Electronic Supplementary Material (ESI) for Analyst.

Electronic Supporting Information

Fluorescence switch for sequentially and selectively sensing copper (II) and L-histidine in vitro and in living cells

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1. Supplementary Methods

General Methods

All the starting materials were purchased from Adamas or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were reagent grade or better. HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and an in-line diode array UV detector using an Agilent Zorbax 300SB - C18 RP column or an Extend - C18 RP column with CH_3OH (0.1% of TFA) or CH_3CN (0.1% of TFA) and ultrapure water (0.1% of TFA) as the eluent. ^1H NMR and ^{13}C NMR spectra was recorded on a Bruker AVANCE AV400 (400 MHz) using tetramethylsilane as an internal reference, and chemical shifts (δ) was expressed in ppm. MALDI-TOF/TOF and ESI mass spectra were obtained on a time-of-flight Ultrflex II mass spectrometer (Bruker Daltonics) and on a Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFisher Corporation) equipped with a standard ESI source, respectively. Fluorescence microscopic images were taken on the IX71 fluorescence microscope (Olympus, Japan). UV-vis spectra were collected on the Cary 300 UV-Vis spectrophotometer.

Fluorescence Test

Fluorescence emissions were recorded on an F-7000 fluorescence spectrophotometer (Hitachi High-Techonologies Corporation, Japan) with excitation and emission wavelengths set to 465 nm and 521 nm, respectively.

Cell Culture

The hepatocellular carcinoma HepG2 cells were obtained from Department of Biochemistry and Molecular Biology, Anhui Medical University. HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Thermo Fisher Scienfitic, United States) supplemented with 10% fetal bovine serum (FBS) (Sijiqing Biological Engineering Materials, Hangzhou, China), streptomycin (100 μg/mL) and 5% CO_2 in humid atmosphere at 37°C. The images were on the IX71 fluorescence microscope (Olympus, Japan).

Cell Imaging

The hepatocellular carcinoma HepG2 cells were seeded into a 12 well plate and incubated at 37 °C in a CO_2 incubator for one day. Then the HepG2 cells were washed three times with phosphate buffered saline (PBS, pH 7.4) and incubated with 10 μM probe **1** in DMEM at 37 °C for 1 h in a CO_2 incubator. The cells were again washed twice with PBS to remove the free **1**. Then incubated in DMEM with 0 μM, 5 μM, 10 μM, 20 μM $CuCl_2$•H_2O for 20 min prior to imaging. HepG2 cells were treated free FITC at a same condition, respectively.

The application based on the complex of **1-Cu^{2+}** for live cell imaging was preliminarily demonstrated. After the HepG2 cells treated with 10 μM probe **1** for one hour at 37 °C, the cells were washed with PBS for three times and then added with 20 μM Cu^{2+} and upon addition various concentrations of histidine (50 μM, 100.0 μM, and 200.0 μM) to the cells for 20 min at 37 °C. Then, the cells were taken out for fluorescence or differential interference contrast (DIC) imaging. All the imaging experiments at each step were repeated twice with each concentration in triplicate.

Cell Uptake

*Calibration of probe **1** in cell lysate*: A volume of 4 mL radioimmuno precipitaton assay (RIPA) buffer containing 1% MDSO (v/v) of HepG-2 cell lysate (2×10^7 cells) was transferred to an ultrafiltration centrifuge tube (10 kDa), and the large biomolecules were separated from the cell lysate by centrifugation at 15, 000 rpm for 10 min at RT. For each measurement made, 90 μL of cell lysate supernatant was added to different concentration of probe **1** in 10 μL RIPA buffer to give the final concentration of probe **1** at 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, and 6.0 μM. After that, the fluorescence spectra of probe **1** at different concentration were measured and a linear fitting (Y = 16.38788+549.22399X, R^2 = 0.997) of the fluorescence intensity at 521 nm with the concentration of probe **1** was obtained. Each experiment was performed three times (n = 3) (Fig. S17).

*Cellular uptake of probe **1***. After incubation of healthy HepG2 cells (1×10^7 cells) with 10 μM **1** in serum-free medium for 1 h, the cells were trypsinized, incubated with 2 mL RIPA buffer, and the large biomolecules were separated from the cell lysate by centrifugation at 15, 000 rpm for 10 min at RT. The supernatant was applied to fluorescence spectrum measurement. After comparing the fluorescence intensity with the calibration fitting curve above, we determined the concentration of probe **1** in 2 mL HepG2 cell lysate to be 17.2 nM (or 0.00345 fmol/cell).

Fluorescence binding constant assay

The binding constant (K_s) assay study of probe **1** with Cu^{2+} was calculated according to calibration curve in Fig. S9. The nonlinear regression equation of fluorescent binding constant of probe **1** with Cu^{2+} was obtained with the equation below:

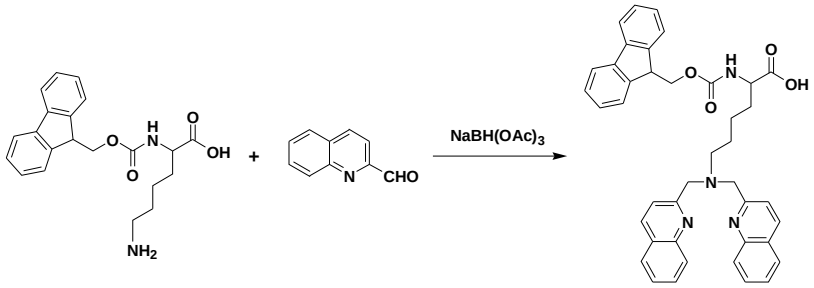
$$Y = Y_0 + \frac{Y_{lim} - Y_0}{2}\left\{1 + \frac{c_M}{c_L} + \frac{1}{K_s c_L} - \left[\left(1 + \frac{c_M}{c_L} + \frac{1}{K_s c_L}\right)^2 - 4\frac{c_M}{c_L}\right]^{1/2}\right\} \qquad (1.1)$$

where Y_0 is the original fluorescence intensity of probe **1** in the absence of Cu^{2+}. Y_{lim} is the limit value of the change in fluorescence. C_L is the concentration of probe **1**. C_M is the concentration of Cu^{2+} added.

2. Syntheses and Characterizations of Fmoc-SAACQ and 1

Preparation of compound Fmoc-SAACQ:

Scheme S1. The synthetic route for compound Fmoc-SAACQ.



Synthesis of Fmoc-SAACQ: To a solution of N-α-Fmoc-L-Lysine (2 g, 5.4 mmol) in 1,2-dichloroethane (20 mL), quinoline-2-aldehyde (1.79 g, 11.34 mmol) in 1,2– dichloroethane (10 mL) was added. After being stirred at room temperature (RT) for 2 hours, the reaction mixture was cooled to 0 ºC and sodium triacetoxy borohydride (2.63 g, 12.42 mmol) was added slowly into the solution in portions. After 2 hours at RT, water was added and the mixture was extracted with dichloromethane (30 mL× 3). The organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated under vacuum. The residue was subjected to chromatographic purification using MeOH-CH_2Cl_2 (15:85) as the eluent (3.03 g, 86 %). MS: calc. M^+= 650.29, obsvd. ESI-MS: 651.38 [$(M+H)^+$]. ^{1}H NMR (400 MHz, d_6-DMSO) δ (ppm) 12.63 (s, 1 H), 8.44-8.42 (d, 2 H), 8.02-8.00 (d, 4 H), 7.88-7.86 (d, 2 H), 7.81-7.77 (m, 2 H), 7.71-7.61 (m, 7 H), 7.41-7.37 (t, 2 H), 7.31- 7.27 (t, 2 H), 4.74 (s, 4 H), 4.30-4.26 (m, 2 H), 4.23-4.18 (m, 1 H), 4.00-3.94 (m, 1 H), 3.18 (s, 2 H), 1.83 (s, 2 H), 1.75-1.70 (m, 1 H), 1.65-1.61 (m, 1 H), 1.43-1.39 (m, 1 H). ^{13}C NMR (101 MHz, d_6-DMSO) δ (ppm) 173.69, 156.14, 151.87, 146.29, 143.80, 143.72, 140.69, 137.68, 130.39, 128.22, 128.05, 127.57, 126.98, 125.18, 121.17, 120.03, 65.51, 57.56, 54.89, 53.55, 46.66, 30.22, 23.12, 22.74 (Fig. S1).

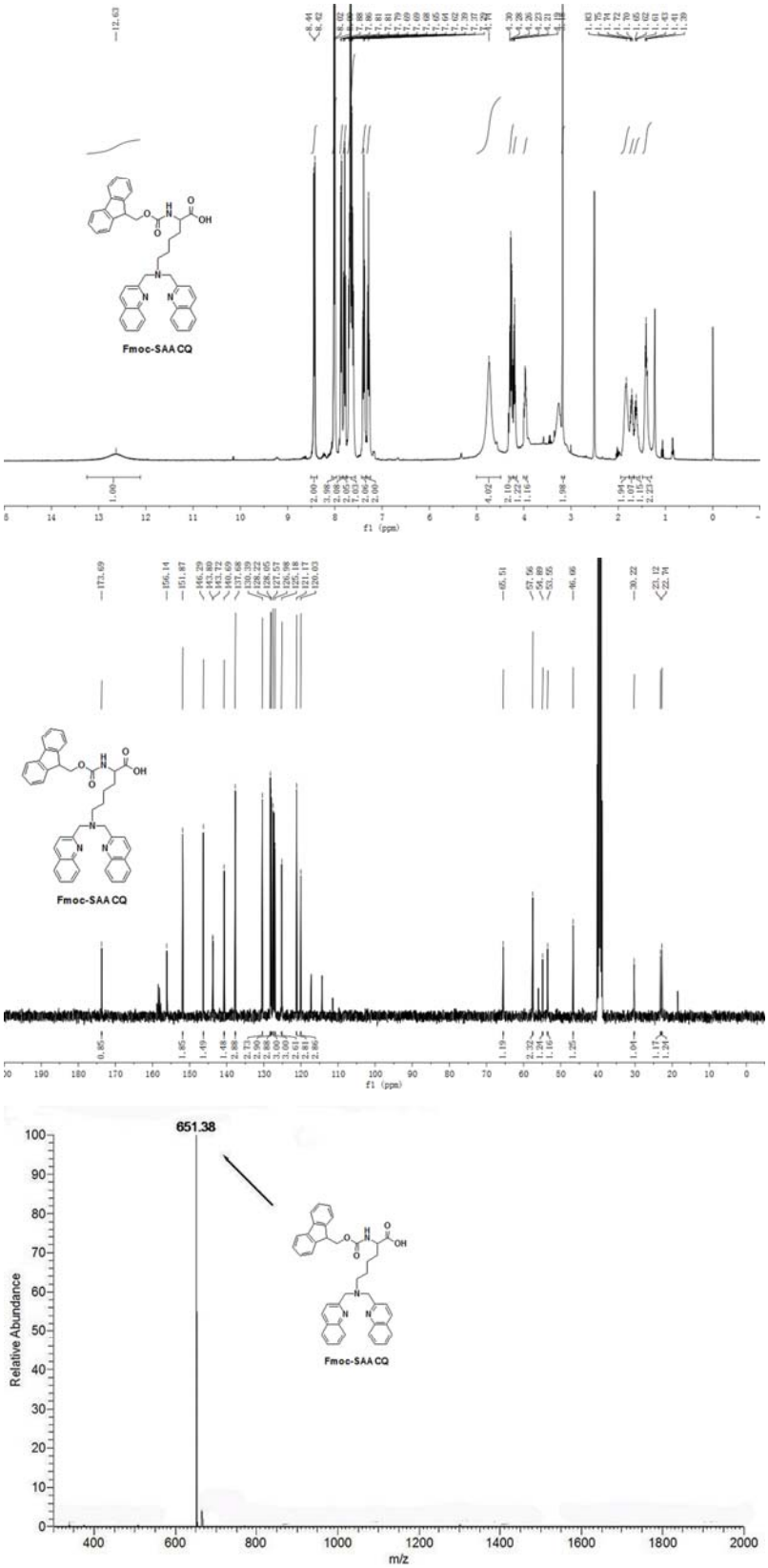
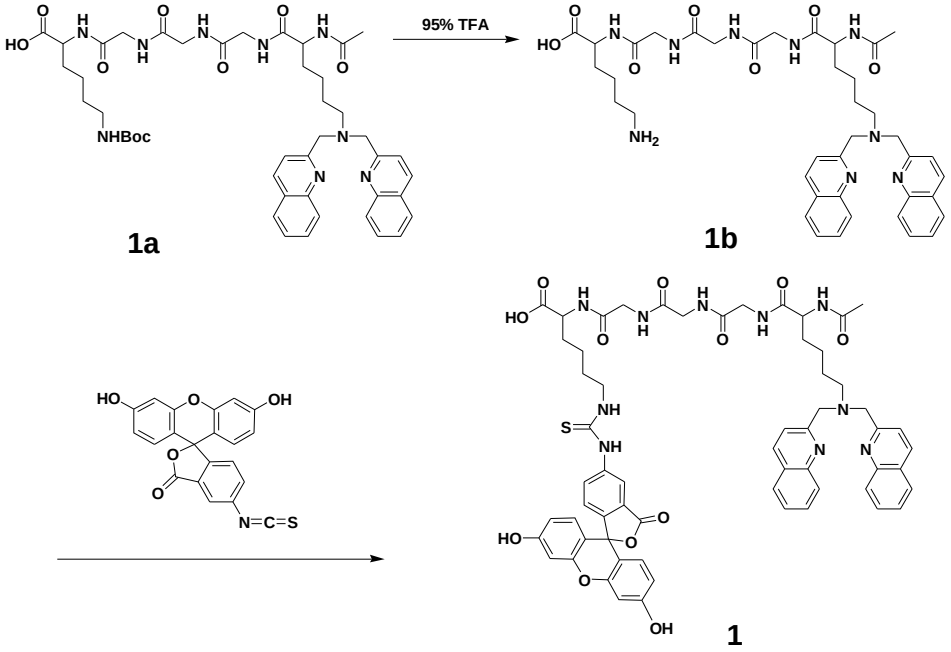


Fig. S1 ^{1}H NMR，^{13}C NMR and ESI-MS spectra of compound Fmoc-SAACQ.

*Preparation of compound **1***:

Scheme S2. Synthetic route for compound **1**.



*Synthesis of **1b***: Peptide Ac-SAACQ-Gly-Gly-Gly-Lys (Boc)-OH (**1a**) was synthesized with solid phase peptide synthesis (SPPS). The Boc protecting group of Compound **1a** was cleaved with 95% TFA in DCM for 3 hours at RT to yield compound **1b** after HPLC purification. MS: $[M+H]^+$ calc. for $C_{40}H_{52}N_9O_7$, 770.3990, obsvd. HR-MALDI-TOF/MS: 770.3990 (Fig. S2).

*Synthesis of **1***: Compound **1b** (30.8 mg, 0.04 mmol) was dissolved in 3 mL of water, and the pH of the solution was adjusted to 8.5 with sodium carbonate. FITC (32 mg, 0.08 mmol) was dissolved in 3 mL of DMSO, and then added to the water solution dropwise. The reaction mixture was stirred for 6 hours. The compound **1** (20.8 mg, yield: 44.9%) was purified by HPLC with water-acetonitrile as the eluent (from 3:7 to 1:9, Table S1). MS: calc. M^+= 1158.43, obsvd. ESI-MS: 1159.72 $[(M+H)^+]$. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 7.08-7.06 (d, 2 H), 7.03 (s, 1 H), 6.75-6.73 (d, 2 H), 6.65-6.63 (d, 2 H), 6.58-6.55 (m, 1 H), 6.51-6.47 (m, 2 H), 6.35-6.31 (m, 2 H), 6.24-6.22 (d, 2 H), 5.91-5.89 (d, 1 H), 5.73-5.71 (d, 2 H), 5.63-5.63 (d, 2 H), 5.50-5.47 (m, 2 H), 3.63 (s, 3 H), 3.15-3.11 (m, 1 H), 3.03-3.00 (m, 1 H), 2.62-2.61 (d, 2 H), 2.59-2.58 (d, 2 H), 2.56 (s, 2 H), 2.31 (s, 2 H), 2.27-2.23 (t, 2 H), 2.02-2.01 (m, 4 H), 0.73-0.69 (m, 2 H), 0.64-0.63 (d, 2 H), 0.40-0.34 (m 2 H), 0.32-0.29 (m, 2 H), 0.17-0.14 (m, 2 H). ^{13}C NMR (101 MHz, CD_3OD) δ (ppm) 173.69, 173.52, 172.37, 170.80, 170.74, 169.97, 150.83, 146.23, 138.27, 130.44, 127.77, 127.72, 127.69, 127.22, 119.79, 101.94, 57.90, 53.36, 51.94, 30.60, 27.75, 23.69, 22.68, 22.42, 21.04 (Fig. S3).

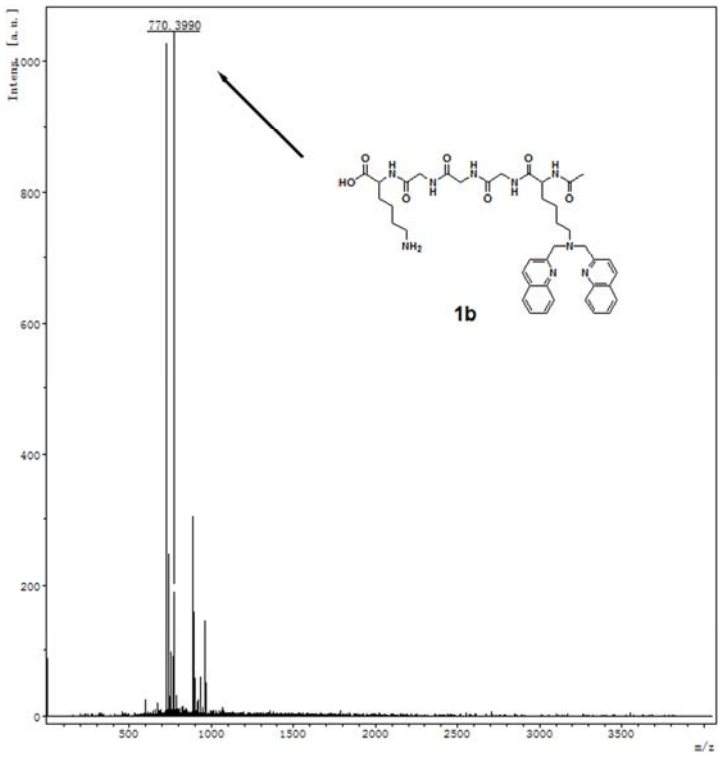


Fig. S2 HR-MALDI-TOF/MS spectra of compound **1b**.

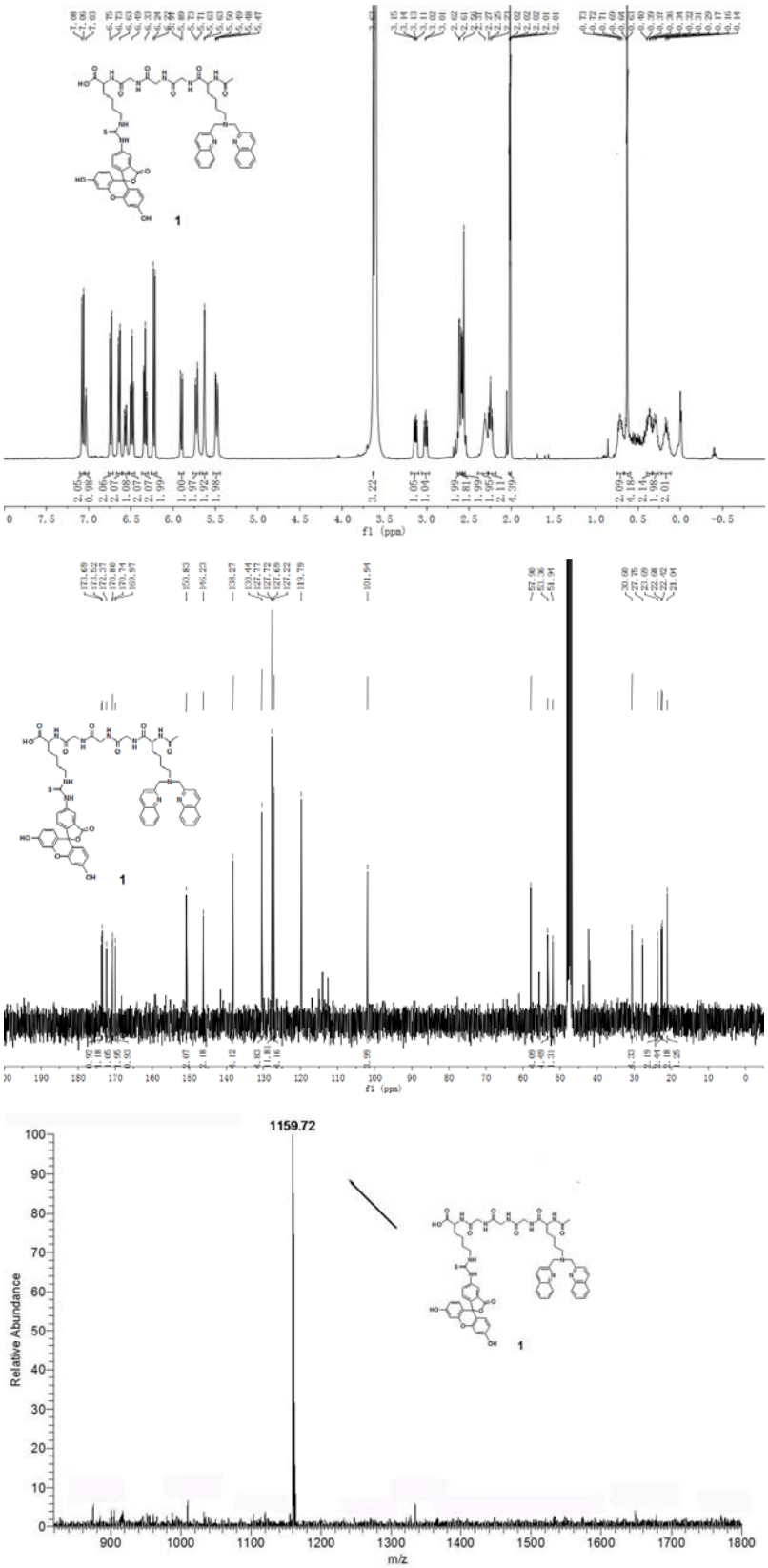


Fig. S3 ^{1}H NMR， ^{13}C NMR and ESI-MS spectra of compound **1**.

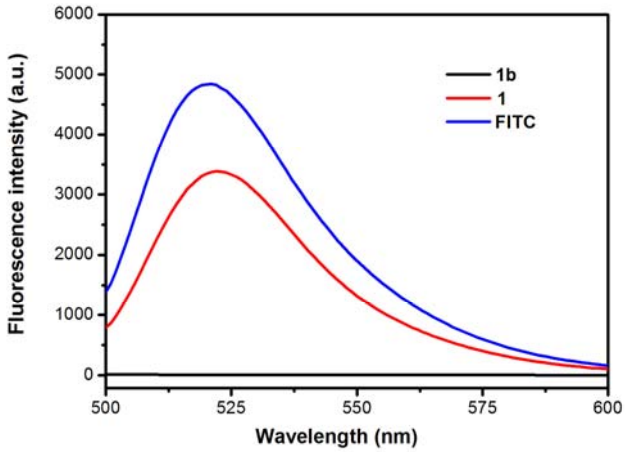


Fig. S4 Fluorescence spectra of 10 uM **1b**, 10 uM **1** and 10 uM FITC in Tris-HCl buffered (pH 7.5, 50 mM) aqueous solution at RT, respectively. λ_{ex} = 465 nm.

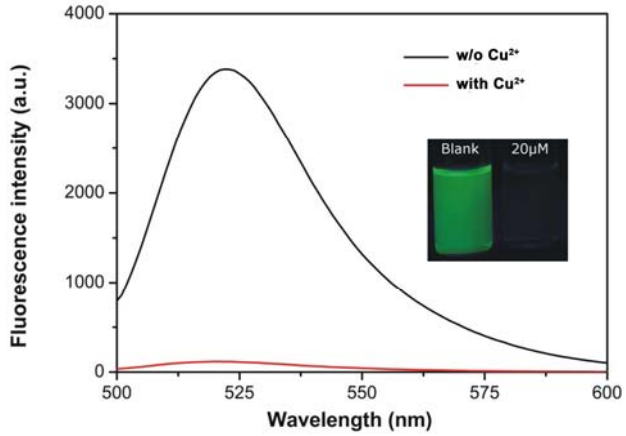


Fig. S5 Fluorescence spectra of probe 10 μM **1** (λ_{ex} = 465 nm) in the presence of 0, or 20 μM Cu^{2+} solutions in Tris-HCl buffered (pH 7.5, 50 mM) aqueous solution at RT. The inset fluorescence photos showing the corresponding change of the fluorescence under a UV lamp.

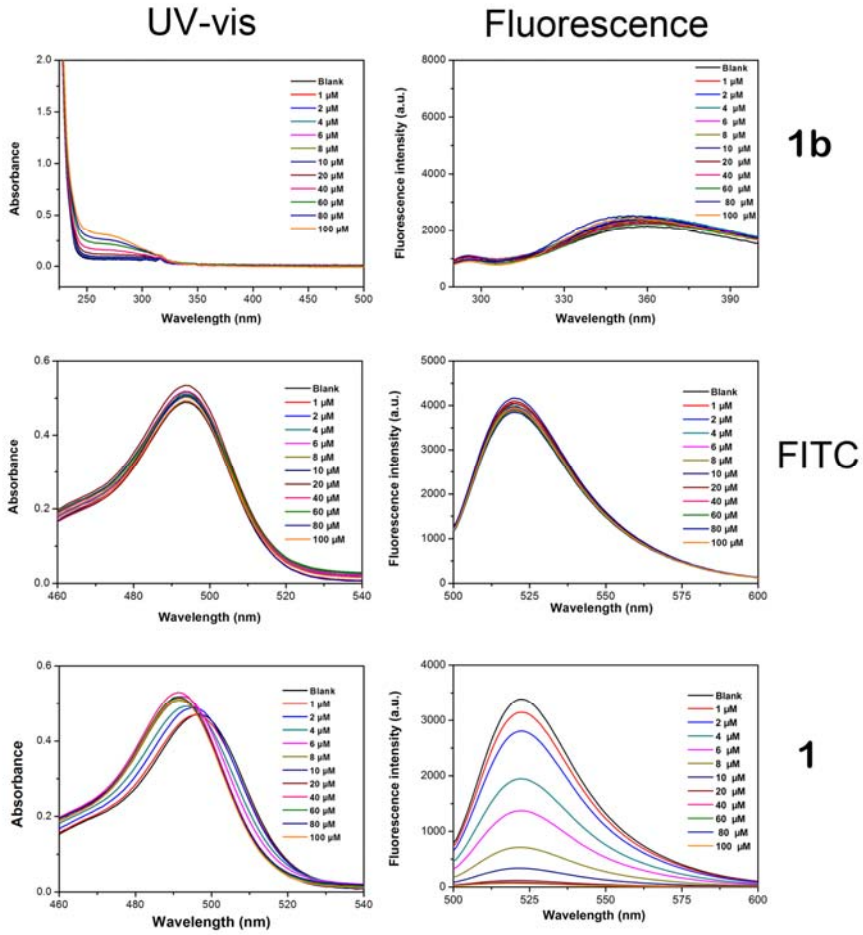


Fig. S6 UV-vis absorption (left) and Fluorescence (right) spectra of 10 μM **1b** (upper row, λ_{ex} = 265 nm), 10 μM FITC (middle row, λ_{ex} = 465 nm), and 10 μM **1** (lower row, λ_{ex} = 465 nm) upon addition of various concentrations of Cu^{2+} (0, 1, 2, 4, 8, 10, 20, 40, 60, 80, and 100 μM) in Tris-HCl buffered (pH 7.5, 50 mM) aqueous solution at RT.

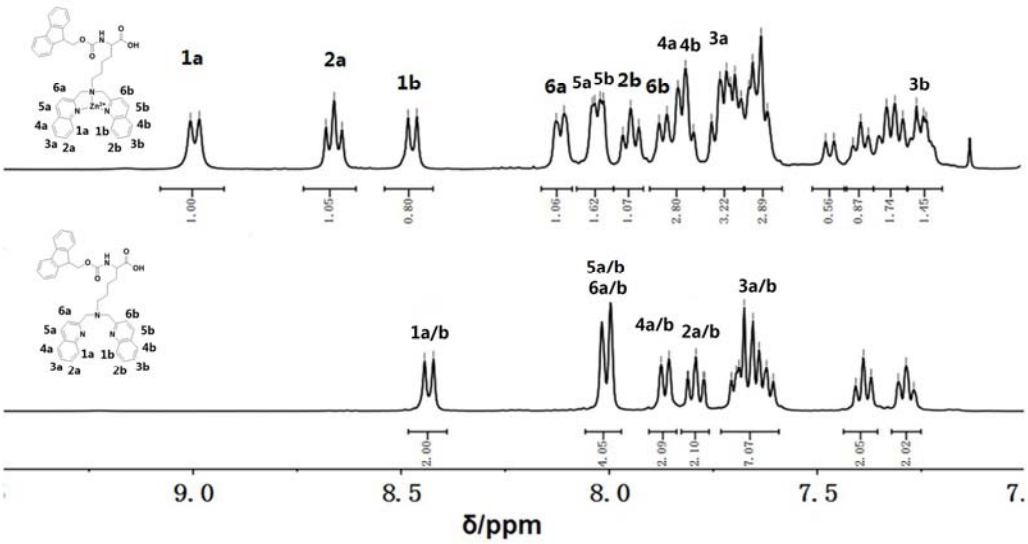


Fig. S7 ^{1}H NMR spectra (400 MHz) of Fmoc-SAACQ (bottom) and Fmoc-SAACQ upon addition of Zn^{2+} (top) in d_6-DMSO.

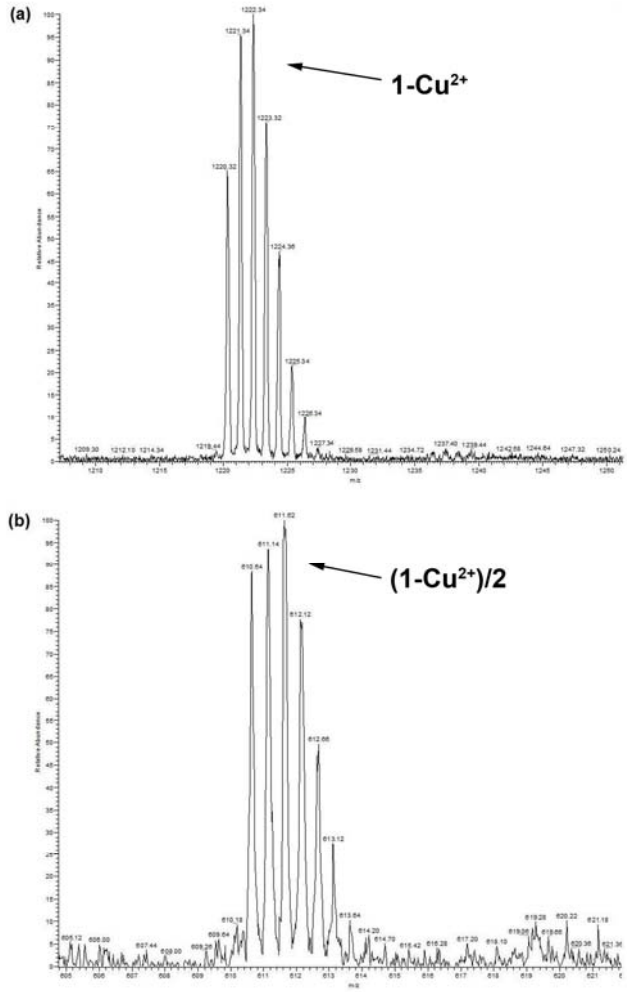


Fig. S8 ESI-MS spectra of compound **1** added with Cu^{2+} solution in Tris-HCl buffered (pH 7.5, 50

mM).

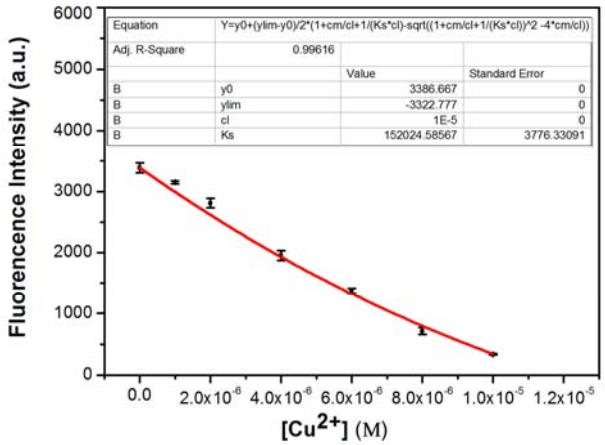


Fig. S9 The nonlinear regression fitting curve of the fluorescence intensity of **1** towards [Cu^{2+}] with equation 1.1.

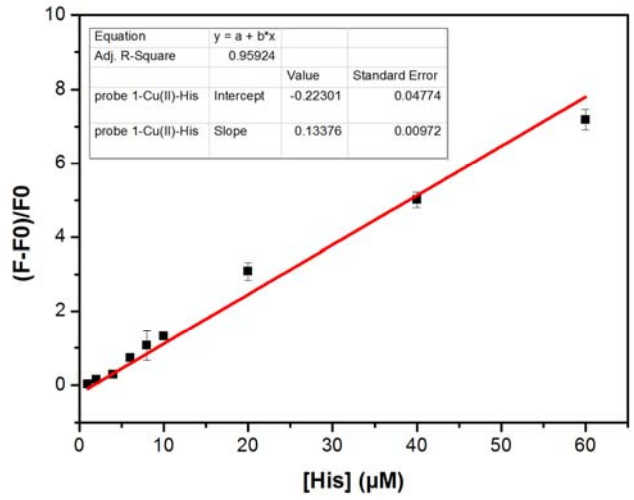


Fig. S10 The fitted calibration line corresponding to Figure 3a.

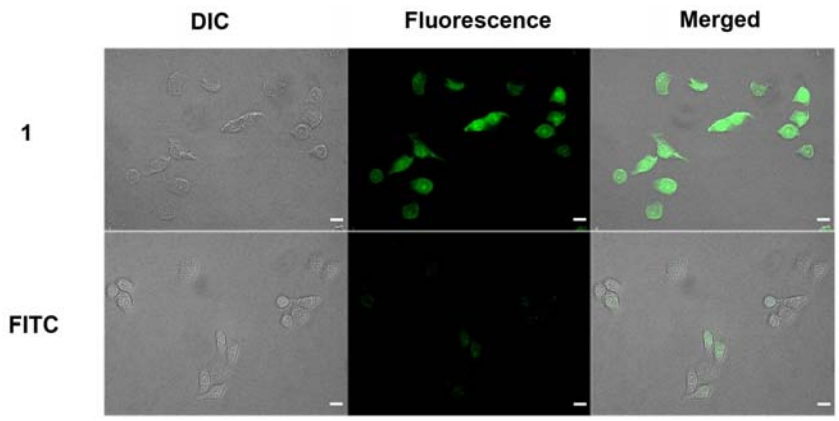


Fig. S11 Differential interference contrast (DIC) images (left), fluorescence images (middle, EGFP channel), and merged images (right) of HepG2 cells incubated with 10 μM probe **1** (upper row) or 10 μM FITC (lower row) in serum-free medium for 1 h at 37 °C, washed with PBS for three times prior to imaging, respectively. Scale bar: 20 μm.

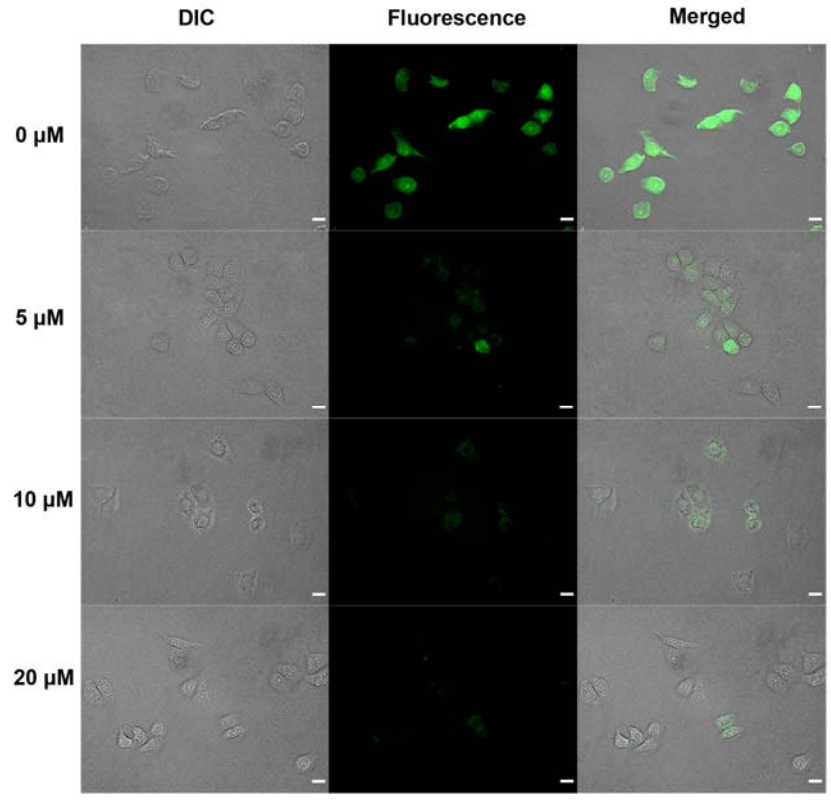


Fig. S12 Differential interference contrast (DIC) images (left), fluorescence images (middle, EGFP channel), and merged images (right) of HepG2 cells incubated with 10 μM of probe **1** in serum-free medium for 1 h at 37 °C, washed with PBS for three times, then incubated with 0, 5, 10, and 20 μM of Cu^{2+} in serum-free medium for 20 min at 37 °C prior to imaging, respectively. Scale bar: 20 μm.

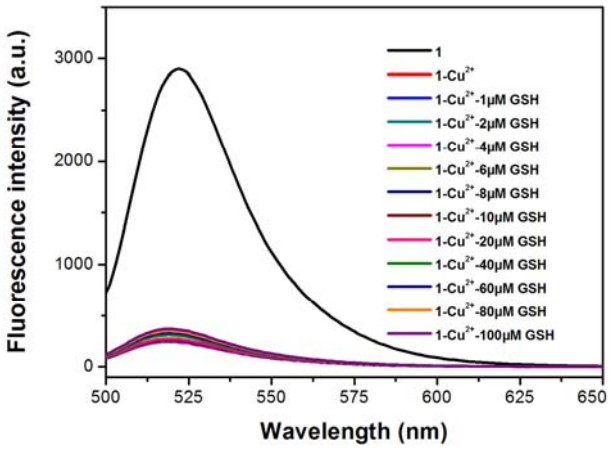


Fig. S13 Fluorescence spectra of **1-Cu^{2+}** complex (10 μM **1** and 20 μM Cu^{2+}) upon addition of various concentrations of GSH (0, 1, 2, 4, 8, 10, 20, 40, 60, 80 and 100 μM) in Tris-HCl buffered aqueous solution (pH 7.5, 50 mM) at RT.

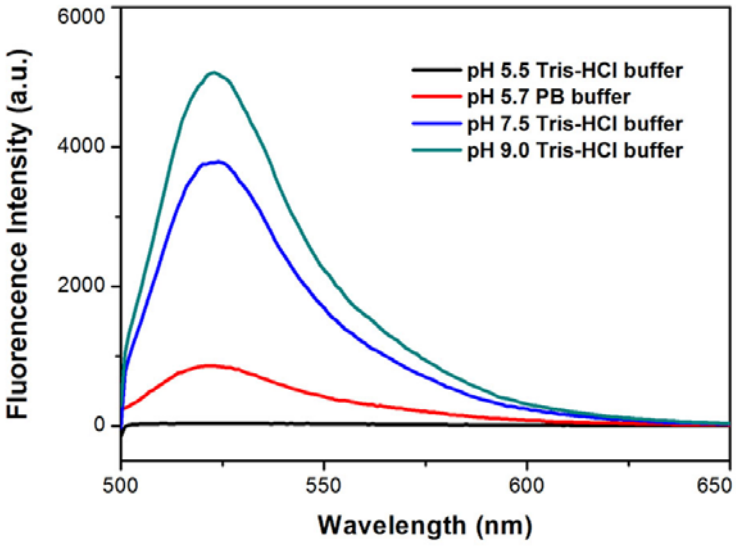


Fig. S14 Fluorescence spectra of 10 μM probe **1** in buffers at different pH value and RT.

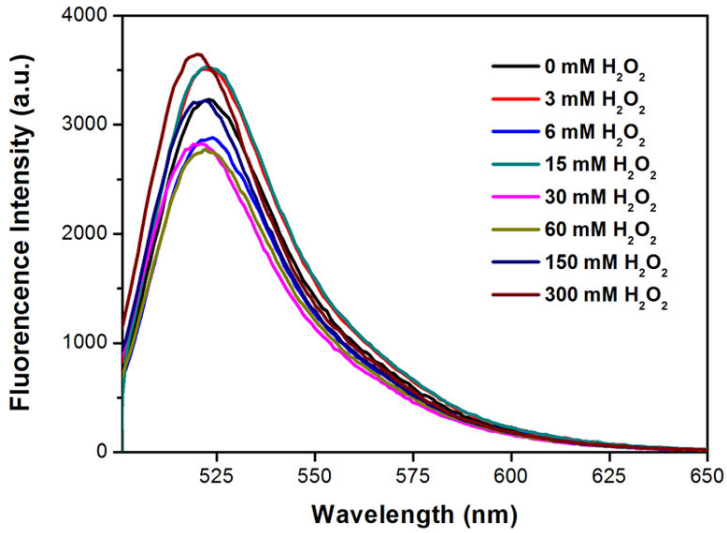


Fig. S15 Fluorescence spectra of 10 μM **1** upon addition of various concentrations of H_2O_2 (0, 3, 6, 15, 30, 60, 150 and 300 mM) in Tris-HCl buffered aqueous solution (pH 7.5, 50 mM) at RT.

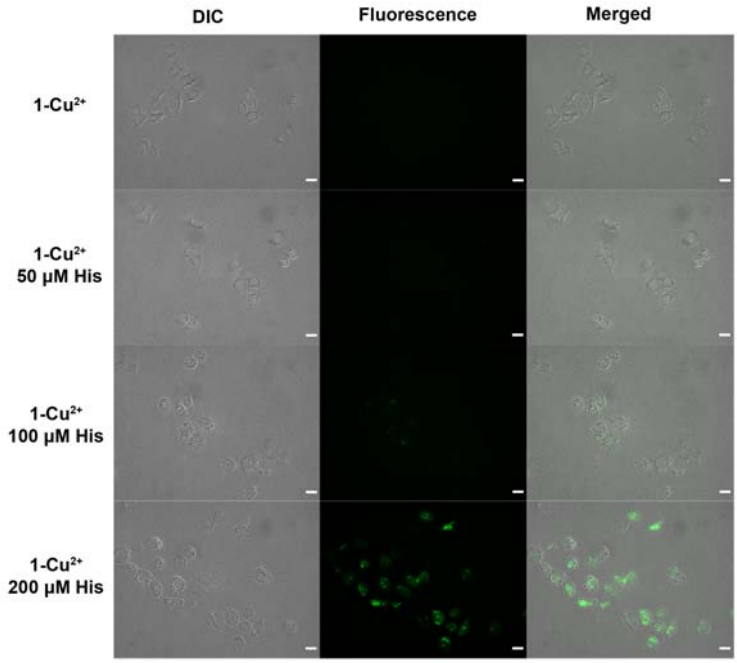


Fig. S16 Differential interference contrast (DIC) images (left), fluorescence images (middle, EGFP channel), and merged images (right) of HepG2 cells incubated with 10 μM of probe **1** in serum-free medium for 1 h at 37 °C, washed with PBS for three times, incubated with 20 μM Cu^{2+} in serum-free medium for 20 min at 37 °C, then incubated with 0, 50, 100, and 200 μM of L-His in serum-free medium for 20 min at 37 °C prior to imaging, respectively. Scale bar: 20 μm.

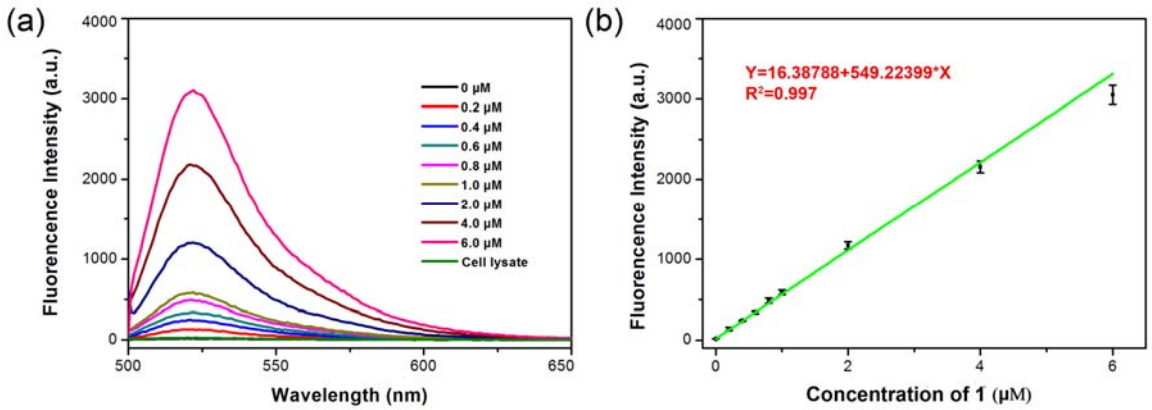


Fig. S17 (a) Fluorescence spectra of **1** at various concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0 and 6.0 μM in cell lysate (λ_{ex} = 465 nm) at RT. (b) Correlation of $FI_{521\ nm}$ in part a with probe **1** concentration between 0 and 6.0 μM. The error bar represents the standard deviation of three measurements.

Table S1. HPLC condition for the purification of compound **1**.

Time (minute)	Flow (ml/min.)	H_2O %	CH_3CN %
0	3.0	70	30
3	3.0	70	30
35	3.0	10	90
37	3.0	10	90
38	3.0	70	30
40	3.0	70	30

Table S2. Binding constants (K_s/ M^{-1}) of **1** and proteins (or peptides) towards Cu(II).

	Binding constant (K_s/ M^{-1})	pH
1 with Cu(II)	1.52×10^5	7.5
Cu(I) specific chelator BCA binding peptides and proteins[1]	1×10^9 - 1×10^{12}	7.4
Peptide-Cu(I) complexes with copper transport (Ctr) protein[2]	1×10^6 - 7×10^6	7.4
Cu(II) to human Amyloid-β (Aβ)[3]	1×10^5 - 1×10^9	7.4
Copper with alpha synuclein (αS)[4]	1.0×10^6 - 6.0×10^5	7.5
Cu(II) with ‘‘prion protein’’ (PrP^C)[5]	5.5×10^4 - 1.72×10^7	7.4
Cu(II) with $_L$-histidine[6]	1×10^8 - 1.3×10^{10}	7.4

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