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

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1. General Methods.

The commercially available chemicals were used without further purification. All of solvents used in experiments were analytical-reagent grade. ^{1}H and ^{13}C NMR spectra were measured on the Bruker ADVANCE III 400 M Hz, Varian INOVA 600 M Hz or Varian Mercury plus 300 M Hz instruments using TMS as an internal standard. Mass spectra were determined on a Bruker esquire 6000 spectrometer or a Bruker micrOTOF mass spectrometer. Fluorescence spectra were recorded on a Hitachi F-7000 spectrophotometer equipped with quartz cuvettes of 1 cm path length. All pH measurements were made with a pH-10C digital pH meter.

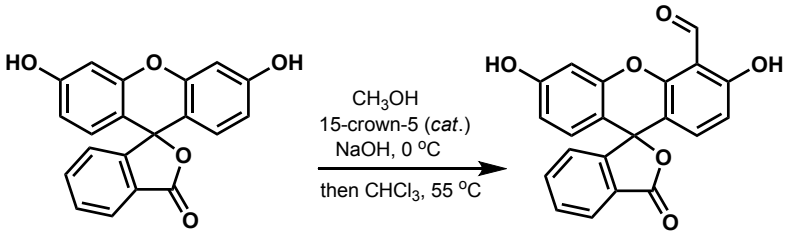
Various stock solutions (10.0 mM) of the perchlorate salts of Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, Ag^{+}, Fe^{3+}, Cr^{3+}, Al^{3+}, Ga^{3+} and Cu^{2+}; *L*-Arg, *L*-Ser, *L*-Phe, *L*-Val, *L*-Pro, *L*-Tyr, *L*-Gln, *L*-Asn, Gly, *L*-Trp, *L*-Glu, *L*-Ile, *L*-Asp, *L*-His, *L*-Ala, *L*-Thr, *L*-Lys, *L*-Met, *L*-Leu, *L*-Cys, *DL*-Hcy, and GSH in deionized water were prepared. Stock solutions of **1** and **2** (10.0 mM) were also prepared in deionized water. Test solutions were prepared by placing 2.0 μL of the probe stock solution into a test tube, and then diluting to 2.0 mL with HEPES buffer (pH = 7.0) solution, followed by the addition of an appropriate aliquot of each metal ion or amino acid's stock solution. For all measurements, fluorescence spectra were obtained by excitation at 491 nm. The excitation slit width was 2.5 nm whereas the emission slit width was 1.0 nm. Fluorescence quantum yields were determined in solution, using fluorescein (Φ = 0.85 in 0.1 M NaOH) as a standard.[1] Fluorescence emission spectra were recorded per 3 min at room temperature.

The HepG2 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (fetal bovine serum), 2 mM of glutamine, penicillin (100 units/mL), and streptomycin (100 units/mL) under an atmosphere of 5% CO_2 and 95% air at 37 °C. These cells were then seeded in a 12-well plate at a density of 1.0 ×

[1] K. Komatsu, Y. Urano, H. Kojima and T. Nagano, *J. Am. Chem. Soc*., 2007, **129**, 13447–13454.

10^4 cells per well in culture media. Before the experiments, they were immediately rinsed with PBS buffer, and then were treated with **1** (100.0 μM) in culture media for 3 h at 37 °C in a humidified incubator. For the control experiment, the HepG2 cells were treated with 500.0 μM of *N*-ethylmaleimide (NEM) in culture media for 30 min at 37 °C in a humidified incubator. After washing with PBS, the cells were further incubated with **1** (100.0 μM) in culture media for 3 h and Cu^{2+} for 15 min, respectively. The bright field and fluorescence images were acquired with Floid cell imaging station (Life Technology).

2. Synthesis and characterization of monoaldehyde-functionalized fluorescein



This procedure was adapted from a known literature.[2] To a 250 mL three-neck round-bottom flask were added fluorescein (5.0 g, 15.0 mmol) and MeOH (12 mL) at room temperature. The whole system was then cooled to 0 °C, followed by the addition of a mixture of NaOH aqueous solution (40 g, 50%) and 15-crown-5 (60 μL) within 5 min. The resulting mixture was stirred for 10 min, and then allowed to warm gradually in an oil bath. $CHCl_3$ (20 mL) was added dropwise while the reaction temperature was maintained at 55 °C. The reaction mixture was further stirred for 10 h at this temperature, and then cooled to room temperature. The mixture is acidified with H_2SO_4 (15 mL, 10 M), and the purple-black precipitate appeared. This solid was filtered and dried and purified by flash column chromatography (DCM/EtOAc = 85:15) on silica gel to afford the crude monoaldehyde-functionalized fluorescein, which could be recrystallized in acetone (10 mL) to give pure sample (708 mg, 13%) as a pale-yellow solid. Mp: 272–276 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 11.89 (s,

[2] W. Wang, O. Rusin, X. Xu, K. K. Kim, J. O. Escobedo, S. O. Fakayode, K. A. Fletcher, M. Lowry, C. M. Schowalter, C. M. Lawrence, F. R. Fronczek, I. M. Warner, R. M. Strongin, *J. Am. Chem. Soc.*, 2005, **127**, 15949–15958.

1H), 10.63 (s, 1H), 10.29 (s, 1H), 8.02 (d, J = 7.5 Hz, 1H), 7.81 (d, J = 7.2 Hz, 1H), 7.73 (d, J = 7.2 Hz, 1H), 7.32 (d, J = 7.5 Hz, 1H), 6.95 (d, J = 9.0 Hz, 1H), 6.85 (s, 1H), 6.71 (d, J = 9.0 Hz, 1H), 6.61 (brs, 2H) ppm. ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 192.9, 168.5, 162.9, 159.6, 152.4, 152.2, 150.9, 136.5, 135.8, 130.3, 129.0, 125.9, 124.8, 124.0, 113.5, 113.4, 109.7, 109.2, 109.1, 102.7, 81.8 ppm. ESI−MS: m/z 361.1 $[M + H]^+$.

^{1}H, ^{13}C NMR and ESI-MS copies of monoaldehyde-functionalized fluorescein.

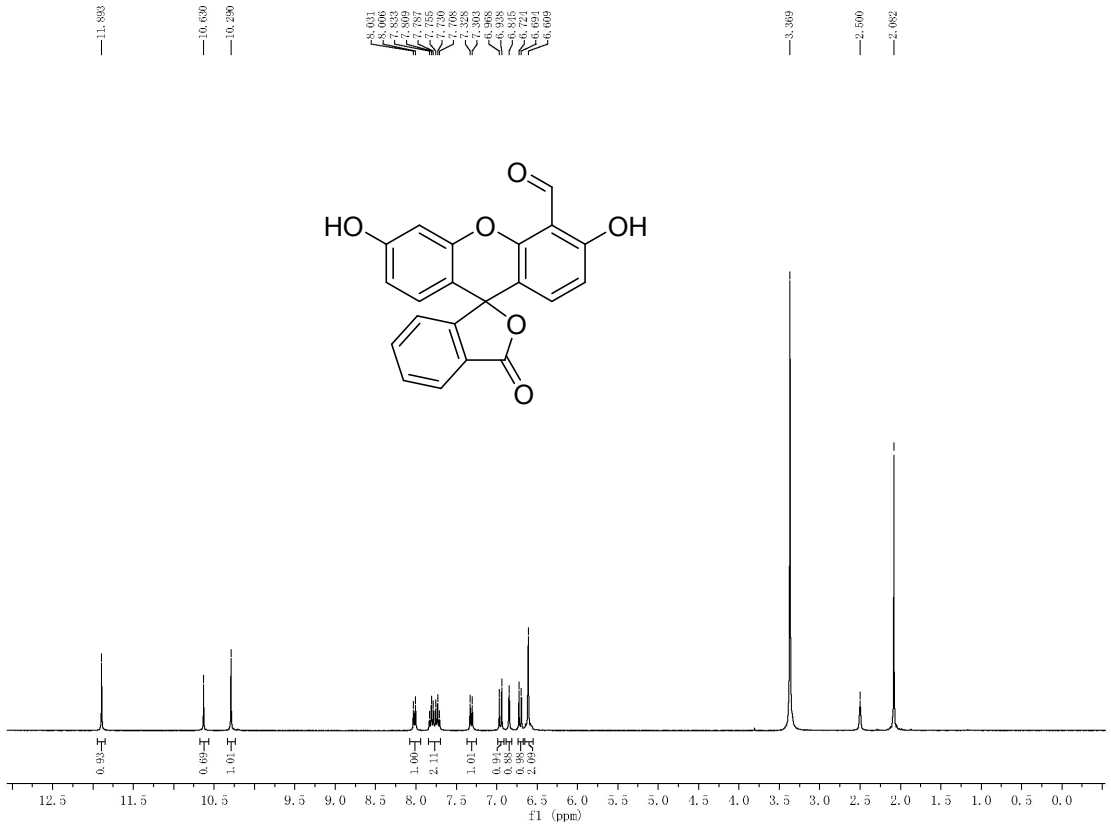


Figure S1. ^{1}H NMR spectrum (300 MHz, DMSO-d_6) of monoaldehyde-functionalized fluorescein.

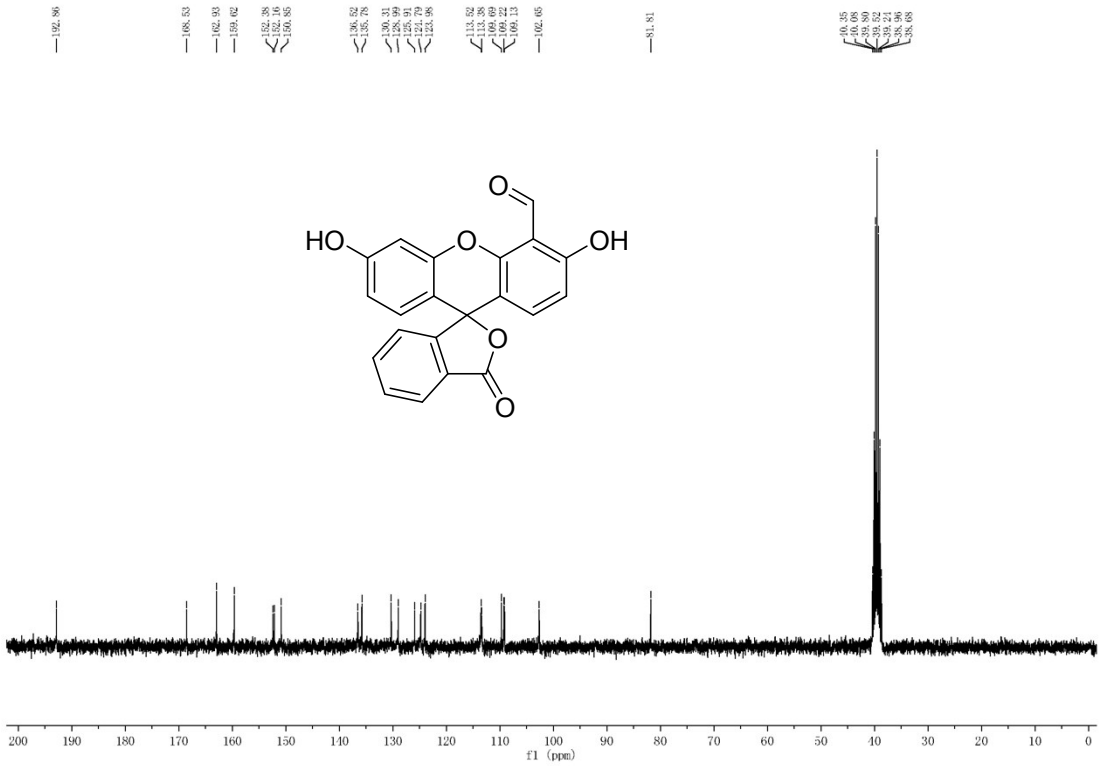


Figure S2. ^{13}C NMR spectrum (75 MHz, DMSO-d_6) of monoaldehyde-functionalized fluorescein.

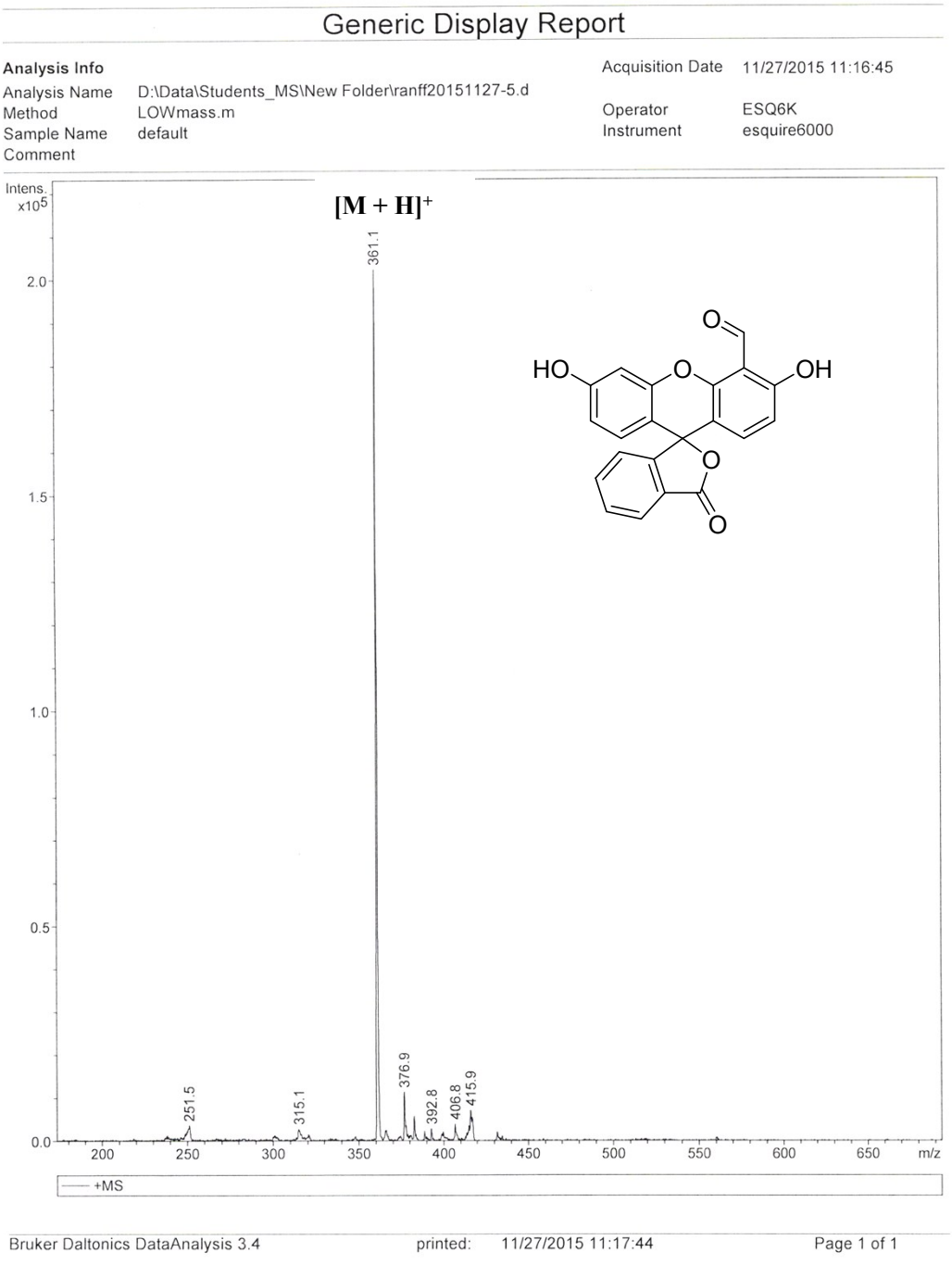


Figure S3. ESI-MS spectrum of monoaldehyde-functionalized fluorescein.

3. ^{1}H, ^{13}C NMR and ESI-MS copies of Probe 1 and Probe 2

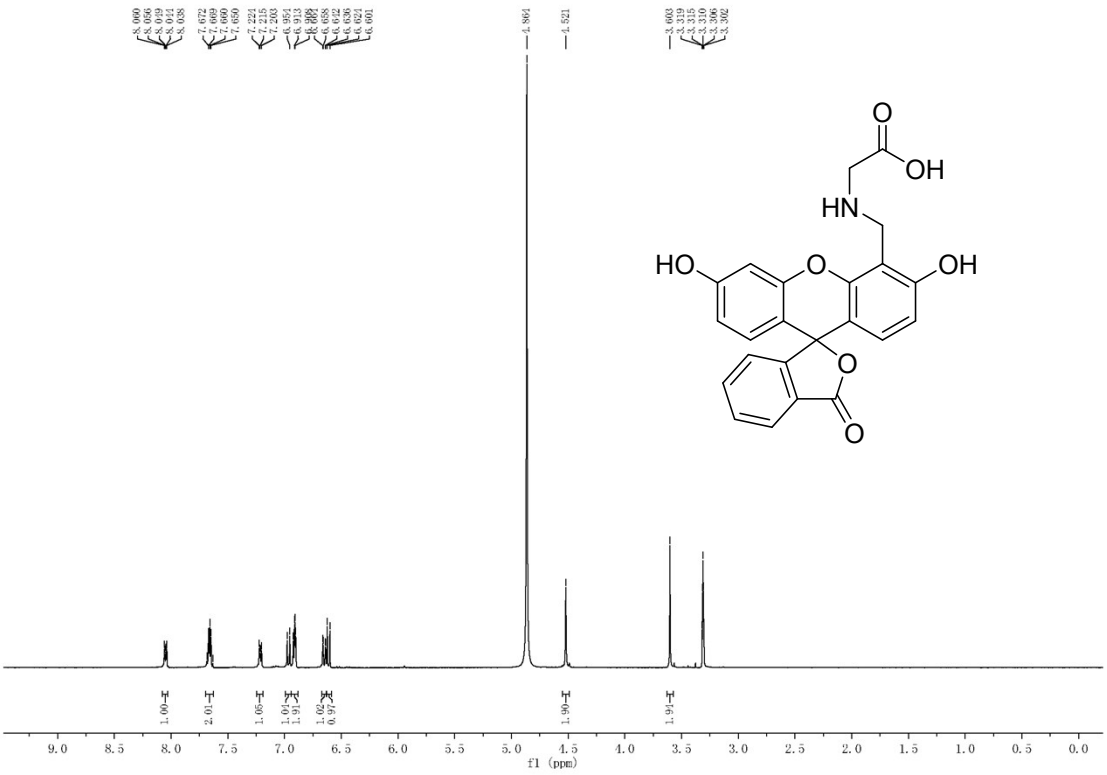


Figure S4. ^{1}H NMR spectrum (400 MHz, MeOH-d_4) of **1**.

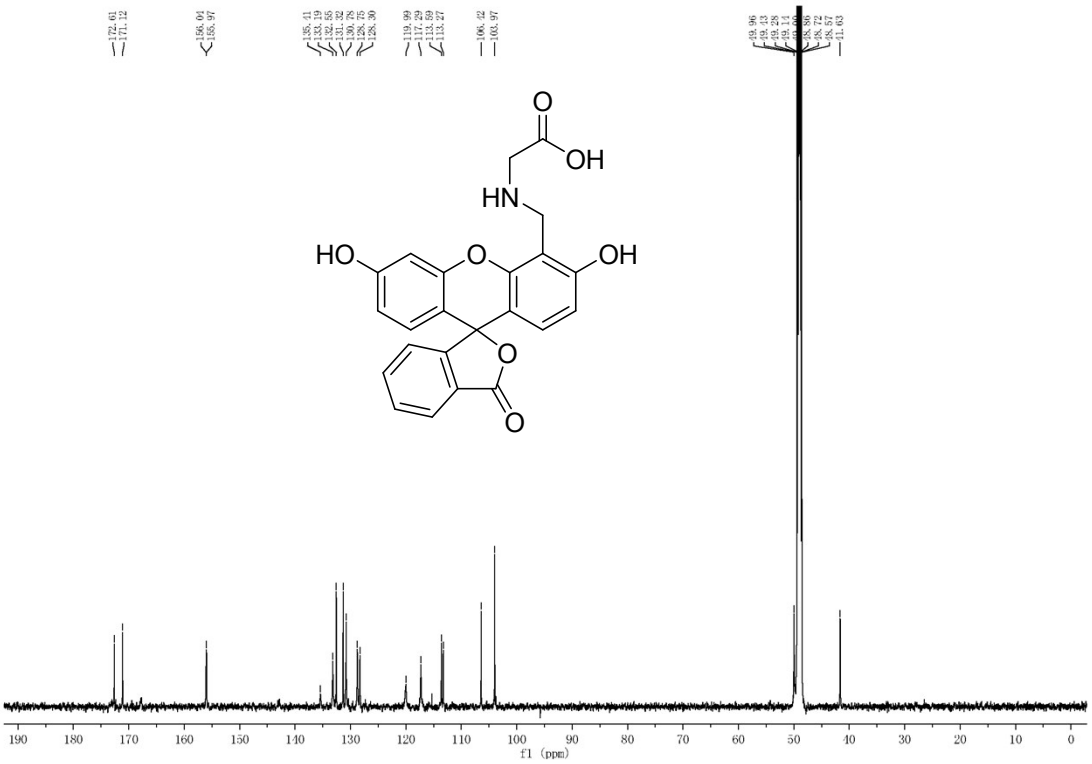


Figure S5. ^{13}C NMR spectrum (150 MHz, MeOH-d_4) of **1**.

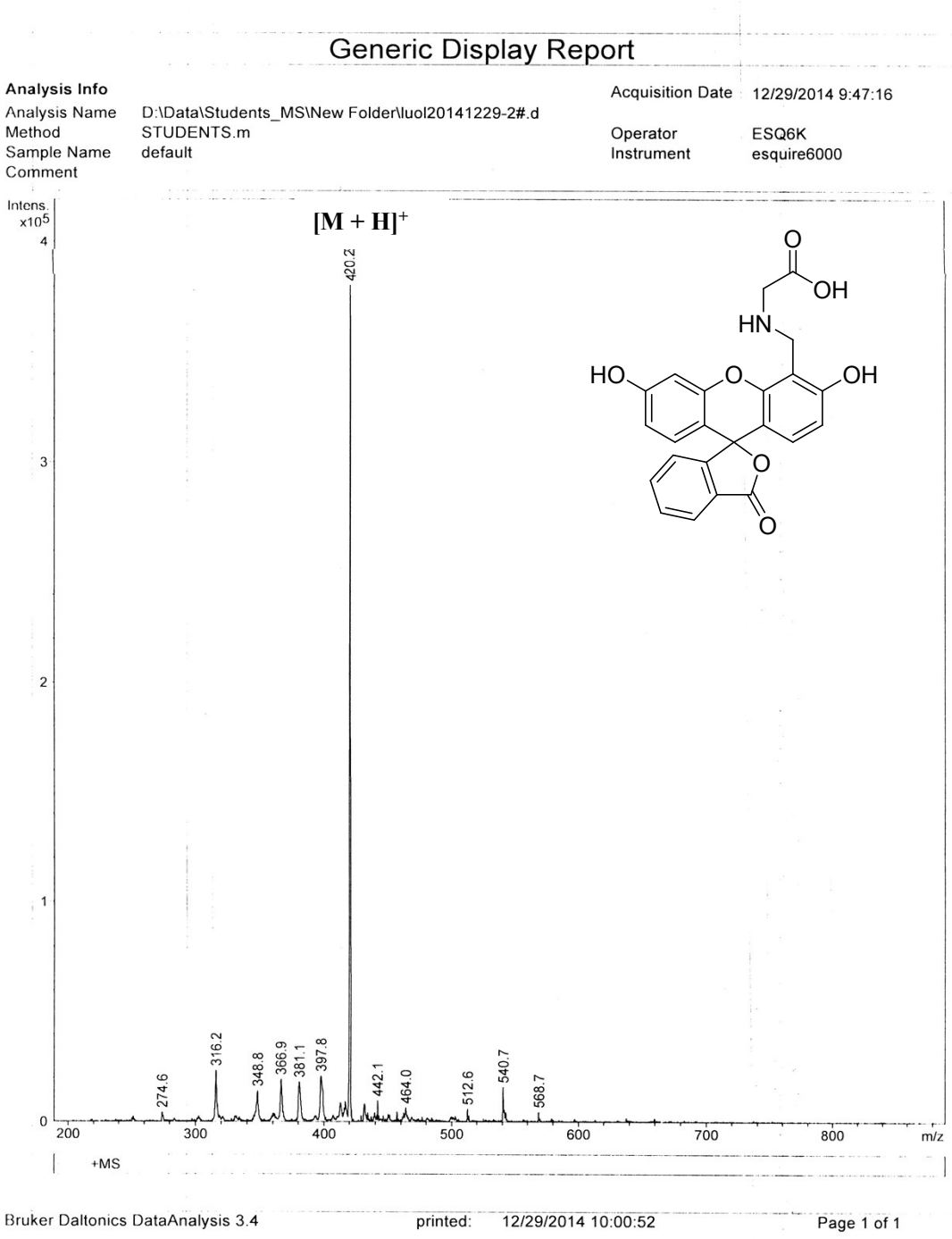


Figure S6. ESI-MS spectrum of **1**.

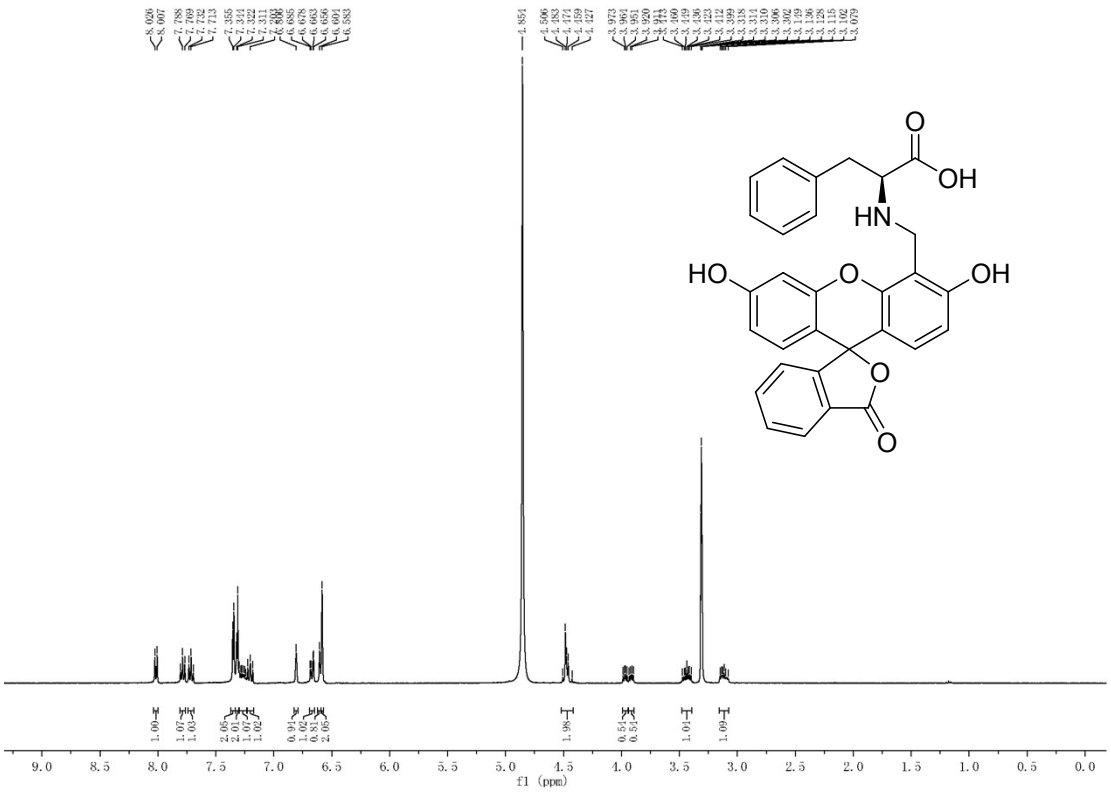


Figure S7. ^{1}H NMR spectrum (400 MHz, MeOH-d_4) of **2**.

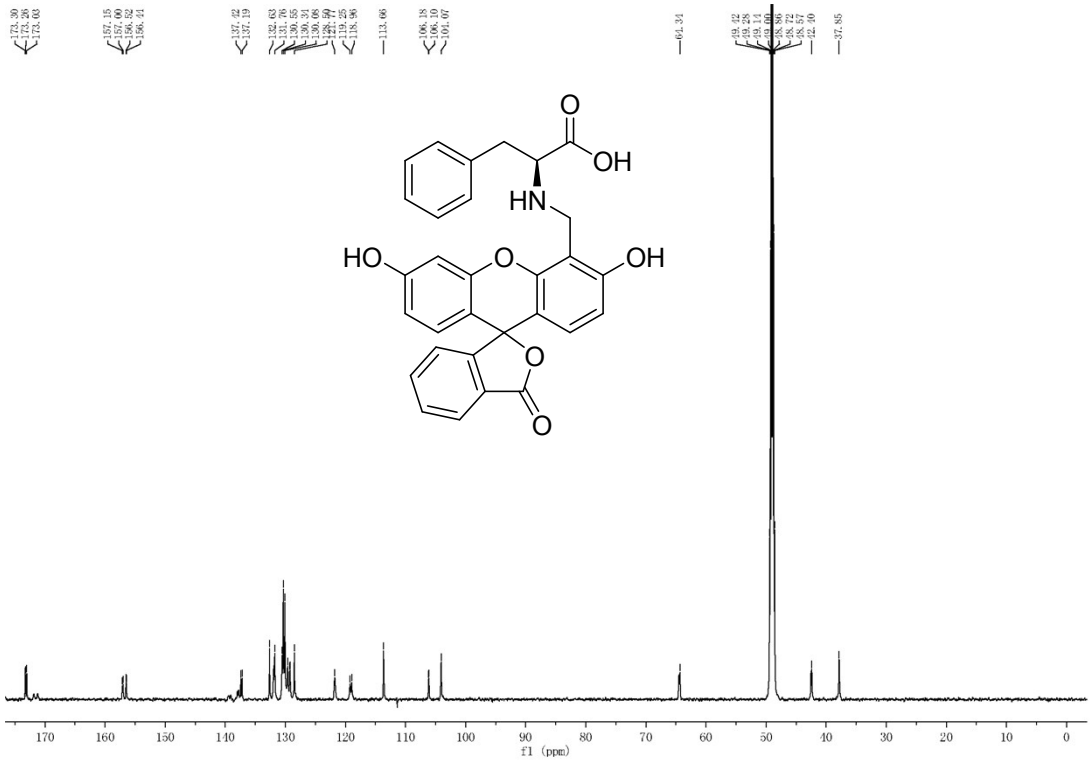


Figure S8. ^{13}C NMR spectrum (150 MHz, MeOH-d_4) of **2**.

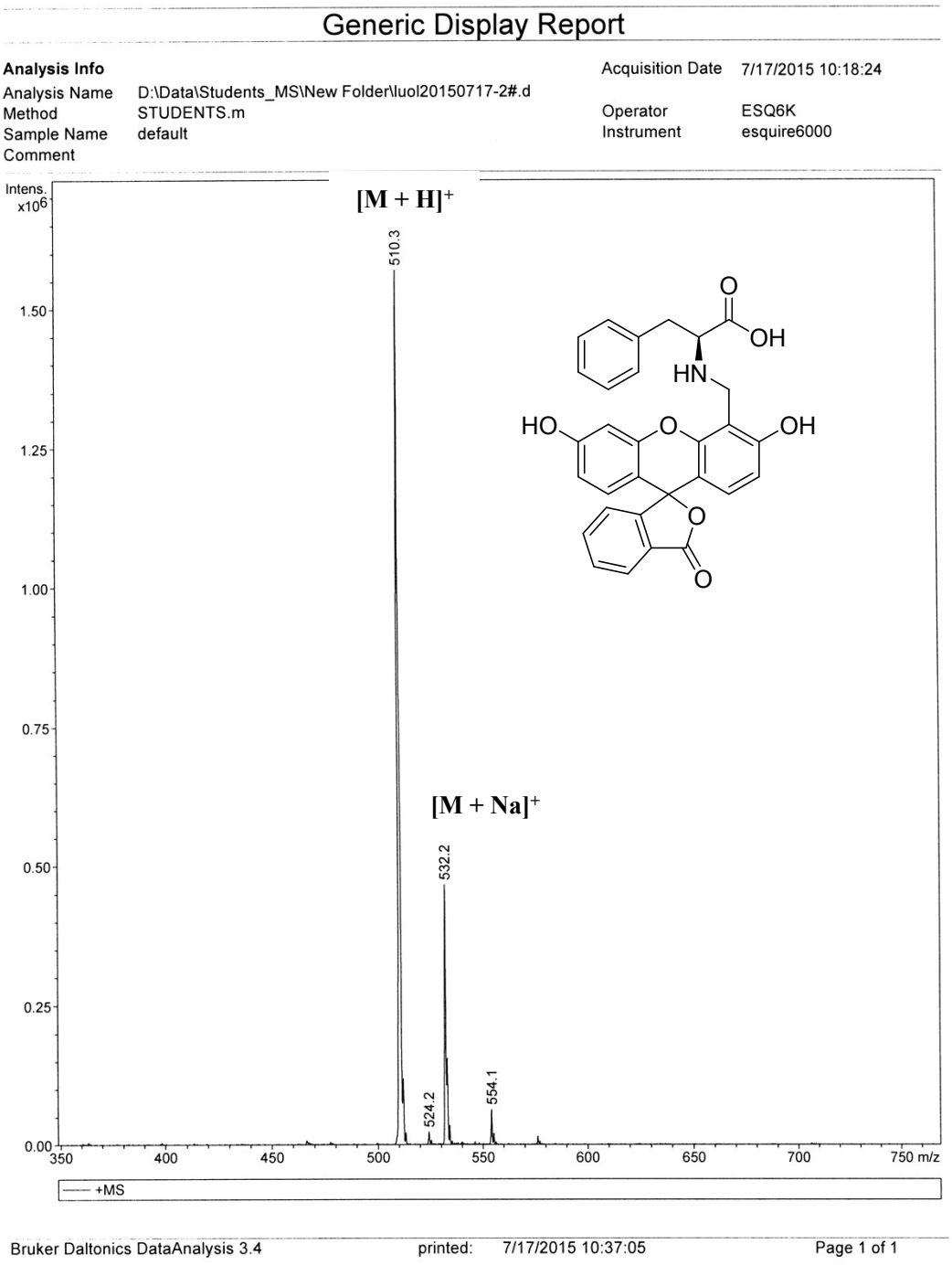
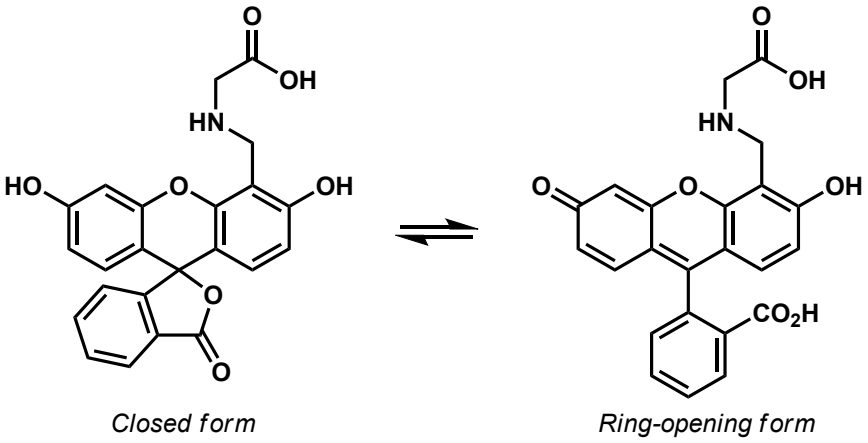


Figure S9. ESI-MS spectrum of **2**.

4.The pH dependence of the fluorescence intensity change.



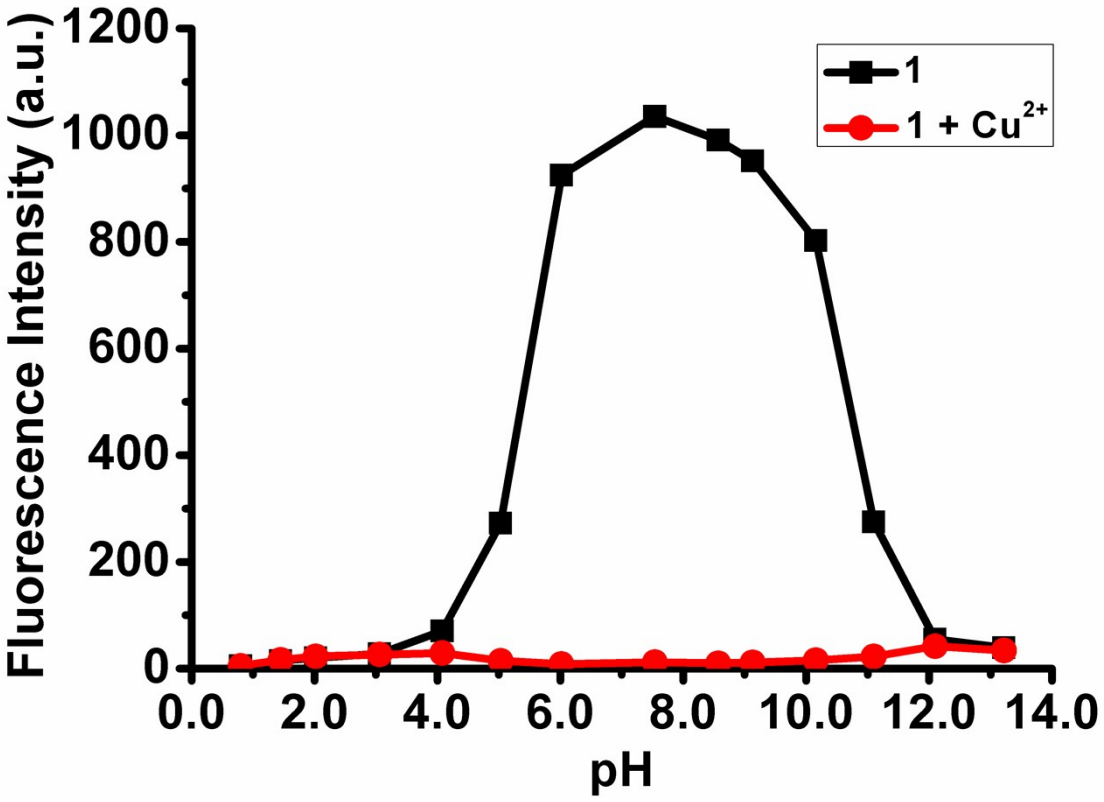


Figure S10. Fluorescence intensity of **1** (10.0 μM) in HEPES solution of different pH in the absence and presence of 10.0 μM Cu^{2+} (λ_{ex} = 491 nm).

5. Job's and Benesi-Hildebrand plots of 1 with Cu^{2+} in HEPES buffer solution.

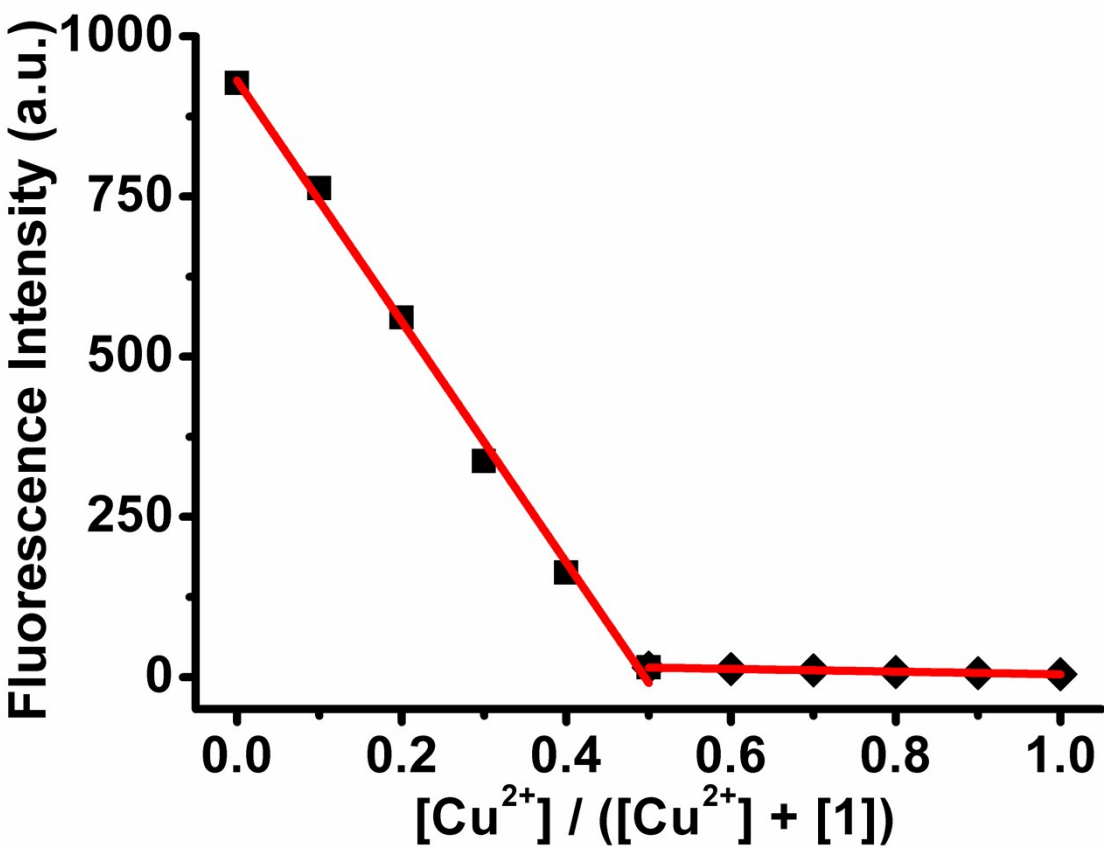


Figure S11. Job's plot for **1** with Cu^{2+} in HEPES buffer (pH = 7.0) solution.

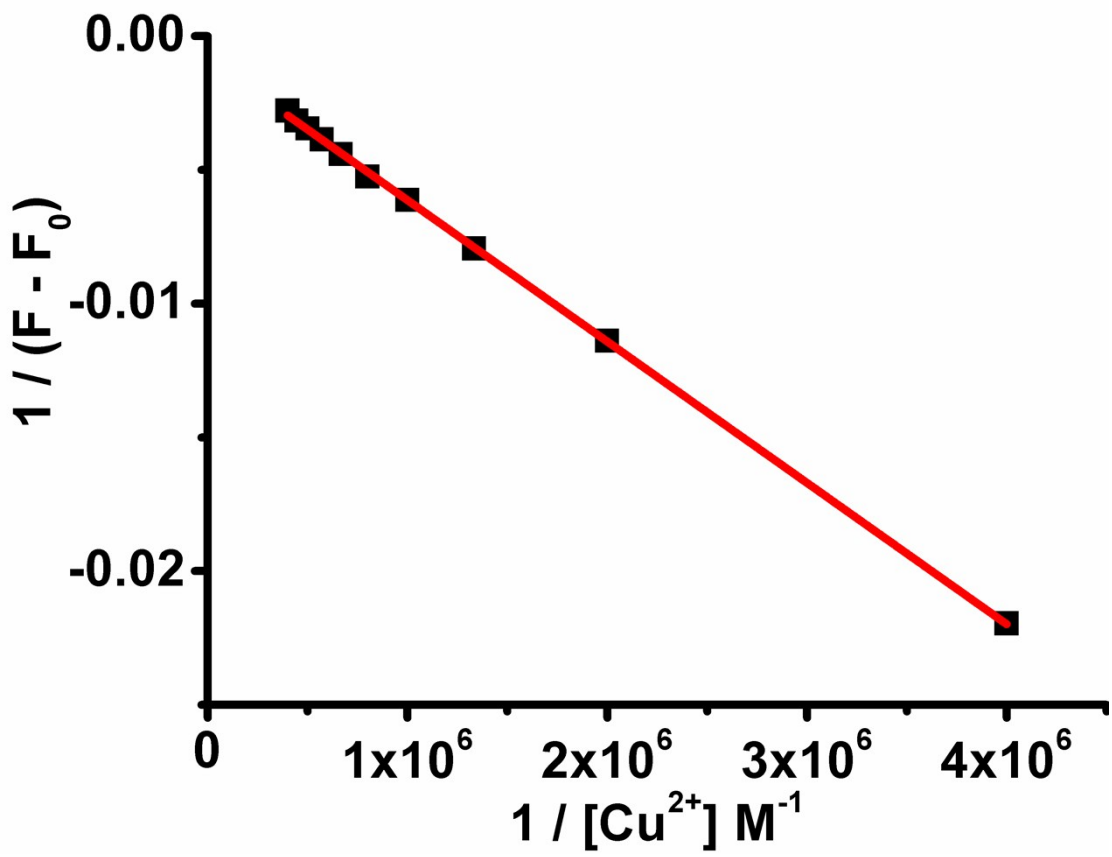


Figure S12. Benesi-Hildebrand plot of **1**, assuming 1:1 stoichiometry for association between **1** and Cu^{2+} in HEPES buffer (pH = 7.0) solution.

The binding constant was determined using a reported procedure for a 1:1 binding mode. The result of the analysis as follows:
Equation: Y = A +B × X
Y = – 8.47077E-4 – 5.28413E-9×X
R = 0.9997
K = A / B = 1.60 × 10^5 M^{-1}

6. ESI-TOF MS spectrum of 1 with Cu^{2+}.

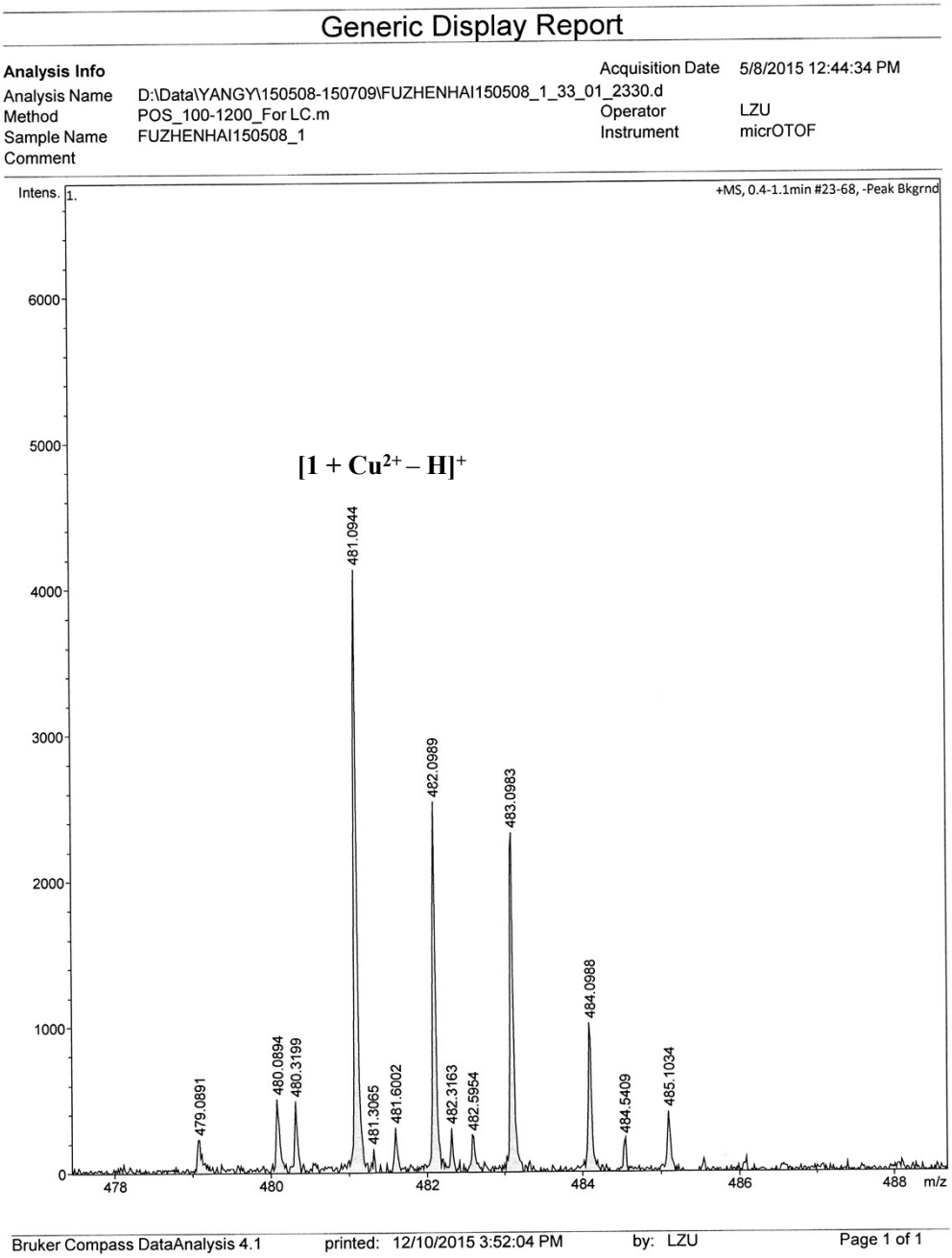


Figure S13. ESI-TOF MS spectrum of **1** with Cu^{2+} in HEPES buffer (pH = 7.0) solution.

7. Fluorescence responses.

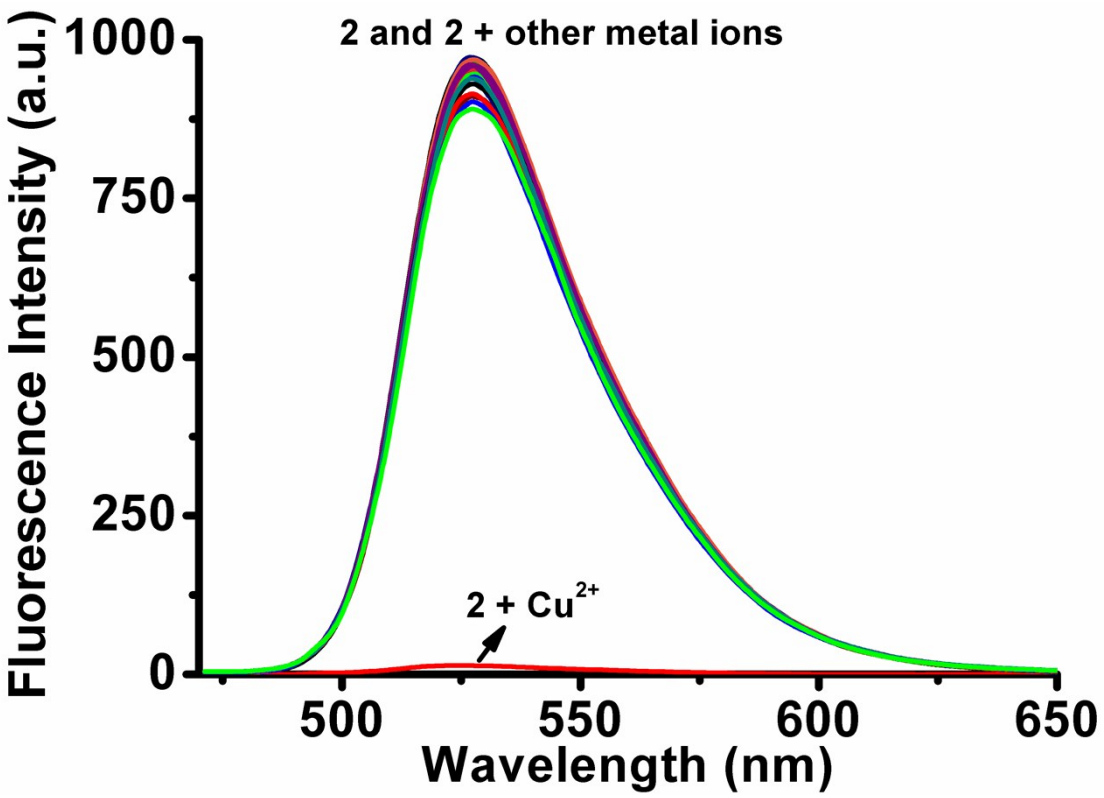


Figure S14. Fluorescence responses of **2** (10.0 μM) in HEPES buffer (pH=7.0) solution with 10.0 μM of Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, Ag^{+}, Fe^{3+}, Cr^{3+}, Al^{3+}, Ga^{3+} and Cu^{2+}.

Fluorescence responses of monoaldehyde-functionalized fluorescein to various metal ions in HEPES buffer solution.

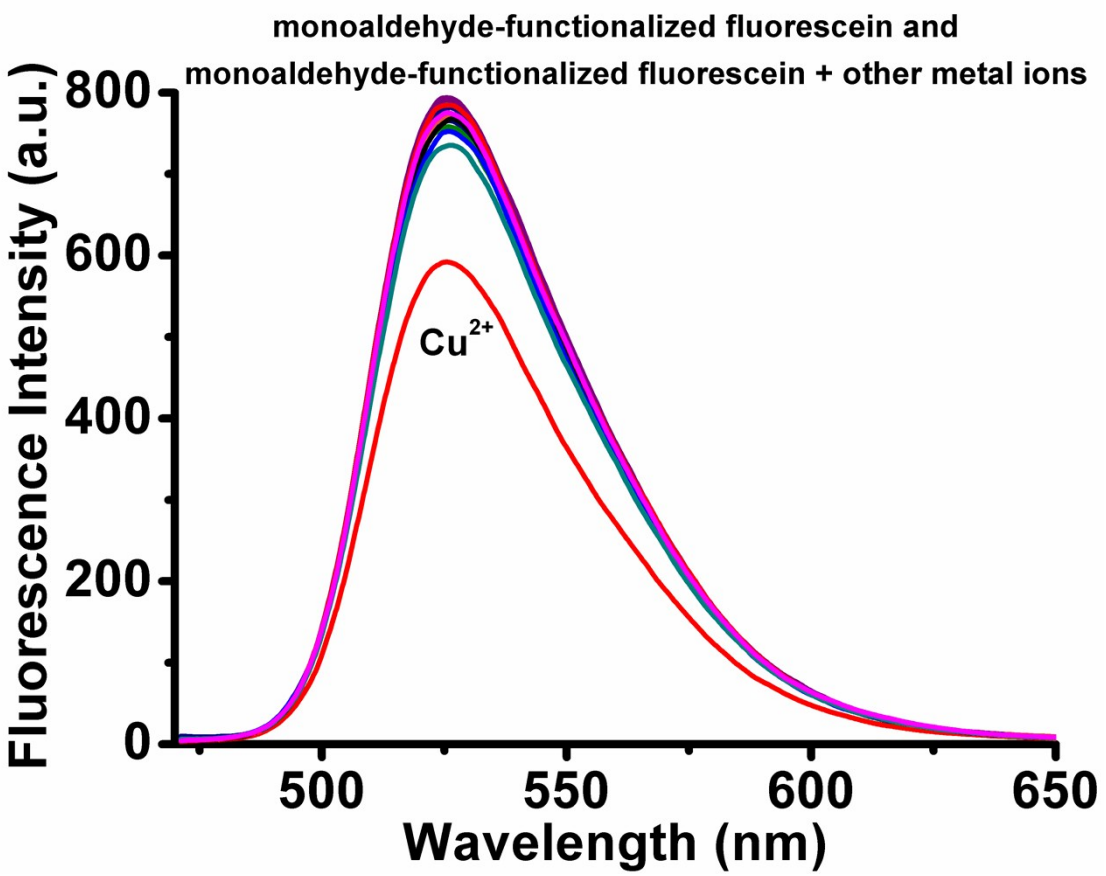


Figure S15. Fluorescence responses of monoaldehyde-functionalized fluorescein (10.0 μM) in HEPES buffer (pH=7.0) solution with 10.0 μM of Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, Ag^{+}, Fe^{3+}, Cr^{3+}, Al^{3+}, Ga^{3+} and Cu^{2+}.

The detection limit of the 1 with Cu^{2+} in HEPES buffer solution.

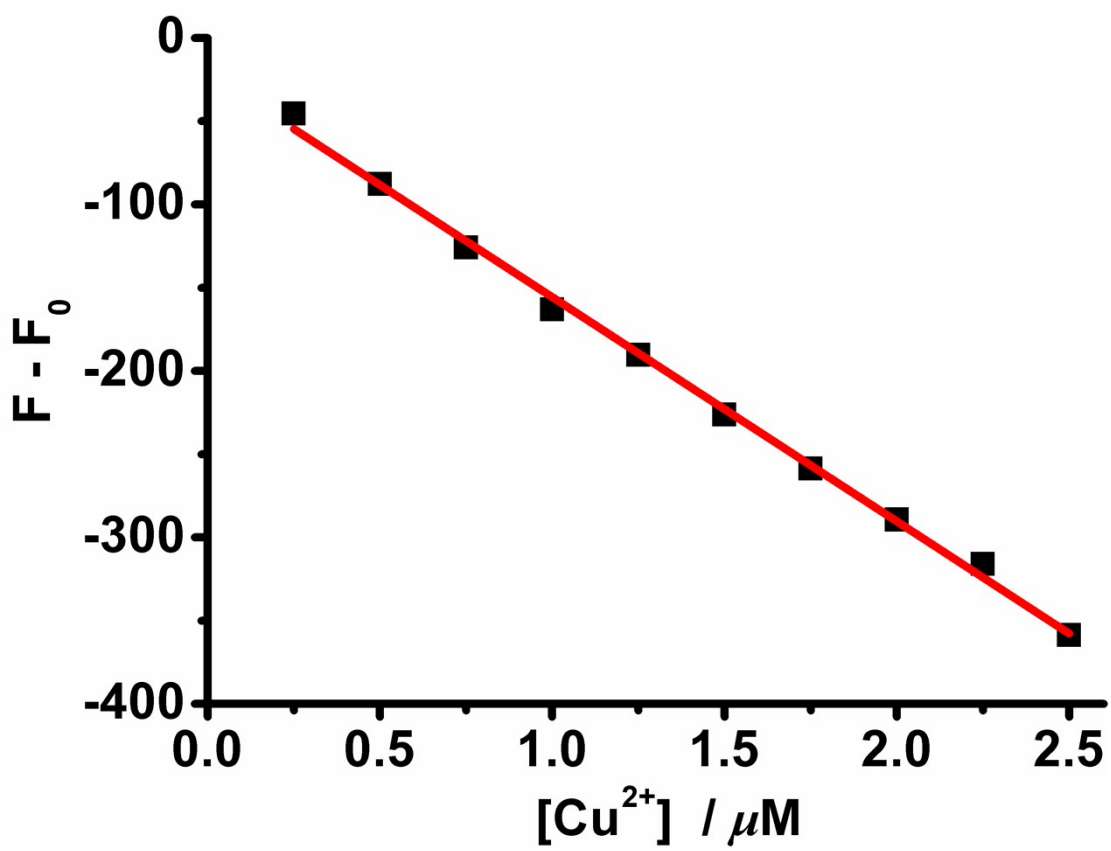


Figure S16. Plot of the intensity at 525 nm for a mixture of **1** and Cu^{2+} in HEPES buffer (pH=7.0) solution in the range 0–2.5 μM (λ_{ex} = 491 nm).

The result of the analysis as follows:

Linear Equation: Y = −20.94992 − 134.68125 × X R = 0.9972

S = 1.35 × 10^8 $\delta = \sqrt{\frac{\sum\left(F_0 - \overline{F_0}\right)^2}{N-1}} = 12.2844 (N = 10)$ K = 3

LOD = K × δ / S = 2.736 × 10^{-7} M

F_0 is the fluorescence intensity at 525 nm of **1**.

Plot of fluorescence intensity against time for 1 upon addition of Cu^{2+}.

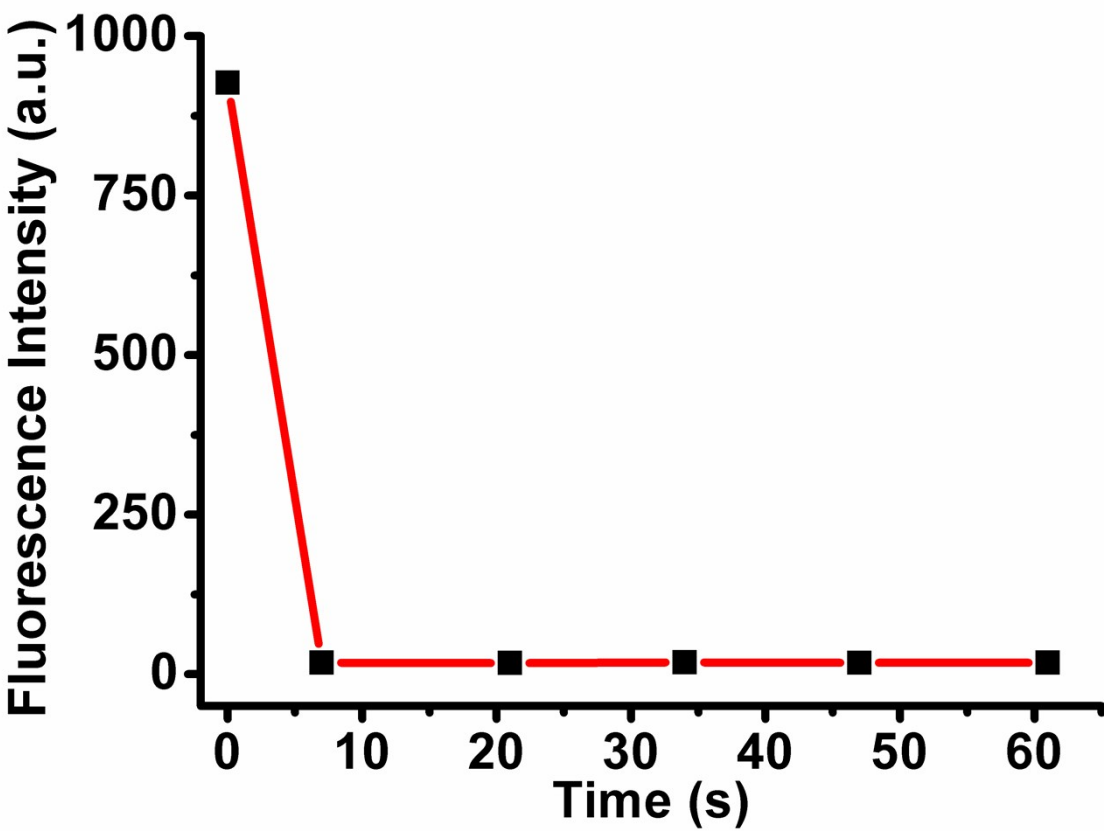


Figure S17. Plot of fluorescence intensity against time for **1** upon addition of 1.0 equiv of molar ratio of Cu^{2+} in HEPES buffer (pH = 7.0) solution.

The fluorescence titrations of 1 + Cu^{2+} with Hcy or GSH.

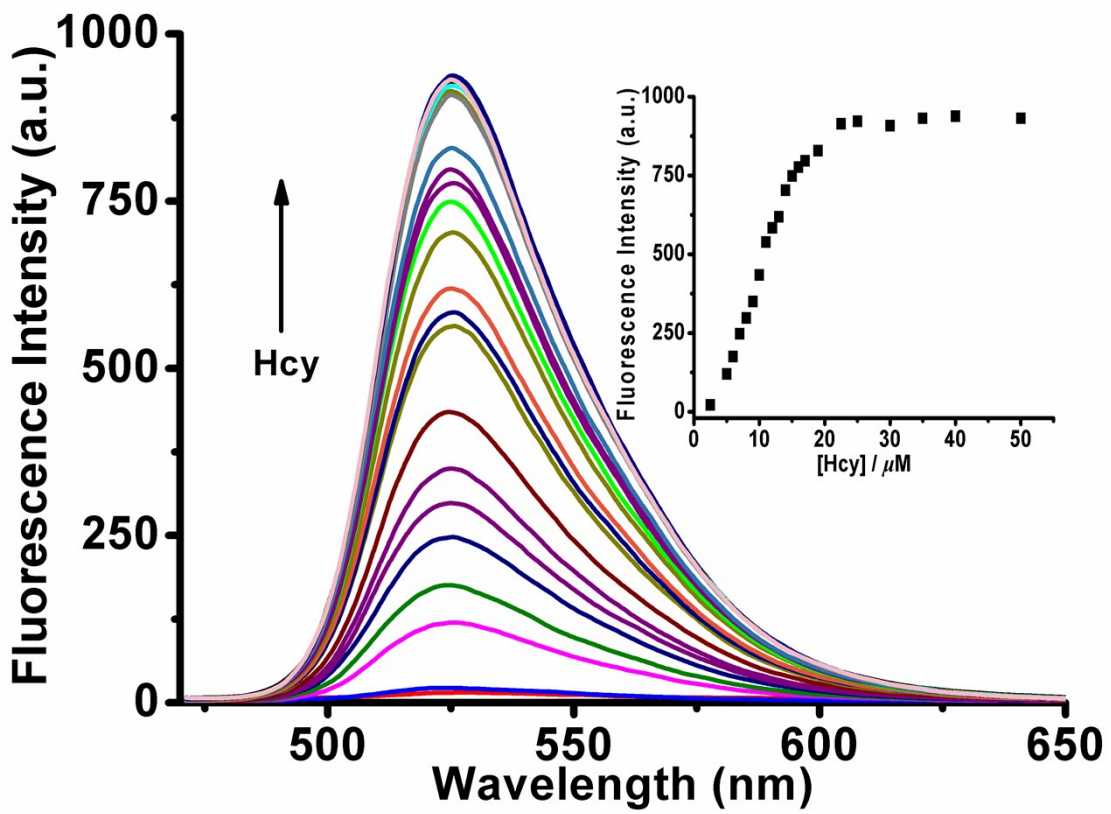


Figure S18. Fluorescence spectra of **1** + Cu^{2+} (10.0 μM + 10.0 μM) upon the addition of Hcy in HEPES buffer (pH = 7.0) solution (λ_{ex} = 491 nm). [Hcy] = 0, 2.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0, 15.0, 16.0, 17.0, 19.0, 22.5, 25.0, 30.0, 35.0, 40.0, 50.0 μM. Inset: Fluorescence intensity at 525 nm as a function of Hcy concentration.

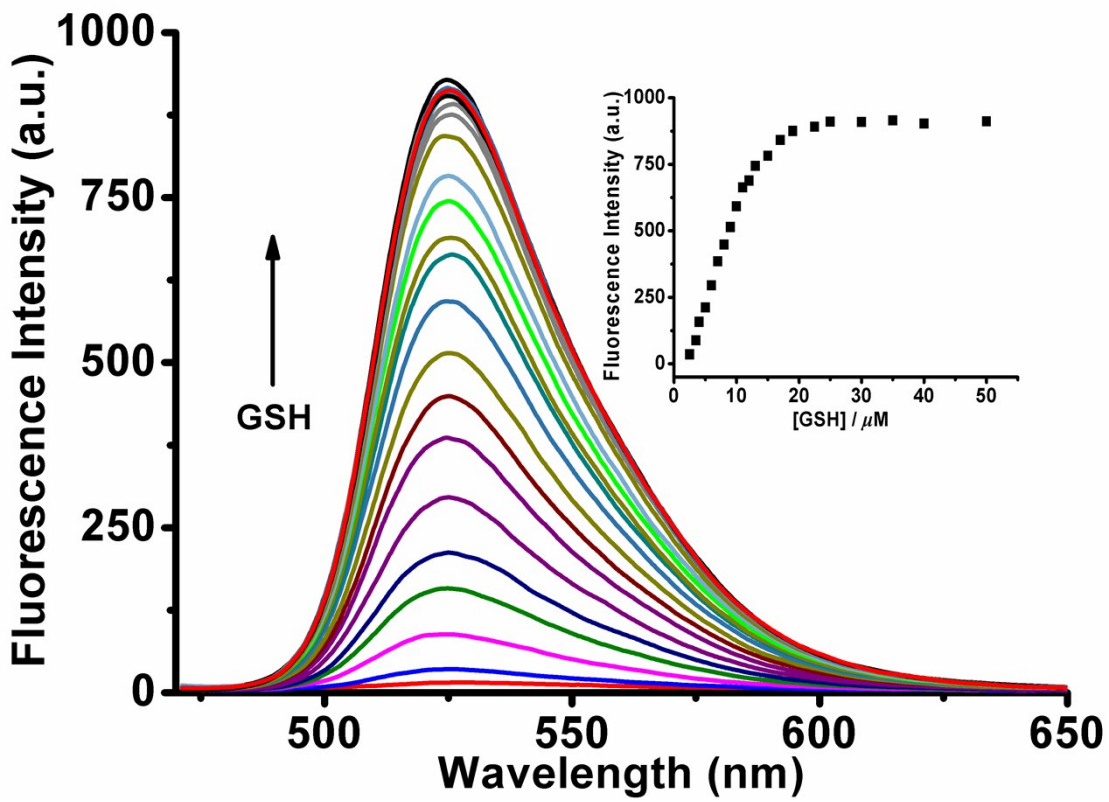


Figure S19. Fluorescence spectra of **1** + Cu^{2+} (10.0 μM + 10.0 μM) upon the addition of GSH in HEPES buffer (pH = 7.0) solution (λ_{ex} = 491 nm). [GSH] = 0, 2.5, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 15.0, 17.0, 19.0, 22.5, 25.0, 30.0, 35.0, 40.0, 50.0 μM. Inset: Fluorescence intensity at 525 nm as a function of GSH concentration.

8. ESI-MS spectra of 1 + Cu^{2+} upon the addition of Cys, Hcy or GSH.

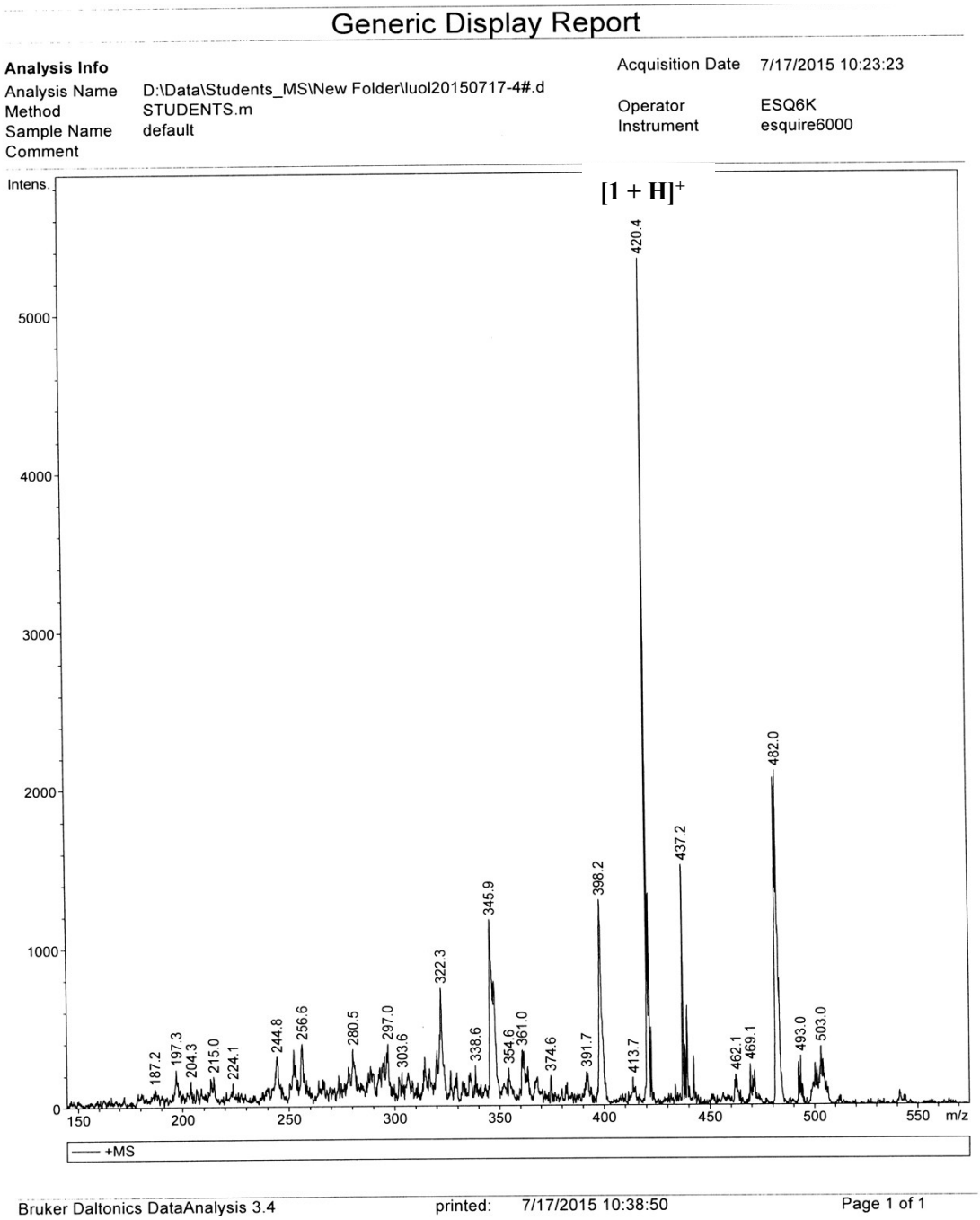


Figure S20. ESI-MS spectrum of **1** + Cu^{2+} upon the addition of Cys.

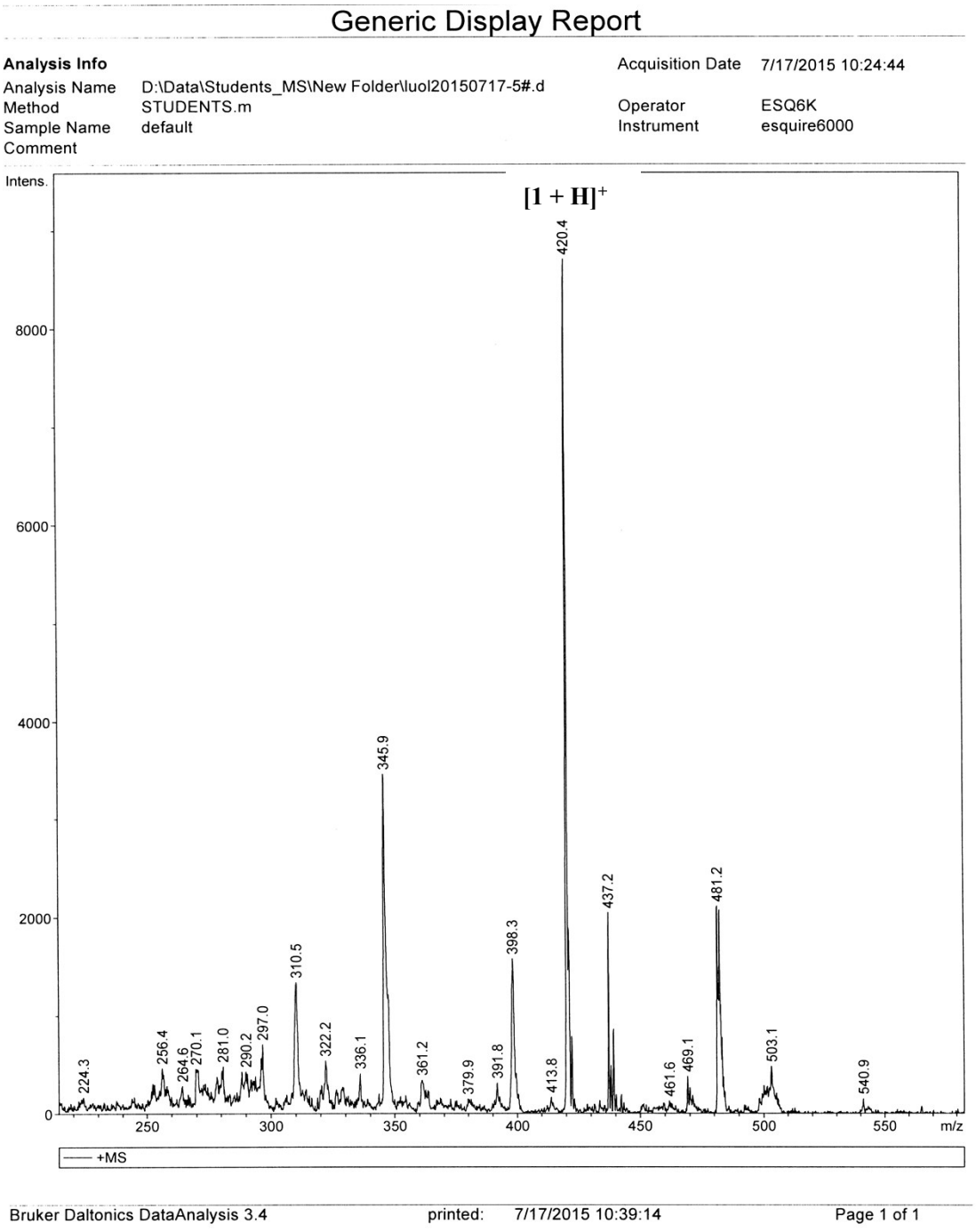


Figure S21. ESI-MS spectrum of **1** + Cu^{2+} upon the addition of Hcy.

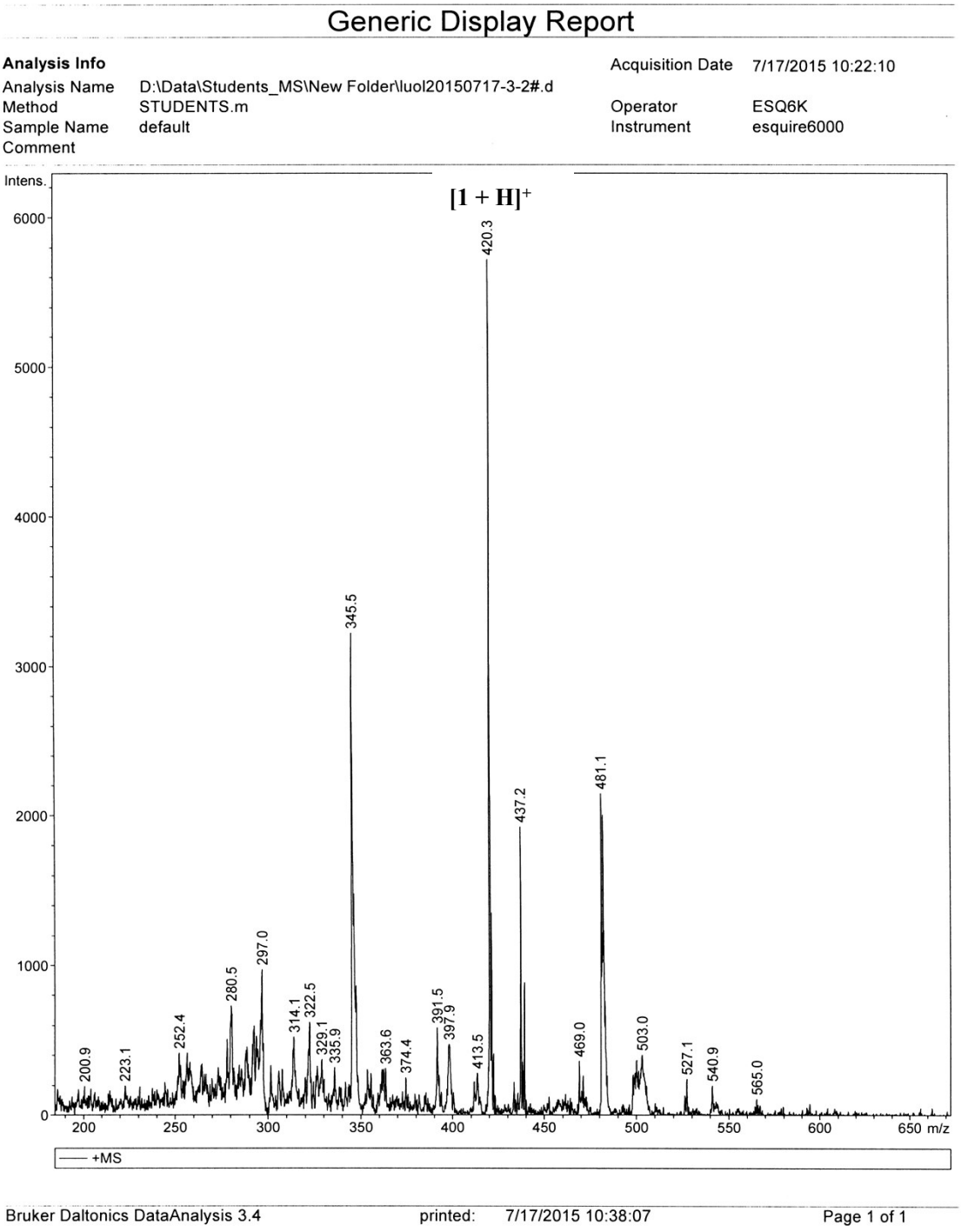


Figure S22. ESI-MS spectrum of **1** + Cu^{2+} upon the addition of GSH.

9. Linear concentration range of 1 + Cu^{2+} with Cys, Hcy or GSH in HEPES buffer solution.

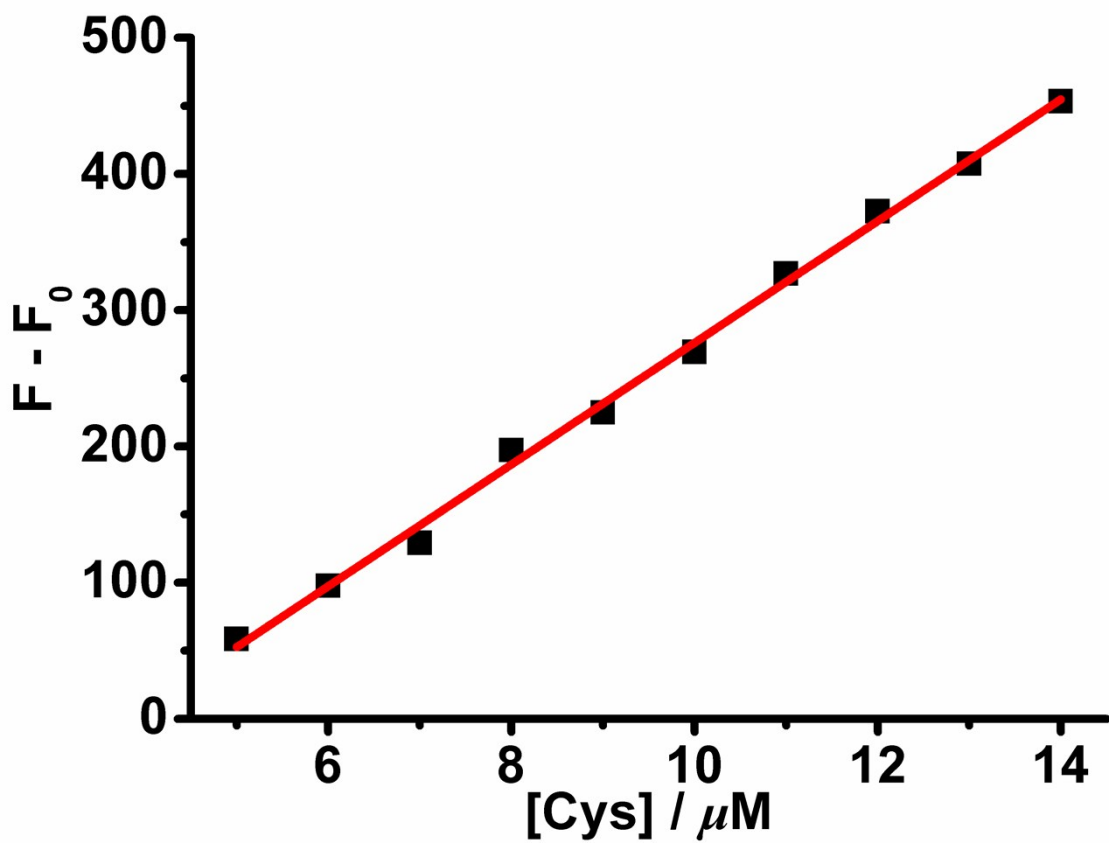


Figure S23. Linear concentration range of **1** + Cu^{2+} (10.0 μM + 10.0 μM) with Cys in HEPES buffer (pH = 7.0) solution (λ_{em} = 425 nm).

The result of the analysis as follows:

Linear Equation: $Y = -170.83677 + 44.68643 \times X$ $R = 0.9966$

$S = 4.47\times10^{7}$ $\delta = \sqrt{\frac{\sum\left(F_0 - \overline{F_0}\right)^2}{N-1}} = 1.7276 (N = 10)$ $K = 3$

$LOD = K \times \delta / S = 1.160 \times 10^{-7}$ M = 0.116 μM

F_0 is the fluorescence intensity at 525 nm of **1** + Cu^{2+}.

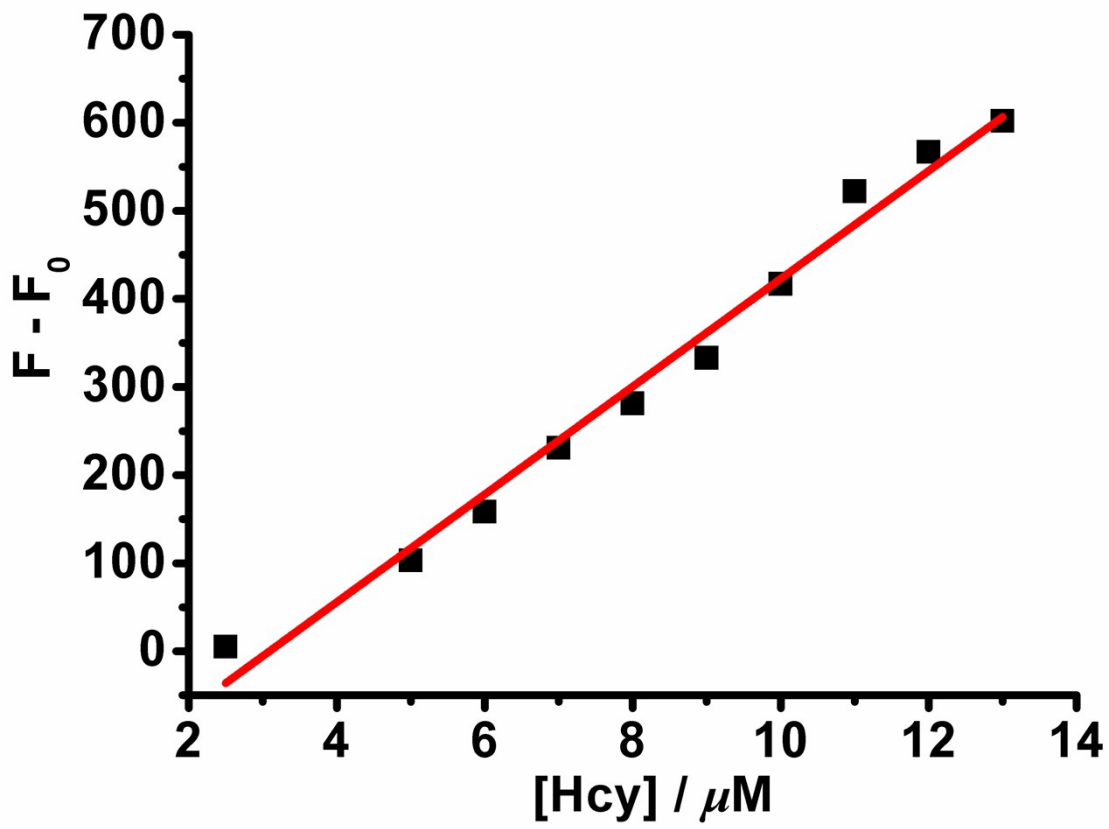


Figure S24. Linear concentration range of **1** + Cu^{2+} (10.0 μM + 10.0 μM) with Hcy in HEPES buffer (pH = 7.0) solution (λ_{em} = 425 nm).

The result of the analysis as follows:

Linear Equation: $Y = -189.10488 + 61.19751 \times X$ $R = 0.9834$

$S = 6.12 \times 10^7$ $\delta = \sqrt{\frac{\sum(F_0 - \overline{F_0})^2}{N-1}} = 0.7425(N = 10)$ $K = 3$

$LOD = K \times \delta / S = 3.640 \times 10^{-8}$ M = 0.0364 μM

F_0 is the fluorescence intensity at 525 nm of **1** + Cu^{2+}.

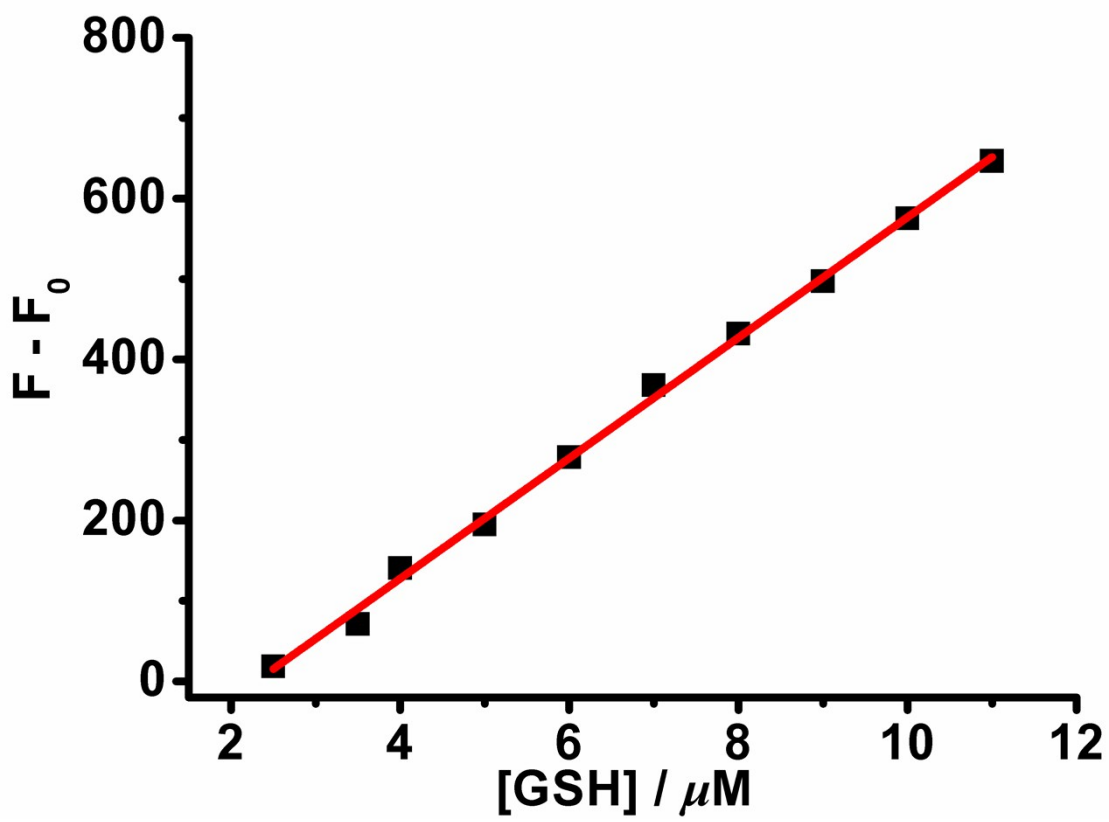


Figure S25. Linear concentration range of **1** + Cu^{2+} (10.0 μM + 10.0 μM) with GSH in HEPES buffer (pH = 7.0) solution (λ_{em} = 425 nm).

The result of the analysis as follows:

Linear Equation: $Y = -171.72846 + 74.84355 \times X$ $R = 0.9975$

$S = 7.48 \times 10^7$ $\delta = \sqrt{\frac{\sum\left(F_0 - \overline{F_0}\right)^2}{N-1}} = 0.6098(N = 10)$ $K = 3$

$LOD = K \times \delta / S = 2.444 \times 10^{-8}$ M = 0.0244 μM

F_0 is the fluorescence intensity at 525 nm of **1** + Cu^{2+}.

10. The selectivity of 1 + Cu^{2+} for Hcy or GSH.

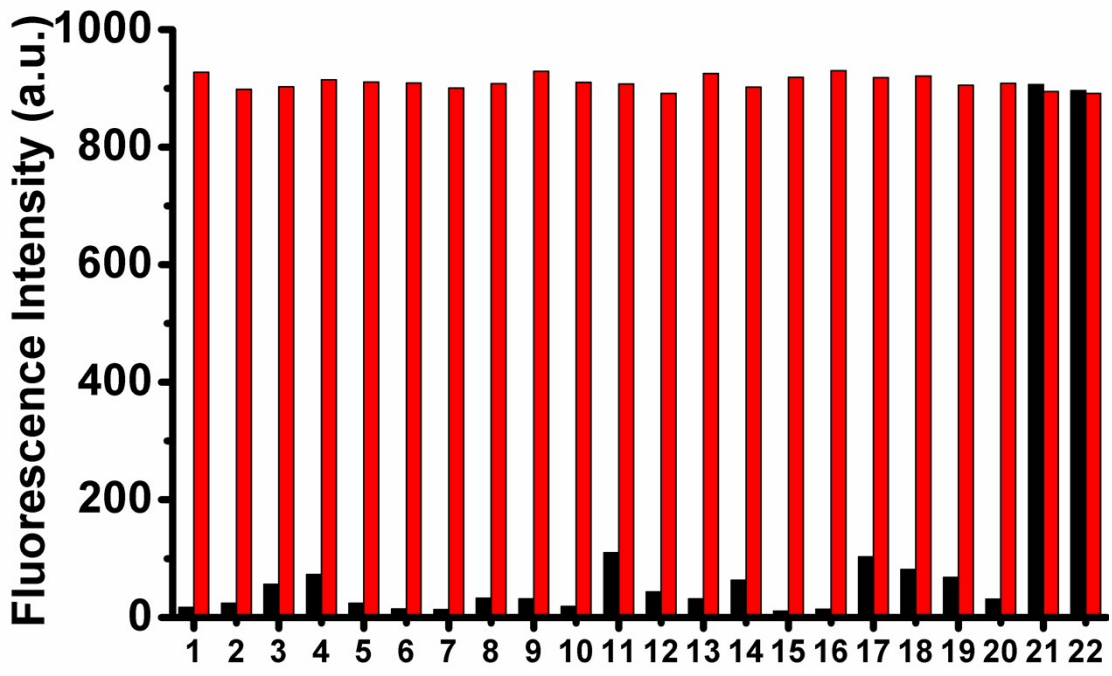


Figure S26. The selectivity of **1** + Cu^{2+} (10.0 μM + 10.0 μM) (λ_{em} = 525 nm). The black bars represent the emission intensity of **1** + Cu^{2+} in the presence of other amino acids (125.0 μM). The red bars represent the emission intensity that occurs upon the subsequent addition of 50.0 μM of Hcy to the above solution. From 1 to 22: none, Arg, Ser, Phe, Val, Pro, Tyr, Gln, Asn, Gly, Trp, Glu, Ile, Asp, His, Ala, Thr, Lys, Met, Leu, Cys, GSH.

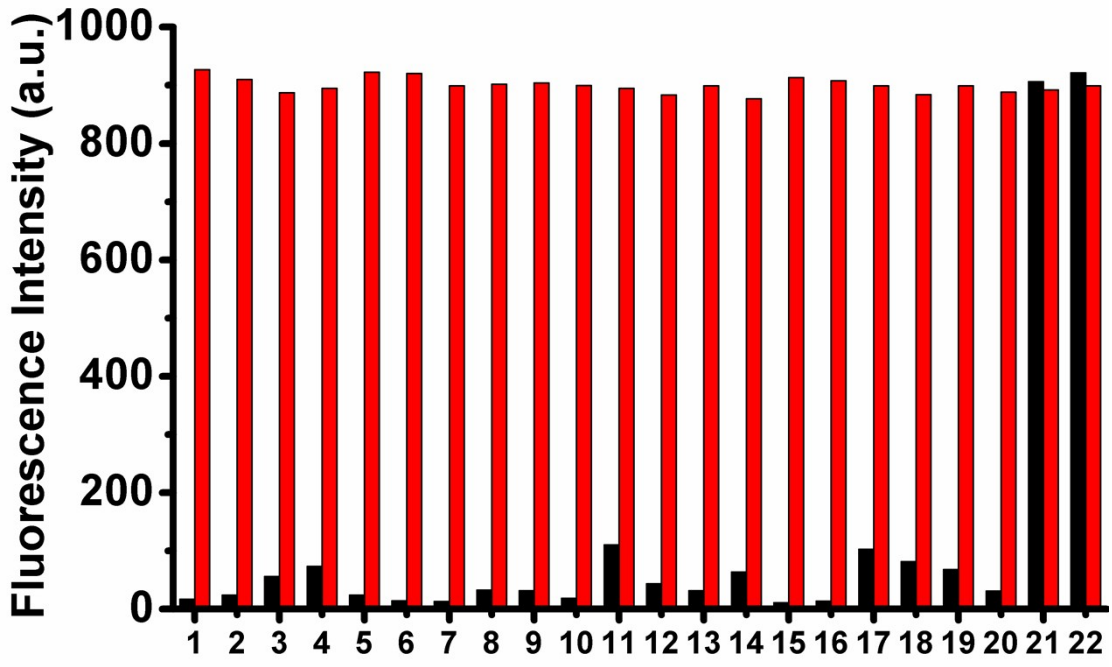


Figure S27. The selectivity of **1** + Cu^{2+} (10.0 μM + 10.0 μM) (λ_{em} = 525 nm). The black bars represent the emission intensity of **1** + Cu^{2+} in the presence of other amino acids (125.0 μM). The red bars represent the emission intensity that occurs upon the subsequent addition of 50.0 μM of GSH to the above solution. From 1 to 22: none, Arg, Ser, Phe, Val, Pro, Tyr, Gln, Asn, Gly, Trp, Glu, Ile, Asp, His, Ala, Thr, Lys, Met, Leu, Cys, Hcy.

11. Optimized structures, cartesian coordinates and computed total energies of probe 1 and 1 + Cu^{2+}.

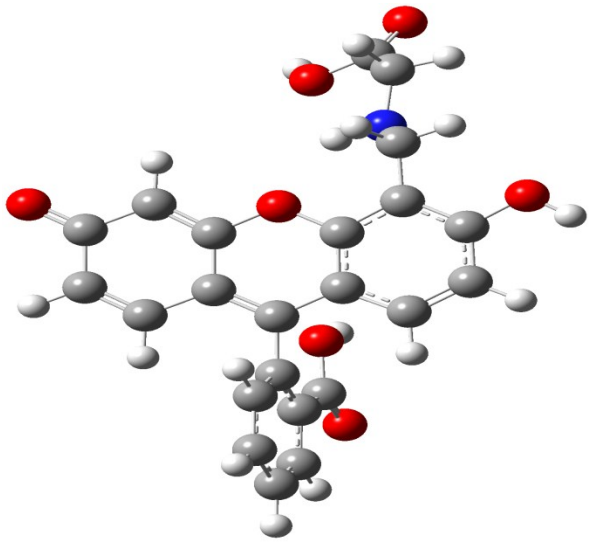


Figure S28. Optimized structure of **1**.

B3LYP/6-311+G in gas phase, E = – 1467.96943053 a.u.

charge = 0 Multiplicity = 1

C	1.5163409744	3.8052394644	-0.4109409205
C	1.9947951380	2.5396791550	-0.3415238848
C	1.1287166496	1.3874517078	-0.4995719995
C	-0.2770946969	1.6793009606	-0.7171489481
C	-0.7798727024	2.9367447270	-0.7927171520
C	0.0936189830	4.0873826300	-0.6445350918
C	1.5621016908	0.0809936081	-0.4274062836
C	-0.7404771514	-0.7072691355	-0.8166607061
C	0.6227938411	-1.0061630552	-0.6087199650
C	0.9835461992	-2.3698255273	-0.5853998862
C	0.0303059512	-3.3599652633	-0.7569339405
C	-1.3159637307	-3.0106708378	-0.9548492139
C	-1.7322216924	-1.6755249167	-0.9920608076
H	2.1628728397	4.6638829951	-0.2930941151
H	3.0467043107	2.3619362898	-0.1653500887
H	-1.8332626280	3.1071886600	-0.9561944524
H	0.3206087498	-4.4029740257	-0.7374335762
C	5.1790293306	-0.5457465215	-1.3305185453
C	5.7680622286	-0.8554127368	-0.1022166052

C	4.9885875649	-0.8581169120	1.0496695115
C	3.6151826281	-0.5550086739	1.0014328538
C	3.0139533232	-0.2408016919	-0.2414240169
C	3.8178715299	-0.2453487042	-1.3931757076
H	5.7725801379	-0.5364267749	-2.2348881311
H	6.8219735257	-1.0890809762	-0.0427839813
H	5.4214815086	-1.0900604475	2.0119102224
H	3.3628110771	-0.0056864091	-2.3446831985
C	2.9099300293	-0.5864884662	2.3077997033
O	3.4418228273	-0.9013711789	3.3791500645
C	-3.1768735600	-1.2872867420	-1.2004358209
N	-3.7391916129	-0.6387139910	-0.0019307294
H	-3.2606347662	0.2264646409	0.2241228901
C	-5.1920521387	-0.4582872114	-0.0478574517
C	-5.7008041032	0.2892118733	1.1539010351
O	-6.7371426537	0.0469219494	1.7691592379
O	-4.8800031772	1.3511952403	1.4797509196
H	-5.5327054850	0.0991809726	-0.9412657901
H	-5.6900870314	-1.4265461147	-0.0637358841
H	-3.2551374031	-0.6409939598	-2.0899527978
H	-3.7515231997	-2.1888035899	-1.4007056278
O	-0.3364774390	5.2754886486	-0.7099336518
O	-2.2969052066	-3.9790238576	-1.1377667616
O	-1.1730480165	0.6113506608	-0.8533697801
O	1.5830049750	-0.2297640117	2.2515177708
H	2.0174471174	-2.6435984149	-0.4336747163
H	1.1830208281	-0.2682309103	3.1427640440
H	-5.2211667896	1.8412555163	2.2533962638

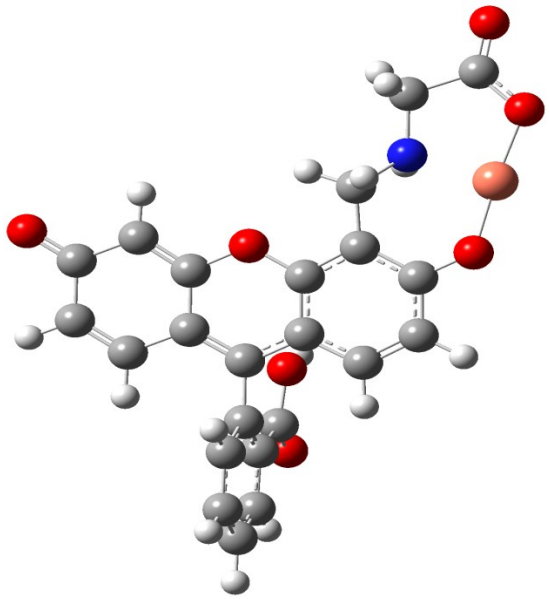


Figure S29. Optimized structure of 1•Cu^{2+} complex.

B3LYP/gen in gas phase, E = – 1662.93824785 a.u.

charge = 0 Multiplicity = 2

C	3.8355998769	3.3535153252	-0.0441949229
C	3.7254987243	2.0 072219386	-0.1494170608
C	2.4374668333	1.3556440913	-0.2786098534
C	1.2847103981	2.2347903427	-0.2842293018
C	1.3682664191	3.5837304762	-0.1819236144
C	2.6616980731	4.2365629481	-0.0537254072
C	2.2666374236	-0.0110749996	-0.3739653027
C	-0.1734998281	0.2935027518	-0.5143443357
C	0.9424432638	-0.5709837086	-0.5053213617
C	0.6803082914	-1.9572705873	-0.6164106205
C	-0.6076292736	-2.4407861525	-0.7171470738
C	-1.7126434658	-1.5569957610	-0.7287643915
C	-1.4916995870	-0.1561520826	-0.6355800906
H	4.7964324389	3.8388445140	0.0532403355
H	4.6083958511	1.3838418657	-0.1380129810
H	0.4832668950	4.2011384190	-0.1942186910
H	-0.8097565731	-3.4984993806	-0.7923275572

C	5.1110447659	-2.0044212326	-1.8636301233
C	5.6957350690	-2.6134430214	-0.7511679107
C	5.1637932532	-2.3846628107	0.5126535171
C	4.0438500722	-1.5524973841	0.6924171202
C	3.4511268090	-0.9292134683	-0.4310668384
C	4.0030799593	-1.1730493650	-1.6994047609
H	5.5145370456	-2.1707267633	-2.8531345042
H	6.5564364391	-3.2567960029	-0.8675607470
H	5.5997020778	-2.8390687508	1.3902164269
H	3.5550611665	-0.6970685945	-2.5606690051
C	3.5870323429	-1.3984988507	2.0950766548
O	4.1645448082	-1.8824327251	3.0750937385
C	-2.6305646118	0.8286237811	-0.7204972457
N	-3.7019861510	0.5402323354	0.3011823377
H	-3.2862837827	0.4796423848	1.2297470487
C	-4.8933324328	1.4509570201	0.3118554140
C	-6.1822124931	0.6766983917	0.6551702773
O	-7.1798196703	1.2745747728	1.0632670660
O	-6.1167596609	-0.6441037169	0.4260518005
H	-4.7594413696	2.2766041608	1.0085011022
H	-5.0079210931	1.8701284890	-0.6888772609
H	-2.2549515299	1.8410706878	-0.5811013280
H	-3.1190949831	0.7764759628	-1.6963651984
O	2.7778983347	5.4900899586	0.0445990238
O	-2.9549879555	-2.0732372938	-0.8236304656
O	0.0040225047	1.6683735963	-0.4069703334
O	2.4310971972	-0.6668418534	2.2441247148
H	1.5113937433	-2.6471624787	-0.6108891776
H	2.2014730670	-0.5969558822	3.1911005014
Cu	-4.4741286825	-1.2984313474	-0.1376796141

13. The controlled experiment of bioimaging in living cells.

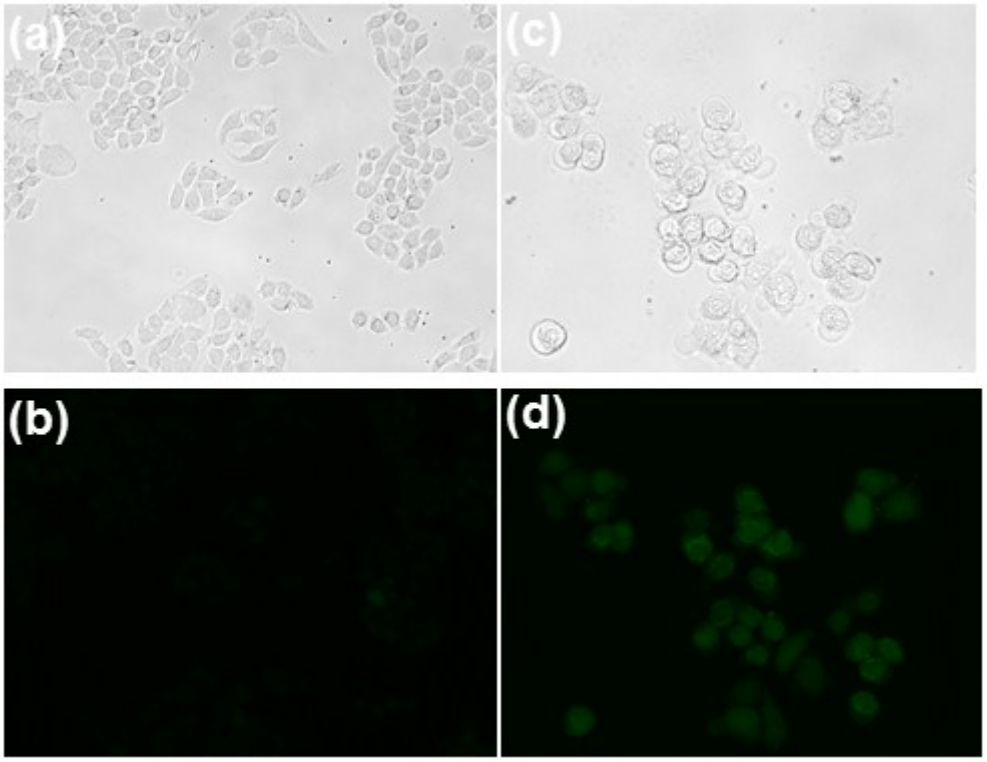


Figure S30. (a) bright field and (b) fluorescence images of HepG2 cells pretreated with 500.0 μM of NEM for 30 min and then incubated with **1** (50.0 μM) for 3 h, and finally incubated with Cu^{2+} (10 equiv) for 15 min. (c) bright field and (d) fluorescence images of HepG2 cells pretreated with 500.0 μM of NEM for 30 min and incubated with **1** (50.0 μM) for 3 h, and then incubated with Cu^{2+} (10 equiv) for 15 min, and finally incubated with GSH (50.0 μM) for 15 min.