incorporating coumarin fluorophores within the benzyl dihydrazone moiety, herein, we have developed a Cu-specific fluorescence sensor, **CB1** (Scheme 1). Its coordination conformation and high affinity for typical *N*,*O*-chelators may provide a platform to coordinate Cu^{2+} ions selectively.[11] Theoretical studies on the optical behavior of **CB1** reveal the frontier orbitals shown in Scheme 1.[12] The HOMO and HOMO−3 are the lone pair orbitals of the nitrogen atoms in the benzyl dihydrazone moiety, while HOMO−1, HOMO−2, LUMO and LUMO + 1 are conjugated π orbitals. The strongest absorption band of **CB1** was computed to be at 477 nm and assigned to a π → π* excitation with minor ET (15%) contributions. Several electron transfer (ET) states, characterized by ET from the nitrogen lone pair orbitals to the LUMO or LUMO + 1, lie below the LE state by 0.16–0.64 eV, suggesting a possible PET fluorescence quenching process.

The reaction of 7-diethylaminocoumarin-3-aldehyde with benzyl dihydrazone gave compound **CB1**. It displayed a typical absorption band of a coumarin chromophore in the visible region, centered at 468 nm (log ε = 4.64), consistent with calculations. As predicted by the calculations, **CB1** displayed a weak fluorescence band with a corresponding emission maximum at 534 nm (ϕ = 0.004) in an aqueous solution when excited at 468 nm. Upon adding Cu^{2+}, the fluorescence intensity of **CB1** increased by *ca.* 70-fold (ϕ = 0.30, Fig. 1). **CB1** thus was one of the brightest Cu^{2+}-binding sensors in aqueous media to date.[13] Since the Cu^{2+}-binding did not change the absorbance spectra significantly, the maintenance of the emission wavelength with the significant luminescence enhancement possibly suggested a PET mechanism.[14] Binding analysis using the method of continues variations (Job's plot) and the linear fitting of the luminescence titration curve established that a 1 : 1 Cu^{2+}-**CB1** complexation species was responsible for the observed fluorescence enhancement, and the association constant for Cu^{2+} binding to **CB1** was calculated as 3.67×10^6 M^{-1}.[15]

The ESI-MS spectrum of **CB1** in the presence of Cu^{2+} exhibited two main peaks at m/z = 377.65 and 854.24, assignable to $[Cu\text{-}\mathbf{CB1}]^{2+}$ (m/z_{calc} = 377.62) and $[(Cu\text{-}\mathbf{CB1})\text{-}(ClO_4)]^{+}$ (m/z_{calc} = 854.19), respectively, referring to the exact comparison of the intense peaks with the simulation on the basis of natural isotopic abundances. This is consistent with the formation of 1 : 1 stoichiometric host–guest complex species in solution. Titration of **CB1** with other metal ions in aqueous media at neutral pH revealed its excellent selectivity towards divalent copper (Fig. 1). Cu^{2+} was the only cation among the test metal ions to induce a fluorescence



Scheme 1 The chemical structure and proposed binding mode of **CB1** to Cu^{2+}, showing the possibility of blocking the PET process upon metal binding.

[a] *State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian, 116012, China. E-mail: cyduan@dlut.edu.cn*
[b] *State Key Laboratory of Molecular Reaction Dynamics, Dalian Institute of Chemical Physics, Dalian, 116023, China. E-mail: fanhj@dicp.ac.cn*

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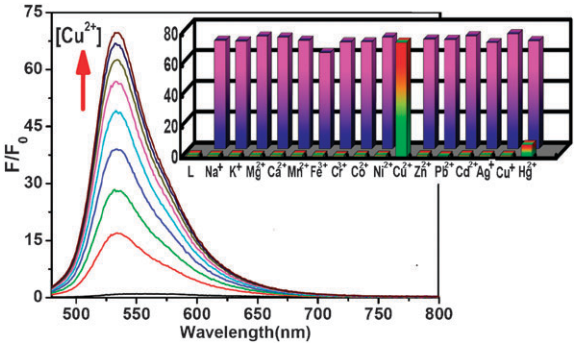

Fig. 1 The fluorescence response of compound **CB1** (20 μM) upon adding increasing concentrations of Cu^{2+} and various other metal ions (insert). Green and yellow bars represent the final integrated fluorescence response over the initial integrated emission in the presence of 0.20 mM of various metal ions. Violent bars represent the subsequent addition of 0.1 mM of Cu^{2+} to the solution. The excitation was at 468 nm. The emission was integrated at 534 nm in $CH_3CN:H_2O$ (1 : 1, v/v).

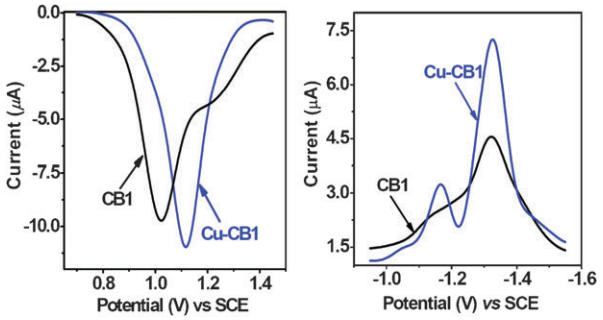

Fig. 2 DPV of **CB1** (0.5 mM) in acetonitrile solution and **CB1**(0.5 mM) in the presence of Cu^{2+} (0.5 mM) (named as Cu-**CB1**). Electrolyte 0.1 M $n$$Bu_4NPF_6$. The scan rate was +25 and −25 mV s^{-1} for the oxidization and reduction processes, respectively.

enhancement. The emission profiles of **CB1** or Cu^{2+}-binding **CB1** did not change in the presence of mmol amounts of alkali and alkaline earth metals, Fe^{3+} and Zn^{2+} (2 mM), indicating the excellent selectivity for Cu^{2+} over these abundant cellular cations. Other metal ions, including Mn^{2+}, Cr^{3+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Hg^{2+}, as well as Pb^{2+} and Ag^{+}, produced no discernible change in emission intensity for the **CB1** probe and did not interfere with its Cu^{2+} response. Especially notable was the observed selectivity for Cu^{2+} over Cu^{+}, rendering the probe potentially valuable for studying specific copper-mediated redox events in biological systems.

Interestingly, **CB1** (50 nM) featured a detection limit for Cu^{2+} of at least down to 7.81×10^{-9} M (0.5 ppb), with the emission increasing 23% fold directly when **CB1** was employed at 50 nM. This detection limit is low enough for Cu^{2+} detection in aqueous media and many biological systems. The addition of an aqueous solution of Na_2S (0.1 mM) to solutions of the **CB1**-Cu^{2+} species[16] diminished the fluorescence significantly to the initial value of the free probe, demonstrating the possible reversibility of the Cu^{2+} binding process. **CB1** also responded to Cu^{2+} in the pH range 5.5 to 8.5, with the fluorescent intensity varying by less than 10%, facilitating the detection of Cu^{2+} in aqueous media at physiological pH values. These results demonstrate that **CB1** is an excellent Cu^{2+} chemosensor with high sensitivity and selectivity, as well as favorable spectroscopic properties.

Different potential voltammetry (DPV) of **CB1** in acetonitrile solution exhibited an oxidized potential of the electron donor $E_{\frac{1}{2}}(D^+/D)$ and a reduction potential of the luminophore $E_{\frac{1}{2}}(A/A^-)$ of 1.04 and −1.34 V (*vs.* SCE), respectively (Fig. 2). The free energy, ΔG_{ET}, of the PET reaction was calculated (eqn (1)) as −0.12 eV,[17] based on a free energy change (ΔG^{0-0}) between the ground state and the vibrationally-related excited state of 2.5 eV.‡ Importantly, the Cu^{2+} binding caused an obvious increase (0.12 V) of $E_{\frac{1}{2}}(D^+/D)$ and a small decrease of $E_{\frac{1}{2}}(A/A^-)$ (0.01 V), respectively. The free energy, ΔG_{ET}, thus increases significantly, demonstrating the possibility of blocking the PET quenching process.

$$\Delta G_{ET} = E_{\frac{1}{2}}(D^+/D) - E_{\frac{1}{2}}(A/A^-) - \Delta G^{0-0} - w_p \qquad (1)$$

The optimized geometry of **CB1**-Cu^{2+} shows that the Cu^{2+} ion is coordinated by two nitrogen atoms of the benzyl dihydrazone and two oxygen atoms of the coumarin fluorophore. The nitrogen lone pair orbitals are strongly stabilized by the Cu^{2+} binding, while the energies of the ligand π orbitals only change slightly. As a result, the highest four occupied orbitals in **CB1**-Cu^{2+} are all ligand π orbitals, and the nitrogen lone pair orbitals are outside of the highest 20 occupied orbitals. The electron transfer from the nitrogen lone pair to the π orbitals becomes much more difficult, and the PET process in **CB1** is disabled. Furthermore, given the fact that the excited π electron (LUMO) and the singly-occupied metal d orbital (SOMO) are well separated, electron transfer between the two orbitals is expected to be kinetically hindered.

CB1 is well-suited to fluorescence imaging in living cells. As determined by laser scanning confocal microscopy imaging experiments (Fig. 3), staining HeLa cells with 5 μM **CB1** for 15 min at room temperature gave no intracellular fluorescence. After rinsing three times with PBS, the cells were supplemented with 5 μM Cu^{2+} for another 30 min, and an increase in intracellular fluorescence was observed. Bright field measurements after copper and **CB1** treatment confirmed that cells were viable throughout the imaging experiments. These results thus clearly indicate that **CB1** is cell-permeable and can respond to the luminescence changes in intracellular Cu^{2+} within living cells in high sensitivity (*ca.* 0.3 ppm). Control experiments without dye gave no background fluorescence, and the pre-treatment of dye-supplemented cells with Cu(I) salts (50 μM) for another 30 min at room temperature showed faint intracellular fluorescence. Nevertheless, the addition of oxide agent Ce(IV) (50 μM) to the dye-supplemented cells with Cu(I) salts caused an observed intracellular fluorescence, indicating the possibility of imaging the valence variation of copper in living cells.

In summary, we have presented a turn-on fluorescent probe for Cu^{2+}, **CB1**, by incorporating coumarin fluorophores within the benzyl dihydrazone moiety that is among the brightest Cu^{2+} binding sensors in aqueous media reported to date. Due to its excellent sensitivity, high selectivity, good water solubility and favorable spectroscopic properties, **CB1** could act as a efficient sensing probe for the detection of Cu^{2+} in living cells, over biologically-relevant metal ions, including Cu^{+}. The design strategy and remarkable photophysical properties of

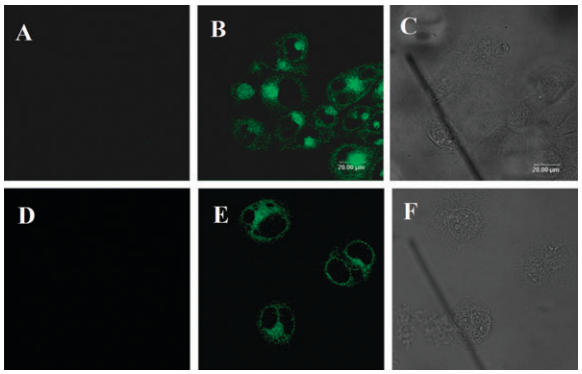

Fig. 3 Confocal fluorescence images of HeLa cells (λ_{ex} = 453 nm; Leica TCS-SP2 confocal fluorescence microscope, 20× objective lens). (A) Cells supplemented with 5 μM **CB1** for 15 min. (B) Cells supplemented with 5 μM **CB1** for 15 min and then incubated with 5 μM Cu^{2+} for 30 min. (C) A brightfield image of the cells in panel (B). (D) Cells supplemented with 5 μM **CB1** for 15 min and then incubated with 50 μM Cu^{+} for 30 min. (E) Cells supplemented with 5 μM **CB1** for 15 min, then incubated with 50 μM Cu^{+} for 30 min and final incubated with 50 μM Ce(IV) for 30 min. (F) A brightfield image of the cells in panel (E).

the probe would help to extend the development of fluorescent probes for sensing HTM ions in living systems.

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Experimental

Instruments and reagents

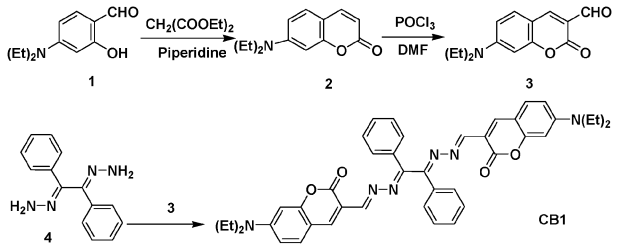
^{1}H and ^{13}C NMR spectra were measured on a Varian INOVA 400 M spectrometer. ESI mass spectra were recorded out on a HPLC-Q-Tof MS spectrometer using methanol as a mobile phase. UV-vis spectra were measured on a HP 8453 spectrometer. Solution fluorescence spectra were measured on an Edinburgh FS 920 instrument. 4-Diethylaminosalicylaldehyde and all cationic compounds were purchased from Acros.

General procedures for spectra acquisition

Stock solutions (2.0×10^{-5} M) of **CB1** were prepared in $CH_3CN:H_2O$ (1 : 1, v/v). The cationic solutions were all aqueous and had a concentration of 2.0×10^{-2} M for spectral analysis. Each time, a 2 mL solution of **CB1** was added to a quartz cell of 1 cm optical path length, and different stock solutions of the cations were gradually added to it using a micropipette. The volume of the cationic stock solution added was less than 100 μL, with the purpose of keeping the total volume of testing solution as unchanged as possible. The excitation wavelength was 468 nm.

Cell culture

The HeLa cells were grown in H-DMEM (Dulbecco's Modified Eagle's Medium, High Glucose) supplemented with 10% FBS (Fetal Bovine Serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. Cells (5×10^{8} L^{-1}) were plated on 18 mm glass cover slips and allowed to adhere for 24 h.



Scheme 2 The synthesis procedure of **CB1**.

Procedure for the synthesis of CB1

A methanol solution of 7-diethylaminocoumarin-3-aldehyde[18] (2.0 mmol, 0.49 g) and benzyl dihydrazone (0.24 g, 1 mmol) were mixed and refluxed for 4 h (Scheme 2). The red precipitate that formed was filtered, washed with methanol and dried under vacuum. Yield **CB1**: 73%. Anal calc. for $C_{42}H_{40}N_6O_4$: H 5.82, C 72.81, N 12.13. Found: H 5.93, C 72.70, N 12.01%. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.77 (s, 2 H), 8.08 (s, 2 H), 7.86 (d, 4 H, J = 8.0 Hz), 7.39 (m, 6 H), 7.27 (d, 2 H, J = 6.8 Hz), 6.55 (d, 2 H, J = 6.8 Hz), 6.42 (s, 2H), 3.41 (q, 8 H, J = 7.0 Hz), 1.20 (t, 12 H, J = 7.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 164.5, 161.7, 157.5, 155.9, 151.8, 141.3, 134.4, 131.0, 130.6, 128.7, 127.8, 113.3, 109.5, 108.9, 97.1, 45.0, 12.5.

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‡ The energy was estimated from the normalized absorption and emission spectra.

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