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A reversible and highly selective two-photon fluorescence “on-off-on” probe for biological Cu²⁺ detection

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Two-photon active probe for physiological copper (Cu²⁺) is expected to play an important role in the process of biological metabolism. Herein, a novel Schiff base derivative (E)-2,2'-((4-((4-(diethylamino)-2-hydroxybenzylidene) amino) phenyl) azanediy) bis(ethan-1-ol) (**L**) with remarkable two-photon activity was developed and synthetically investigated. **L** presents high selectivity and sensitivity for sensing Cu²⁺ in ethanol/HEPES buffer (v/v, 1:1), accompanying with the fluorescence switching “off” and subsequently “on” in the addition of EDTA. The mechanism for the detection of Cu²⁺ is further analyzed according to ¹HNMR titration, mass spectra and theoretical calculations. Furthermore, since the probe **L** possesses well photophysical properties, excellent biocompatibility and low cytotoxicity, it is successfully applied to tracking Cu²⁺ in the cellular endoplasmic reticulum by two-photon fluorescence imaging, exhibiting its potential value of practical applications in biological systems.

1. Introduction

Recently, development fluorescent chemosensors for transition metal ions has been attached considerable attentions due to their potential applications in chemical and biological processes¹⁻⁵. In human body, Cu²⁺, as the third most abundant transition metal ion, plays important roles in biological systems, and keeping its appropriate homeostasis for the growth, development and fitness of living organisms⁶⁻⁸. However, abnormal level of Cu²⁺ in the human body causes severe neurological diseases such as Parkinson's disease and Alzheimer's disease⁹⁻¹¹. Moreover, copper pollution is becoming more widespread attributed to the increasing dosage of Cu-containing

materials in industry and agriculture. Consequently, the detection of Cu²⁺ in environment and biological samples becomes essential.

So far, many Cu²⁺ fluorescent probes have attracted great concern because of their high sensitivity and selectivity¹²⁻²³. However, most of the designed probes are only available for one-photon fluorescence microscopy (1PM) with short excitation wavelengths and suffering from background interference. Thus, how to effectively track Cu²⁺ in biological systems with low tissue auto-fluorescence and photo-damage still remains challenge. As an alternative, two-photon microscopy (2PM), a powerful imaging tool for biomedical research, exhibits remarkable advantages over 1PM, including increased penetration ability, reduced photodamage and photobleaching²⁴. Recently, Tian's group²⁵ presented a reversible two-photon (2P) fluorescence probe (**HL**) for Cu²⁺ in HEPES buffer. In that work, the terminated quaternary ammonium salt moieties in the probe were supposed to be the main contributions to the mitochondria targeting ability of **HL**. To some extent, it means that the

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varied terminated moieties possibly lead to different specificity. Herein, a novel Schiff-base based fluorescent probe with hydroxyl moieties was then synthesized according to the literature²⁶, as well as the similar sensing behaviours towards Cu^{2+} were expected. 2,2'-((4-aminophenyl)azanediyl)diethanol was readily used to react with 4-(diethylamino)-2-hydroxybenzaldehyde to generate the proposed Schiff-base compound (**L**). The further studies demonstrated that **L** could selectively coordinate with Cu^{2+} with a distinct fluorescence quenching and subsequently a fluorescence recovering by the addition of EDTA. Furthermore, probe **L** exhibited suitable 2PA property, which could track Cu^{2+} in cellular endoplasmic reticulum with good photo-stability, offering a powerful tool for tracking Cu^{2+} in biological systems.

Scheme 1 The synthetic route for probe **L**.

2. Synthesis of **L**

A mixture of 2,2'-((4-aminophenyl)azanediyl)bis(ethanol-1-ol) (**M1**)²⁷ (0.39 g, 2 mmol) and 4-(diethylamino)-2-hydroxybenzaldehyde (**M2**) (0.40 g, 2 mmol) were dissolved in 50 mL dry ethanol, and then added two drops glacial acetic acid, refluxed for 12 h. The mixture was cooled to room temperature and filtered, washed twice with ethanol. The crude product was recrystallized with acetonitrile. Yield: 80 %. ¹H-NMR (d_6 -DMSO, 400 MHz, ppm) δ : 13.96 (s, 1H), 8.60 (s, 1H), 7.23 (t, $J = 14.1$ Hz, 1H), 7.18 (d, $J = 8.4$ Hz, 2H), 6.71 (d, $J = 8.4$ Hz, 2H), 6.28 (t, $J = 13.4$ Hz, 1H), 6.04 (s, 1H), 4.74 (s, 2H), 3.55 (d, $J = 3.9$ Hz, 4H), 3.42 (q, $J = 8.0$ Hz, 4H), 3.36 (t, $J = 7.5$ Hz, 4H), 1.11 (t, $J = 6.8$ Hz, 6H). ¹³C-NMR (d_6 -DMSO, 100 MHz, ppm) δ : 162.8, 156.8, 150.6, 146.4, 136.1, 133.2, 121.5, 111.7, 108.8, 103.3, 97.0, 58.1, 53.3, 43.8,

12.53. IR (cm^{-1}): 3177, 2968, 2924, 1613, 1519, 1432, 1357, 1303, 1240, 1130, 1065, 1004, 964, 921, 893, 825, 786, 677, 520. ESI-MS: 372.23 [$\text{M}+1$]⁺.

Figure 1 Crystal structure of **L**, all H atoms were omitted for clarity.

3 Results and discussion

3.1 The synthesis of the probe **L**

The probe **L** was synthesized by the simple and convenient process as displayed in Scheme 1. **M1** was synthesized according to the reported literature²⁷. Then, **M1** was reacted with 4-(diethylamino)-2-hydroxybenzaldehyde to give the probe **L** in ethanol. The probe **L** was characterized using ¹H NMR, ¹³C NMR spectroscopy and mass spectrometry. The molecular structure of probe **L** was also confirmed by single-crystal X-ray diffraction analysis. The crystal data, selected bond lengths and angles were summarized in Table S1 and S2. As shown in Figure 1, the dihedral angle between the two benzene rings is 16.08°, which is linked by C12-N2 (1.417 Å), N2-C11 (1.282 Å) and C11-C8 (1.440 Å) with conjugated bond length. The planarity and the conjugated geometric configuration suggest that the probe **L** possesses highly π -electron delocalized system.

3.2 Recognition of probe **L** toward various metal ions

The UV-vis absorption and one-photon fluorescence spectra of probe **L** towards various metal ions (Ag^+ , Al^{3+} , K^+ , Ba^{2+} , Bi^{3+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cr^{3+} , Fe^{3+} , Hg^{2+} , Zn^{2+} , Li^{2+} , Mg^{2+} , Cu^{2+} , Mn^{2+} , Na^+ , Pb^{2+} , Ni^{2+} , Cu^+) in ethanol/HEPES buffer (v/v, 1:1) were depicted in Figure 2. As displayed in Figure 2a, the UV-vis absorption

spectra of **L** (12.5 μM) exhibited one absorption band at 440 nm. When adding the above metal ions (4 equiv.) respectively, only Cu^{2+} gave a remarkable response with a new absorption peak appearing at 390 nm, which demonstrated the selective binding affinity of **L** toward Cu^{2+} . In addition, strong spectral response of **L** towards Cu^{2+} was also determined in the fluorescence titration experiments. **L** displayed 8.5-fold decrease of fluorescence intensity at 528 nm, whereas no obvious spectral changes in the presence of other metal ions (**Figure 2b**). The reason can be explained by the fact that Cu^{2+} has quenching capabilities due to its unfilled d shell and paramagnetic nature especially in aqueous media. Due to the unfilled d orbital of Cu^{2+} , after being chelated by compound **L**, the d-d transitions of the **L**-Cu assemble would be largely prohibited with the lowest energy, leading to luminescence quenching²⁸.

Figure 2 UV-vis absorption (a) and one-photon fluorescence (b) spectra of **L** (12.5 μM) in the presence of (4 equiv.) different metal ions in ethanol/HEPES buffer (pH=7.42) (v/v, 1:1).

Anti-interference experiment was then carried out to investigate the interferences of different metal ions on the Cu^{2+} recognition of **L**. As shown in **Figure S1**, the competitive metal ions failed to cause remarkable fluorescence quenching of **L** except the subsequent addition of Cu^{2+} . Taken together, these commonly coexisting metal ions do not interfere with “on-off” fluorescent response of **L** for detecting Cu^{2+} .

Figure 3 UV-vis absorption spectra (a) and fluorescence spectra of **L** (12.5 μM) upon the addition of Cu^{2+} (0 to 10 equiv.) in ethanol/HEPES buffer (pH=7.42) (v/v, 1:1). (c) Job's plot of **L** with Cu^{2+} according to the method of continuous variation. $[\text{L}] + [\text{Cu}^{2+}] = 20 \mu\text{M}$. (d) Two-photon action cross-section of **L** in DMSO solution.

To evaluate the sensitivity of probe **L** towards Cu^{2+} in ethanol/HEPES buffer (v/v, 1:1), the titration experiments were performed *via* UV-vis absorption and fluorescence spectra. As depicted in **Figure 3a**, probe **L** exhibited a main absorption band at 440 nm. With the increasing concentrations of Cu^{2+} , the absorbance band at 440 nm gradually decreased accompanying with a blue shift. Meanwhile, two new absorption bands appeared at 390 nm and 480 nm, demonstrating a new complex was formed between probe **L** and Cu^{2+} . As expected, the fluorescence intensity of the emission band at 528 nm decreased continually along with 0-10.0 equiv of Cu^{2+} (**Figure 3b**). Further, the reversibility of copper complexation with probe **L** was confirmed by EDTA titration. As shown in **Figure S2**, the UV-vis absorbance and fluorescence spectra of **L** could be largely restored upon the addition of EDTA. The results demonstrated that the binding of probe **L** and Cu^{2+} was chemically reversible.

To further investigate the binding mode, Job's plot and Benesi-Hildebrand plot analysis were performed (**Figure 3c**, **S3** and **S4**). The results indicated that probe **L** binds to Cu^{2+} in a 2:1 stoichiometry. The binding constant of probe **L** and Cu^{2+} was calculated to be $8.5 \times 10^4 \text{ M}^{-1}$ according to

the Benesi-Hilderbrand equation. In addition, the limit of detection (LOD) of **L** towards Cu^{2+} was calculated to be $1.78 \mu\text{M}$ ²⁹. As shown in **Figure S5**, the response time of **L** to Cu^{2+} was also investigated. The results indicated that probe **L** possesses fast response to Cu^{2+} in ethanol/HEPES buffer (v/v, 1:1).

Before further exploring its biological application, the two-photon action cross-section ($\Phi\sigma$) of probe **L** was investigated. As manifested in **Figure 3d**, the maximum $\Phi\sigma$ of **L** was 29 GM at 700 nm. As shown in **Figure S6**, two-photon excited fluorescence spectra exhibited logarithmic plots of the fluorescence integral *versus* input power with a slope of 2.09 as the input laser power increased, indicating a two-photon absorption property and **L** is suitable for detecting Cu^{2+} by using two-photon microscopy technique.

Figure 4 ^1H NMR spectra of **L** (0.01 M) upon titration with 0 equiv, 0.2 equiv, 0.3 equiv, 0.4 equiv, 0.5 equiv, 1 equiv of $\text{Cu}(\text{NO}_3)_2$ (0.1 M) in d_6 -acetone; a proposed 2:1 complex formed between **L** and Cu^{2+} .

3.3 Mechanism studies

To verify the above sensing mechanism, ^1H NMR titration spectra were carried out^{25, 30-31}. As we know, copper (II) almost universally causes peaks to disappear in ^1H NMR spectra upon complex formation due to its paramagnetic nature. As displayed in **Figure 4**, the $-\text{OH}$ proton (Hb) signal and the aromatic ring proton (Hc)

gradually decreased, simultaneously small changes were observed that the $-\text{CH}=\text{N}-$ proton (Ha) signal at 8.60 ppm with the addition of Cu^{2+} . When the quantity of Cu^{2+} increased to 0.5 equiv, the signal of Hc almost disappeared, the changes of which manifested that the N atom of $-\text{CH}=\text{N}-$ bond and the O atom of the phenolic hydroxyl may coordinate with the center Cu^{2+} by a 2:1 binding model, which was further confirmed by the mass spectra. As shown in **Figure S7**, after the addition of Cu^{2+} to the solution, a new peak at $m/z = 800.77$ corresponding to $[\text{LCu-3H}]^+$ appeared. Taken together, we proposed a plausible binding mode of probe **L** responses to Cu^{2+} (**Figure 4**).

To gain an insight into the photophysical property of **L** and **LCu**, TD-DFT calculations were carried out by Gaussian 09 program. The frontier molecular orbitals distributions and the calculated data were listed in **Figure S7** and **Table S3**. As shown in **Figure S8**, for free probe **L**, the HOMO-1 and LUMO is mainly localized on the whole molecule, respectively, which indicated that the HOMO-1 $\rightarrow$ LUMO transition can be assigned as a π - π^* transition. Upon the addition of Cu^{2+} , two new absorption bands at 390 nm and 480 nm can be observed. For **LCu**, the distribution of HOMO-5 is mainly located on the 4-(Diethylamino)salicylaldehyde moiety, and C=N group. The HOMO-1 is primarily located on the one of free ligand with a small distribution on the copper (II) center. The LUMO+1 and LUMO+2 largely occupy the two different ligands. The lowest energy band calculated at 484 nm, which mainly originates from HOMO-1 $\rightarrow$ LUMO+2 transitions, which can be assigned to the LLCT (ligand-to-ligand charge transfer) mixed with MLCT (metal-to-ligand charge transfer). The transition at 394 nm corresponds to the HOMO-5 $\rightarrow$ LUMO+1, which can be assigned to the ICT (intramolecular charge transfer) mixed with LLCT. Accordingly, a turn-off fluorescent probe of sensing Cu^{2+} based on Schiff base derivative has been realized.

3.4 Biological application

To explore the potential biological application of probe **L**, the biocompatibility is essential to evaluate and the MTT assay was used to investigate its cytotoxicity. **Figure S9** showed that after over 24 h of treated with probe **L**, high cell viability was observed at the concentration from 5 μM to 60 μM . The MTT assay results demonstrated that probe **L** exhibited low toxicity against living HepG2 cells and good biocompatibility, which can be served as a potential probe for living cell imaging.

As depicted in **Figure 5**, after incubation with probe **L** (10 μM) for 30 min, clear intracellular fluorescence in cytoplasm was observed in living cell imaging by confocal microscopy. However, the bright green fluorescence of live cells was disappeared when Cu^{2+} (20 μM) were added into the incubation solution. Then upon the addition of EDTA (20 μM) for 30 min, the fluorescence within cells was restored under the same optical window. These facts were in accordance with the sensing results obtained in the solution. Therefore, probe **L** could serve as an intracellular probe for detecting Cu^{2+} in living cells.

Figure 5 Confocal fluorescence images of HepG2 cells treated with 10 μM **L** for 30 min (a). (b) **L** treated HepG2 cells after 30 min labeled with 10 μM $\text{Cu}(\text{NO}_3)_2$ water solution. (c) **LCu** treated HepG2 cells after 30 min labeled with 20 μM EDTA water solution. (d) The relative fluorescence intensities of (a)-(c). Bar = 20 μm

To demonstrate the specific localization of probe **L** in living cells, a colocalization experiments were performed. ER tracker green dye and Hoechst 33342 were used to co-stain HepG2 cells. As depicted in **Figure 6**, The colocalization imaging experiment unambiguously proved that probe **L** specifically localize in the endoplasmic reticulum with Pearson's coefficients $R_r = 0.93$, but no overlapping was observed with Hoechst 33342. It is noteworthy that endoplasmic reticulum is membrane-rich organelle, as probe **L** is lipophilic molecules because **L** has

N,N-azanediyldiethanol and N,N-diethylamine moiety, so we speculated that probe **L** could associate with lipophilic region within the cell. These result indicated that probe **L** was capable of targeting endoplasmic reticulum in living cells.

Figure 6 Determination of intracellular localization of **L** by confocal microscopy. HepG2 cells were treated with **L** (10 μM) ($\lambda_{\text{ex}} = 700 \text{ nm}$, $\lambda_{\text{em}} = 520\text{--}560 \text{ nm}$) for 30 min and then co-incubated with Hoechst 33342 and ER tracker for 30 min at 37 $^\circ\text{C}$, respectively. Bar = 20 μm .

The cellular uptake mechanism of probe **L** was also studied. As shown in **Figure 7**, HepG2 cells were incubated with probe **L** at 4 $^\circ\text{C}$, the fluorescence can be still observed, revealing that probe **L** enter cells *via* a temperature-independent pathway. Pre-treatment of HepG2 cells with endocytosis inhibitors (chloroquine and NH_4Cl) exhibit no effect on the uptake of probe **L**. The results revealed that probe **L** is membrane permeable and penetrates into the cells *via* a temperature-independent pathway, possibly passive infusion.

Considering an ideal cellular probe should operate in a certain pH range for biological applications. The effect of pH ranging from 4.0 to 10.0 on the one-photon fluorescence of probe **L** was investigated in HEPES buffer. **Figure S10** displayed that the probe **L** was insensitive to pH in the range of 6.8–8.3, indicating that **L** is pH-independent in typical physiological environments (6.8–7.4). As shown in **Figure S11**, the photostability of **L** was further investigated, and ER tracker was included as the

control. During ~300 s, the relative fluorescence intensity of probe **L** remained 80%, whereas only 40% fluorescence intensity of ER tracker was observed. The higher photostability of **L** makes it favorable for long-term real-time tracking.

Figure 7 Effect of incubation temperature (37 °C and 4 °C), chloroquine (50 μM) and NH_4Cl (50 μM) on cellular uptake of **L** (10 μM) measured by confocal microscopy. Scale bar = 20 μm

4. Conclusion

In summary, a novel fluorescent probe **L** with high selectivity and sensitivity for sensing Cu^{2+} in an “on-off-on” fluorescent switch was fabricated by using a simple one-step reaction. The mechanism has been investigated both experimentally and theoretically. Importantly, probe **L** exhibits low cytotoxicity, good photo-stability, and specificity to localize in endoplasmic reticulum, indicating probe **L** could serve as a tool to exploit potential functions of Cu^{2+} at subcellular levels. Therefore, we hope that this probe could play a crucial part in physiology and pathology science in the future research.

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