Electronic Supplementary Information (ESI)

A Near-Infrared Fluorescent Probe for Detecting Copper (II) with High Selectivity and Sensitivity and Its Biological Imaging Applications

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1. Apparatus and Materials

1. Apparatus:

Fluorescence spectra were obtained with FLS-920 Edinburgh Fluorescence Spectrometer (Edinburgh, England) with a Xenon lamp and 1.0 cm × 1.0 cm quartz cuvettes. Absorption spectra were measured on a pharmaspect UV-1700 UV-Visible spectrophotometer (Shimadzu, Japan). Infra-red spectra were obtained from a PE-983G IR-spectrophotometer (Perkin-Elmer). Determination of organic elements was performed with Model element analyzer (PE-2400(II), Perkin-Elmer, California, CA). ¹H NMR, ¹³C NMR spectra were taken on a Bruker 600 MHz spectrometer. Molecular weight was gotten from UPLC-LTQ orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The kinetics experiment was obtained from Varian Cary Eclips spectrofluorometer (Australian) with a Xenon lamp at room temperature. All pH measurements were taken with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, China) with a combined glass-calomel electrode.

2. Materials:

2-[4-Chloro-7-(1-ethyl-3,3-dimethyl(indolin-2-ylidene)-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl)-1-ethyl-3,3-dimethyl-3H-indolium (Cy.7.Cl) was prepared in our laboratory. Diethyl iminodiacetate and TPEN were purchased from Sigma-Aldrich. Lyso-Tracker DND-26 was from molecular probes company. All other chemicals were from commercial sources and of analytical reagent grade, unless indicated otherwise. Ultrapure water (18.2 MΩ ·cm, Sartorious, Germany) was used throughout.

2. Synthesis and Characterization of probe

![Chemical structure of probe](image)

2-[bis(2-cthio-2xioethyl)amino]-3-[2-(1-ethyl-3,3-dimethylidolin-2-yldiene)cyclohex-1-enyl|vinyl-1-ethyl-3,3-dim ethyl-3H-indolium(2) Ethyl-tricarbocyanine 1 (638 mg, 1.0 mmol) was dissolved in anhydrous DMF (15 mL) in a round bottom flask, and then diethyl iminodiacetate (1.89g, 10 mmol) was added dropwise. The mixture was stirred at 95°C under argon for 72 hours with progressively monitored by TLC, then cooled to room temperature and added into ether (300 mL) with violent stirring. The obtained blue solid was filtered and dried under vacuum, then purified on silica gel chromatography eluted with ethyl acetate-methanol-hexane 2:1:1. GC-MS (API-ES): m/z Calcd 664.2, found 664.4 [M⁺]. IR (KBr, cm⁻¹): ν = 1723.64 (COO⁻). Elemental Analysis Calcd: C, 63.7; H, 6.9; I, 16.0; N, 5.3; O, 8.1; Found: C, 63.6; H, 7.0; I, 15.9; N, 5.4; O, 8.1.
2-[bis(2-hydroxyamino-2-oxoethyl)amino]-3-[2-(1-ethyl-3,3-dimethylindolin-2-ylidene)cyclohex-1-enylvinyl]-1-ethyl-3,3-dimethyl-3H-indolium (Cy-Cu)

Hydroxylamine hydrochloride (690 mg, 10 mmol) was dissolved in 10 mL water, then 3 mL of 5 M sodium hydroxide was carefully added with cooling of ice bath. Then compound 2 (664 mg, 1.0 mmol) dissolved in 10 mL ethanol was dropped, and the mixture was stirred at 35°C for 12 hours. After that, the mixture was neutralized to pH ≈ 6.0 with hydrochloric acid, extracted with dichloromethane and evaporated under vacuum. The solid was purified on silica gel chromatography with ethyl acetate-methanol-hexane 1:1:1 as the eluent, and then recrystallized in ethanol.

1H NMR (CDCl3, 600 MHz): δ = 1.44-1.49 (t, 6H, CH3), 1.56 (t, 2H, CH2), 1.72 (s, 12H, CH3), 2.00 (d, 2H, OH), 2.76 (t, 4H, CH2), 3.85 (s, 4H, CH2), 4.22-4.24 (q, 4H, CH2), 6.19-6.24 (d, 2H, CH), 6.47-6.51 (d, 2H, CH), 7.14-7.40 (m, 6H, CH), 8.09 (s, 2H, NH), 8.33-8.38 (d, 2H, CH). 13C NMR (CDCl3, 150 MHz): δ = 172.8, 172.6, 169.9, 145.8, 143.7, 143.6, 142.9, 142.1, 141.1, 128.7, 126.9, 126.7, 126.6, 122.3, 120.3, 115.6, 110.9, 102.1, 55.6, 53.6, 49.5, 49.0, 33.6, 27.7, 23.0, 21.3, 14.6. GC-MS (API-ES): m/z Calcd 638.4, found 638.7 [M]+. Elemental Analysis Calcd: C, 59.6; H, 6.3; I, 16.6; N, 9.1; O, 8.4; Found: C, 59.4; H, 6.5; I, 16.5; N, 9.1; O, 8.5.


Ultrapure water was used to prepare all aqueous solutions. The probe showed the maximum excitation at 745 nm and emission at 800 nm. The excitation and emission slits were both set to 5.0 nm. Absorption spectra were recorded at room temperature with 1.0 cm quartz cells. The probe was diluted to 5.0 μM with 1% DMSO as cosolvent, 10 mM HEPES buffers at pH 6.85. Fluorescence quantum yields were determined by reference to Rhodamine B in methanol (Φ = 0.69). The apparent dissociation constant (Kd) was determined from a plot of normalized fluorescence response versus [Cu²⁺]. The data were fitted to the following equation: F = (Fmax[Cu²⁺] + Fmin/Kd)/(Kd + [Cu²⁺])² where F is the observed fluorescence, Fmax is the fluorescence for the Cu²⁺ complex, and Fmin is the fluorescence for the free Cy-Cu dye. All other metal ions tested for metal ion selectivity studies with the exception of Fe²⁺ were from their chloride salts as aqueous solutions. Ammonium ferrous sulfate hexahydrate was used as a source of Fe²⁺. This salt was dissolved in degassed water and was quickly added to a degassed aqueous solution of probe.

4. Biological Treatments

(1) Cell Culture. Human hepatocellular liver carcinoma cell line (HepG2) and Raw264.7 macrophages were maintained following protocols provided by the American Type Tissue Culture Collection. Cells were seeded at a density of 1×10⁶ cells·mL⁻¹ for confocal imaging in high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), NaHCO₃ (2 g/L) and 1 % antibiotics (penicillin /streptomycin,100 U/ml). Cultures were maintained at 37°C under a humidified atmosphere containing 5% CO₂.

(2) Confocal Imaging. The cell, tissue and zebrafish imaging were carried on a TCS SPE confocal laser-scanning microscope (Leica). The excitation wavelength was 633 nm, the collection window is 750-800 nm. The medium was removed and cells were washed with HEPES (0.1 M, pH=7.4) for three times.

(3) Preparation of Rat Hippocampal Slices. Slices were prepared from hippocampus of rat (100-120g). The slices were cut into 100 μm-thick with a ice-frozen slicer (Slee Cryostat). The slices were incubated in artificial cerebrospinal fluid (ACSF) at 37 °C. Some slices were treated with 20 μM Cy-Cu for 2 hours in HEPES buffer solution, others were pretreated with 60 μM Cu²⁺ for an hour followed by incubating with 20 μM Cy-Cu for 2 hours. Slices were washed three times with HEPES and removed on cover-slips.
(4) Preparation of zebrafish. Zebrafish were bred at optimal conditions in sea salt water at 28 °C. The zebrafish were fed with CuCl₂ (10 µM) for an hour, then washed with sea salt water and incubated with Cy-Cu (10 µM) for another hour at 28 °C. Before imaging, the fish were washed three times with sea salt water.

5. Supplementary spectral figure

**Fig. S1** Fluorescence spectra of the probe (5.0 µM). Red lines: probe alone; black lines: probe with 5.0 µM Cu²⁺ (1% DMSO as cosolvent, HEPES pH=6.85). Insert: absorption spectrum of the probe.

**Fig. S2** Effect of pH on the fluorescence intensity of the probe (5.0 µM) with and without Cu²⁺. Black line: without Cu²⁺; Red line: with 5.0 µM Cu²⁺.
**Fig. S3** Effect of the buffer solution concentration on the fluorescence intensity. (HEPES buffer, pH=6.85, 5.0 µM probe, 5.0 µM Cu$^{2+}$)

**Fig. S4** Effect of the probe concentration on the fluorescence intensity. (10 mM HEPES buffer, pH=6.85, 5.0 µM Cu$^{2+}$)

**Fig. S5** Fluorescence intensity at 790 nm versus different pH (pH is adjusted by HCl and NaOH, pH= 0.77, 1.1, 1.45, 1.85, 2.15, 2.35, 2.55, 2.75, 2.95, 3.28, 3.73, 4.11, 4.53, 5.03, 5.23, 5.81, 6.27, 6.66, 7.09, 7.49, 7.91, 8.31, 10, 11.5). The concentration of the probe was 5.0 µM.
**Fig. S6** The pH response to different concentrations of Cu$^{2+}$. (10 mM HEPES buffer, pH=6.85)

**Fig. S7** The saturation curve between the relative fluorescence intensity and the concentrations of Cu$^{2+}$. Insert: a linear correlation ($\lambda_{ex}=745$ nm, 5.0 µM probe, 1% DMSO as cosolvent, 10 mM HEPES buffer, pH = 6.85).

**Fig. S8** Normalized fluorescence response of 5.0 µM probe to Cu$^{2+}$ for $K_d$ value determination at pH=7.0. The points shown are for Cu$^{2+}$ added at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7.5, 10, 15 and 30 µM, respectively. The observed $K_d$ value is $2.7 \pm 0.3$ µM.
**Fig. S9** The disassociation constant ($K_d$) of the complex Cu$^{2+}$ at different pH.

![Fig S9](image)

**Fig. S10** Reversibility of Cu$^{2+}$ binding to the probe upon addition of N, N, N', N'-9-tetra (2-picoly) ethylenediamine (TPEN) with excitation at 745 nm. (a): only probe; (b): fluorescence increase upon addition of 1 equiv Cu$^{2+}$; (c): decrease in fluorescence intensity resulting from addition of 1 equiv TPEN. ($\lambda_{ex} = 745$ nm, 1% DMSO as cosolvent, 10 mM HEPES buffer, pH = 6.85).

![Fig S10](image)

**Fig. S11** (a) Time course for the fluorescence intensity of probe. (b) Time course for the fluorescence intensity of probe response to Cu$^{2+}$. (c) Time course for the fluorescence intensity when TPEN was added to (b) solution.

![Fig S11](image)
To determine the stoichiometry of the Cy-Cu / Cu²⁺ complex, Job’s method was employed. The inflection point was at 0.5, which testified that the probe formed a 1:1 stoichiometry complex with Cu²⁺ (Fig. S12).

**Fig. S12** Job plot of the complexation between the probe and Cu²⁺ (1% DMSO as cosolvent, 10 mM HEPES, pH = 6.85).

**Fig. S13** Confocal fluorescence images of live Raw264.7 macrophages. (a) The cells incubated with 10 µM probe for 10 min at 37 °C. (b) The cells were pretreated with 30 µM Cu²⁺ for 20 min at 37 °C, then were incubated with 10 µM Cy-Cu for 10 min. (c) Bright field image of live Raw264.7 macrophages shown in panel (b), confirming their viability. (d) probe-supplemented cells pretreated with Cu²⁺, then treatment with TPEN. (e) Cells incubated with 30 µM Cu²⁺ for 20 min at 37 °C, then 10µM probe for 10 min. (f) Addition of 50 nM Lyso-Tracker DND-26 for 10 min. (g) Overlay of (e) and (f).
MS of 2

IR of 2
$^{1}$H NMR of Cy-Cu

![NMR Spectrum](image1)

$^{13}$C NMR of Cy-Cu

![NMR Spectrum](image2)
6. References: