Supporting Information

Colorimetric and Near-infrared Fluorescence Turn-on Molecular Probe for Direct and Highly Selective Detection of Cysteine in Human Plasma

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1. Synthesis and characterization

1,4,4'-Trimethyl-2, 2'-dipyridylium iodide. 4,4'-Dimethyl-2, 2'-dipyridyl (1.1054g, 6 mmol) was dissolved in an excess of 5mL of methyl iodide. The mixture was refluxed at 40 °C for 20 hours. The excess methyl iodide was removed by rotary evaporation. The separated precipitate was washed with acetone and hexane. An off-white powder was obtained. This synthesis had a yield of 1.85g (95%) with a melting point of 201-202 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ (TMS, ppm): 2.52 (3H, s), 2.68 (3H, s), 4.21 (3H, s), 7.56 (1H, d, J = 3.2 Hz), 7.82 (1H, s), 8.07 (1H,d, J = 6.2 Hz), 8.16 (1H, s), 8.71 (1 H, d, J = 3.2 Hz), 9.02 (1H, d, J = 6.2 Hz). MS (ESI, MeOH) *m/z* (%): 199.3 $[M]^{+}$, (100%).

M1. To a 30 mL of methanol containing 1,4,4'-trimethyl-2, 2'-dipyridylium iodide (3.3 g, 10 mmol), 200 mL of a methanol solution with 2 molar equivalent amounts of Li-TCNQ (4.22 g, 20 mmol) was added and the resulting green colored mixture was refluxed for 1 hour. Next, 5mL of a methanol solution of 1-N-piperidineethanol (1.3g, 10mmol) was added in small increments over the course of 1 hour. The reaction was monitored using the UV-Vis spectra of the reaction mixture in DMF and typically the reaction can be finished within 24 hours. To do this, a small amount of the reaction mixture was taken out and dissolved in DMF for a UV-Vis measurement. The reaction was stopped until the charge-transfer band of the chromophore (~680nm) was much stronger than the characteristic peaks of the radical anion (~ 430,740 and 830nm). The solution gradually turned to a blue color. After the reaction mixture was cooled to room temperature, the green precipitate was filtered and washed with methanol and ethyl ether.

The final product was a green powder collected in 60% (2.28 g.). ^{1}H NMR (400 MHz, DMSO-d_6) δ (TMS, ppm): 2.48 (3H, s), 4.19 (3H, s), 6.87 (2H,d, J = 8.7 Hz), 7.56 (1H,d, J = 5.5 Hz), 7.58 (2H,d, J = 8.7 Hz), 7.80 (1H, s), 7.88 (1H, s), 8.38 (1H, s), 8.39 (2H, d, J = 6.6 Hz), 8.72 (1H, d, J = 5.5), 9.01 (1H, d, J = 6.6). ^{13}C NMR (75 MHz, DMSO- d_6) δ (TMS, ppm): 152.28, 150.45, 150.00, 149.73, 147.78, 147.55, 128.16, 127.98, 127.13, 126.95, 125.59, 123.80, 123.47, 121.59, 120.36, 119.02, 117.32, 46.80, 21.12. TOF-HRMS (ESI, DMF/Acetonitrile 1:1) *m/z* (%): 376.1615 $[M+H]^+$, 396.1422 MNa^+. IR (KBr, cm^{-1}): 2226, 2173 and 2137 ($\nu_{C\equiv N}$), 1629, 1600 (C=C) . UV – Vis (DMF): λ_{max} (nm): 680 nm.

M1-Cu complex. M1 (0.375 g, 1 mmol) was dissolved in 10 mL of DMF; 5mL of a methanol solution of copper (II) chloride (0.67 g, 5 mmol) was then added drop-wise with continuous stirring for 2 hours. At room temperature, a yellow precipitate was isolated gradually; the complex was filtered and washed with ethanol and anhydrous ethyl ether. It was then dried in an oven at 60℃ until a constant weight was obtained with a yield of 80%. m. p > 280°C. TOF-HRMS (ESI, MeOH) *m/z* (%): 375.042 $[M]^+$ (100%), 884.957 $[2M+CuCl_2]^+$ (20%), 849.119 $[2M + CuCl]^+$ (2%), 407.081 $[2M+Cu]^{2+}$ (30.1%). IR (KBr, cm^{-1}): 2223, 2017 (C≡N). UV – Vis (DMF, λ_{max}): 435 nm.

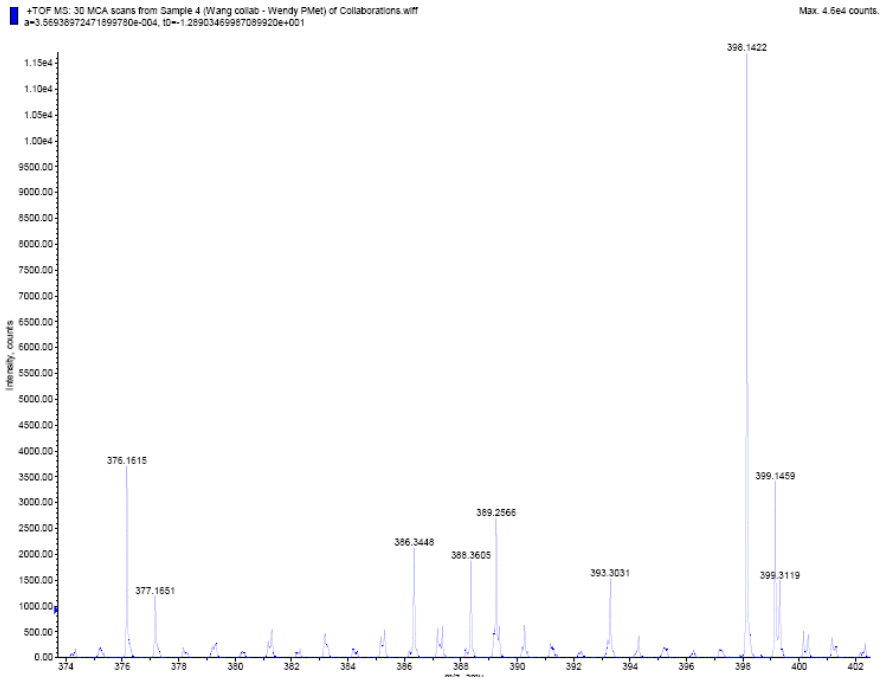


Figure S1.1. ESI mass spectrum of **M1.**

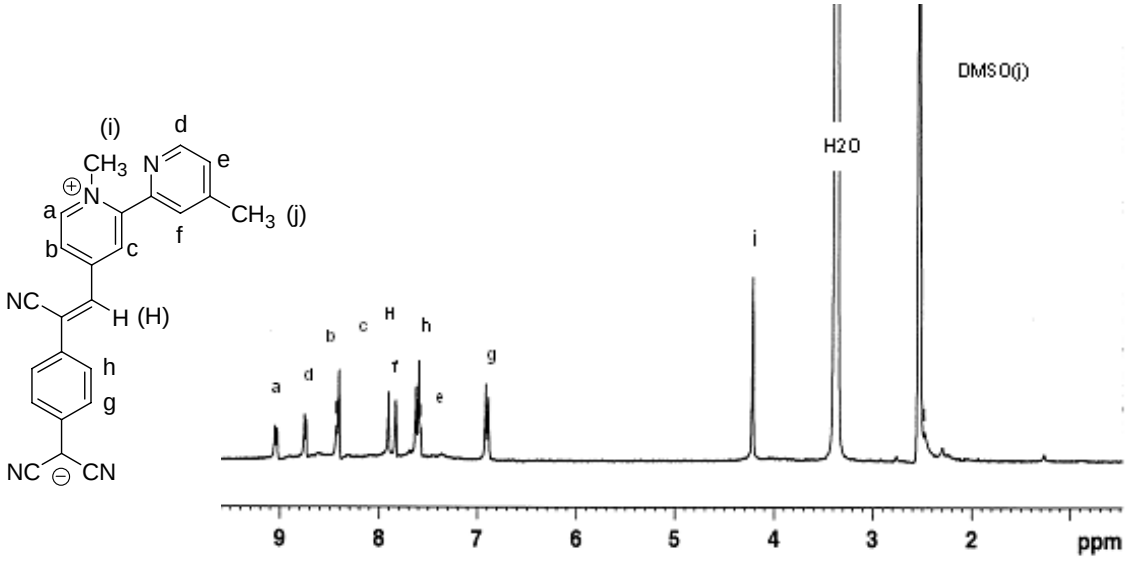


Figure S1.2. ^{1}H NMR (300 MHz) spectrum of **M1** in DMSO-d_6.

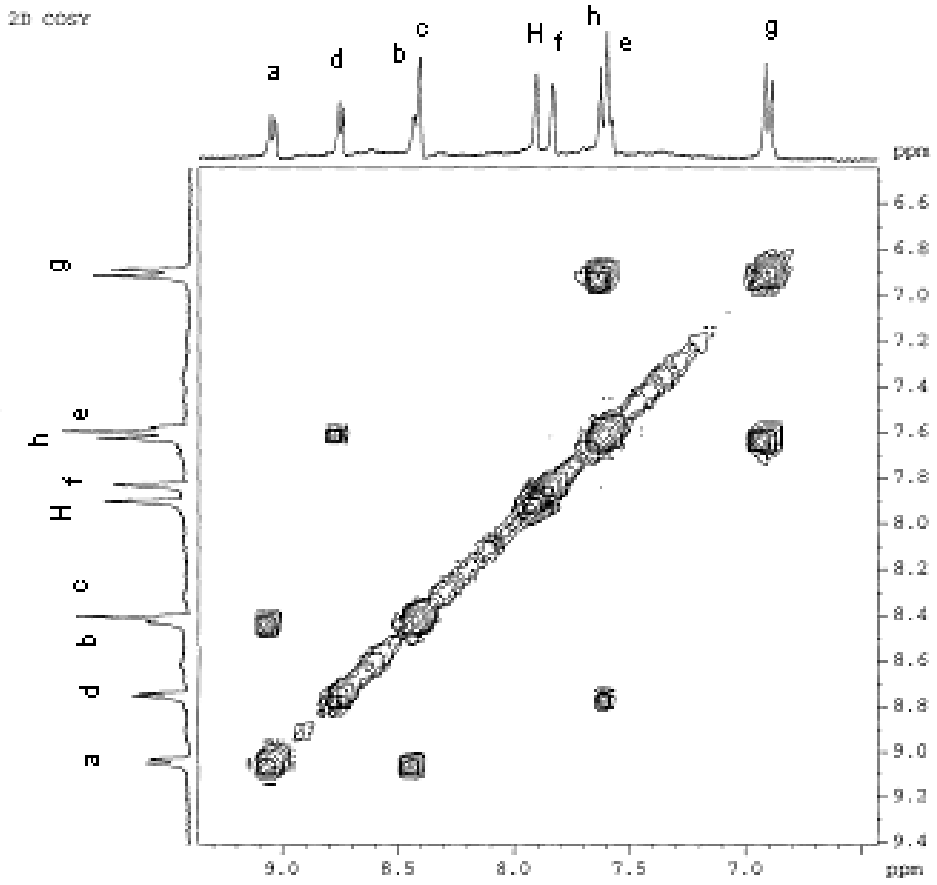


Figure S1.3. ^{1}H NMR (2D COSY) spectrum of aromatic protons of **M1** in DMSO-d_6.

2. Absorbance and fluorescence spectra, quantum yield measurement and negative solvatochromism of M1

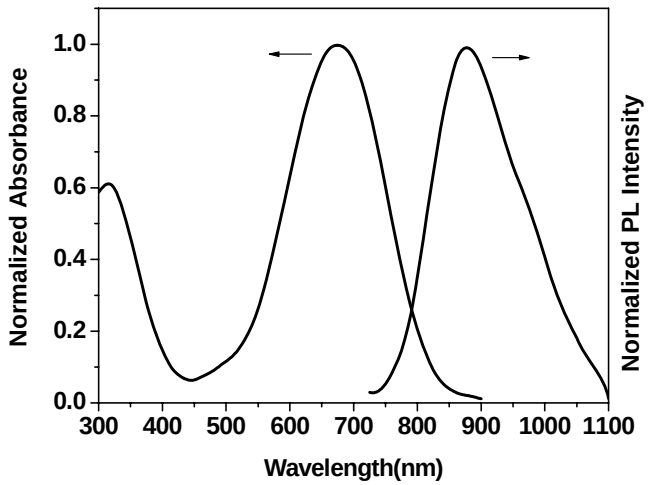


Figure S2.1. Normalized absorption and fluorescence spectra of **M1** in DMF (2 x 10^{-5} M).

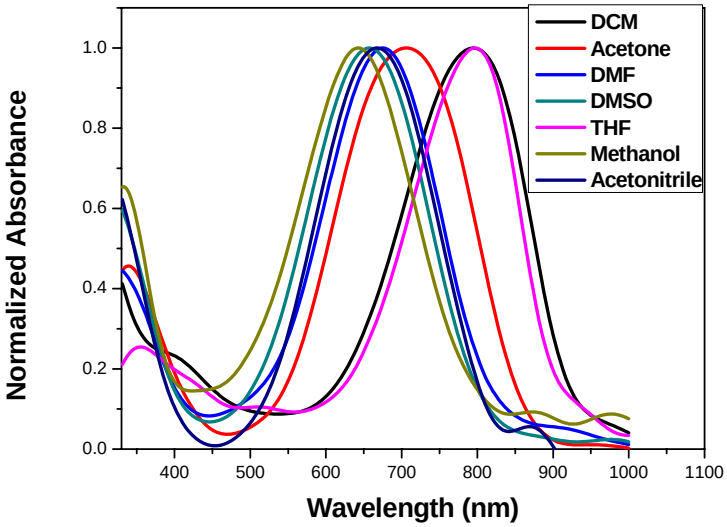


Figure S2.2. UV-Vis-NIR spectra of **M1** in different solvents.

Quantum yields measurement

The fluorescence emission spectra were measured with a PTI spectrofluorometer. The fluorescence emission spectra of **M1** (2 x 10^{-5} M) were measured at 25 °C, with excitation at 630 nm. The fluorescent quantum yield (ΦF) of **M1** were evaluated using a relative method with reference to a luminescence standard, IR-125 (Φ_F = 0.13 in DMSO).[1] The quantum yield of **M1** can be expressed by eq. 1.[2] where Φ is the quantum yield (subscript "r" stands for the reference and "x" for the sample), A is the absorbance at the excitation wavelength, I (λ) is the relative intensity of the exciting light at wavelength λ, n is the refractive index of the solvent for the luminescence, and D is the area (on an energy scale) of the luminescence spectra.

$$Q_x = Q_r\left(\frac{A_r(\lambda_r)}{A_x(\lambda_x)}\right)\left(\frac{I(\lambda_r)}{I(\lambda_x)}\right)\left(\frac{n_x^{\,2}}{n_r^{\,2}}\right)\left(\frac{D_x}{D_r}\right) \quad \text{(eq.1)}$$

The samples and the reference were excited at the same wavelength. The sample absorbance at the excitation wavelength was kept as low as possible to avoid fluorescence errors ($A_{exc} < 0.1$).

3. UV, IR and MS spectra of M1-Cu and binding constant of M1 with Cu^{2+}

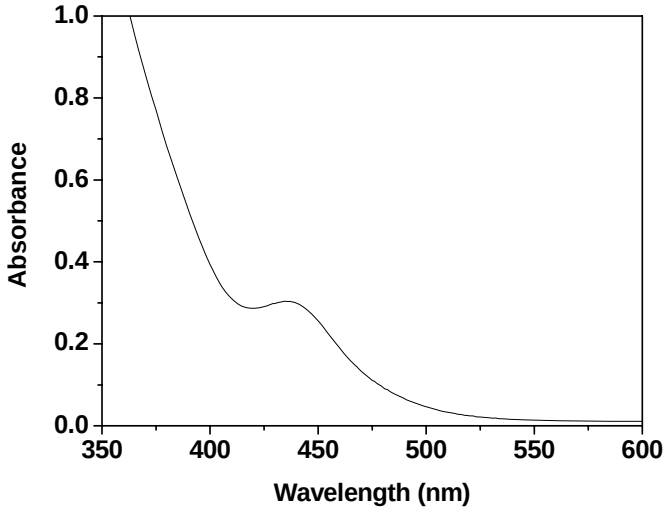


Figure S3.1. UV-Vis spectrum of **M1-Cu**.

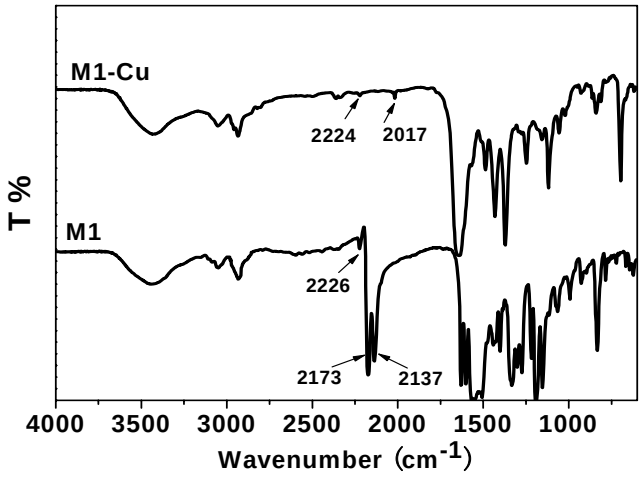


Figure S3.2. Baseline corrected FTIR spectra (4000-600 cm^{-1}) of **M1** and **M1-Cu**.

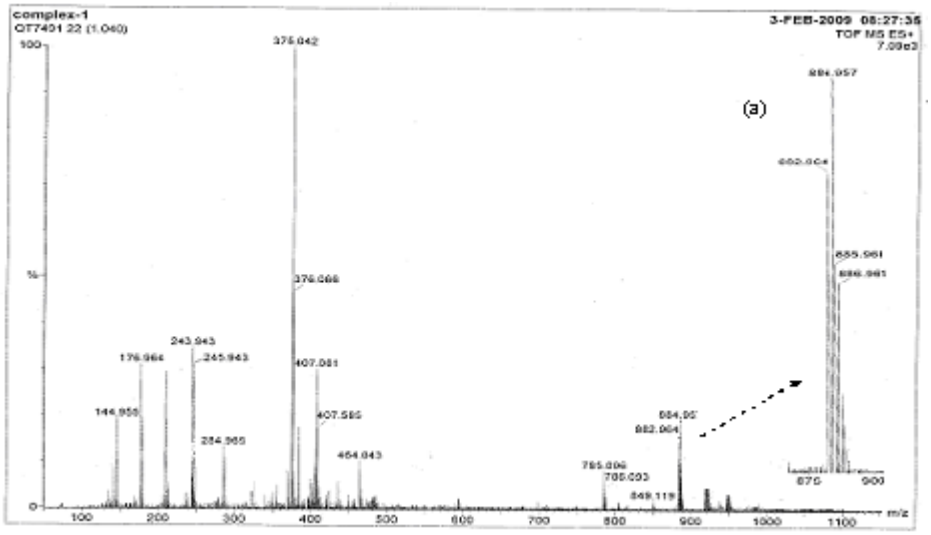


Figure S3.3. ESI mass spectrum of **M1-Cu**. Inset: (a) expanded peak of the complex.

3.3 Determination of binding constant of M1 with Cu^{2+}. To determine the binding strengths of the **M1**-Cu^{2+} donor-acceptor complex, a series of the **M1** donor solutions at a fixed concentration were mixed with the Cu^{2+} acceptor solutions at various concentrations. Absorbance of the resultant mixtures at 680 nm was measured. Fluorescence emission intensity of the resultant mixtures at 875nm was measured. All measurements were carried out in DMF: Methanol = 99:1 (v/v).

3.4 Equation for binding constant of M1 with Cu^{2+}. According to the donor-acceptor binding ratio (2:1) determined by TOF-HRMS of the **M1-Cu** complex, the reaction equation describing the model containing the 2:1 complex can be written:

$2A + B \rightleftharpoons A_2B$ (eq. 2.1)

Where A represents **M1**, B is Cu^{2+} and A_2B is **M1- Cu** complex.

This equilibrium reaction can be expressed in terms of the association constant Ka:

$$K_a = \frac{[A_2B]}{[A]^2[B]} \qquad \text{(eq. 2.2)}$$

When copper is introduced, the system experiences a shift in its equilibrium. The concentrations at equilibrium for all species are:

$$[A_2B] = \frac{(C-Y)}{2} \qquad [A] = Y \qquad [B] = X - \frac{C-Y}{2}$$

Where C is the original concentration of **M1**, (2×10^{-5}M), X is the concentration of Cu^{2+} in the solution, Y is the concentration of the **M1** in the solution at equilibrium and available for fluorescence emission. The formation constant can then be expressed as follows:

$$K_a = \frac{\left(\frac{(C-Y)}{2}\right)}{(Y)^2\left(X - \frac{(C-Y)}{2}\right)} \qquad \text{(eq. 2.3)}$$

This equation can be rearranged into a cubic form:

$$X = \frac{1}{2K_a}\left(\frac{C}{Y^2} - \frac{1}{Y} + C - Y\right) \qquad \text{(eq. 2.4)}$$

Letting $y = \frac{C}{Y^2} - \frac{1}{Y} + C - Y \qquad x = X$

Then, the final equation 2.5 is used to calculate the association constant for the **M1-Cu**:

$$y = 2K_a x \qquad \text{(eq. 2.5)}$$

The fluorescence intensity data were fitted to Eq 2.5, the value of *K*a was determined from the fittings for the fluorescence intensity.

3.5 Binding constant of the M1 with Cu^{2+}. The binding constant of the **M1** with Cu^{2+} was calculated using the linear fit method with Origin 7.5 (Origin-Lab Corporation).

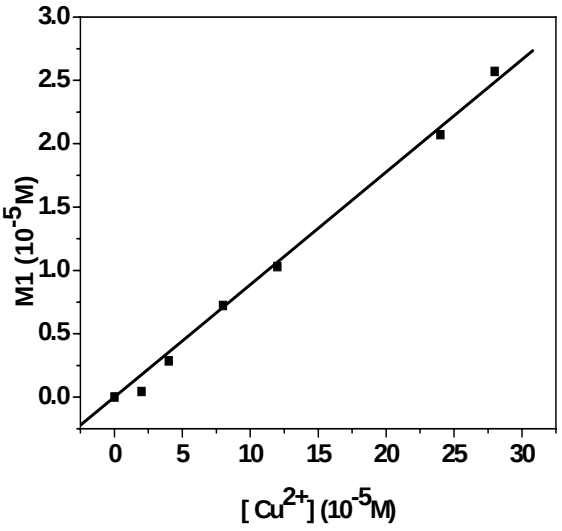


Figure S3.4. Binding constant analysis of **M1** at different concentration of Cu^{2+}.

$Y = B \times X$

Parameter	Value		Error
A	0		0
B	0.0888		0.0019
R	SD	N	P
0.99823	0.07586	7	<0.0001

$2K_a = 0.0888 \times 10^{10}$ $K_a = 4.44 \times 10^8 M^{-2}$.

The binding constant of **M1** with Cu^{2+} in DMF/MeOH was found to be 4.44 x 10^8 M^{-2}.

4. Absorption and fluorescence titration experimental details

Preparation of M1 and Cu^{2+} solution. M1 (1.8 mg) was dissolved in DMF to afford the stock solution with the concentration of 2 x 10^{-4} M. This stock solution was diluted to 2 x 10^{-5} M. To afford a 2 x 10^{-1}M solution, 2 mmol of copper (II) chloride was dissolved in methanol (10 mL).

Preparation of solutions of 18 amino acids. 5 mmol of each amino acid were dissolved in distilled water (25.0 mL) to afford a 2 x 10^{-1}M aqueous solution, except for D-Leucine (8 x 10^{-2} M) and L-tyrosine (8 x 10^{-3} M).

Preparation of solutions of L-cystine, propylamine, ethanethiol, ethyl sulfide, homocysteine, **L-glutathione and N-acetylcysteine.** 5 mmol of each compound was dissolved in distilled water (25.0 mL) to afford a 2 x 10^{-1} M aqueous solution, except for L-cystine (2 x 10^{-3} M).

Fluorescence titration of M1-Cu with L-cystine, propylamine, ethanethiol and ethyl sulfide. A solution of **M1** (2 x 10^{-5} M) was prepared in DMF. The solution of Cu^{2+} (2 x 10^{-1} M, 40 μL) was added to 10 mL of **M1** solution to quench the fluorescence. The solution of **M1-Cu** was placed in a quartz cell (10.0 mm width) and the fluorescence and absorption spectra were recorded. The prepared solutions of L-cystine, propylamine, ethanethiol or ethyl sulfide were introduced in portions up to 40 equiv of Cys (2 x 10^{-1}M) and the fluorescence intensity changes were recorded at room temperature each time (excitation wavelength: 670 nm).

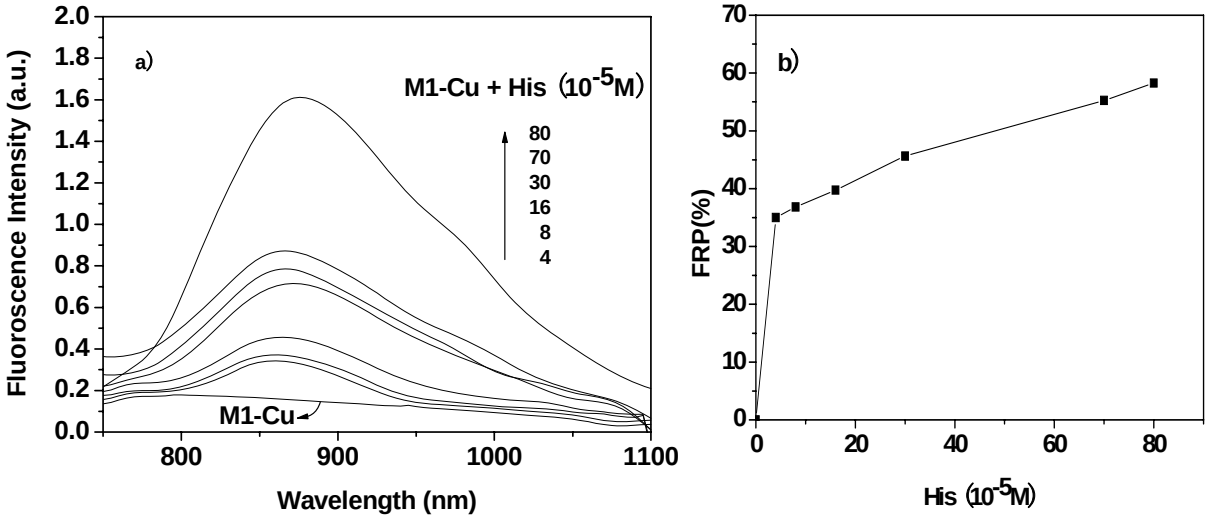


Figure S4.1. a) Changes in the fluorescence emission spectra of **M1**, **M1-Cu** and fluorescence titration of **M1-Cu** with increasing amounts of Histidine (His). The concentration of **M1** is fixed at 2 x 10^{-5}M. Excitation wavelength (nm): 670. b) FRP versus the concentration of His. Each spectrum is acquired 1 min after His addition.

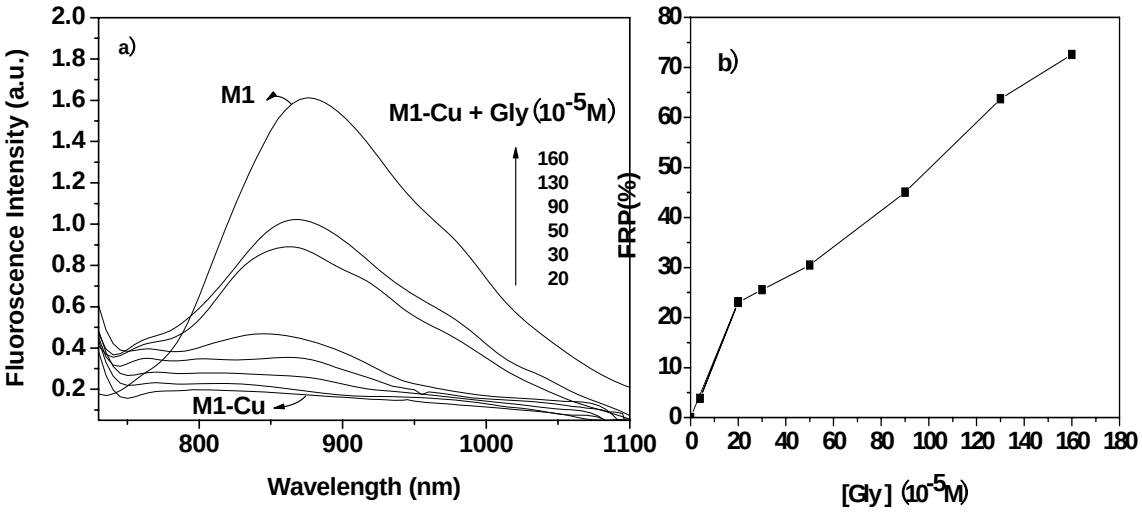


Figure S4.2. a) Changes in the fluorescence emission spectra of **M1**, **M1-Cu** and fluorescence titration of **M1-Cu** with increasing amounts of Glycine (Gly). The concentration of **M1** is fixed at 2 x 10^{-5} M. Excitation wavelength (nm): 670. b) FRP versus the concentration of Gly. Each spectrum is acquired 1 min after Gly addition.

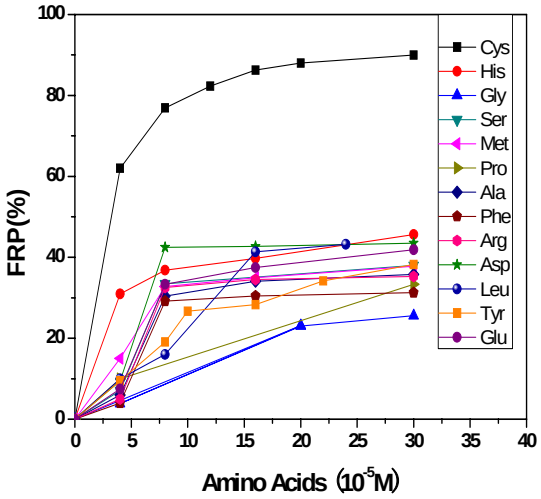


Figure S4.3. FRP with respect to the concentration of different α-amino acids.

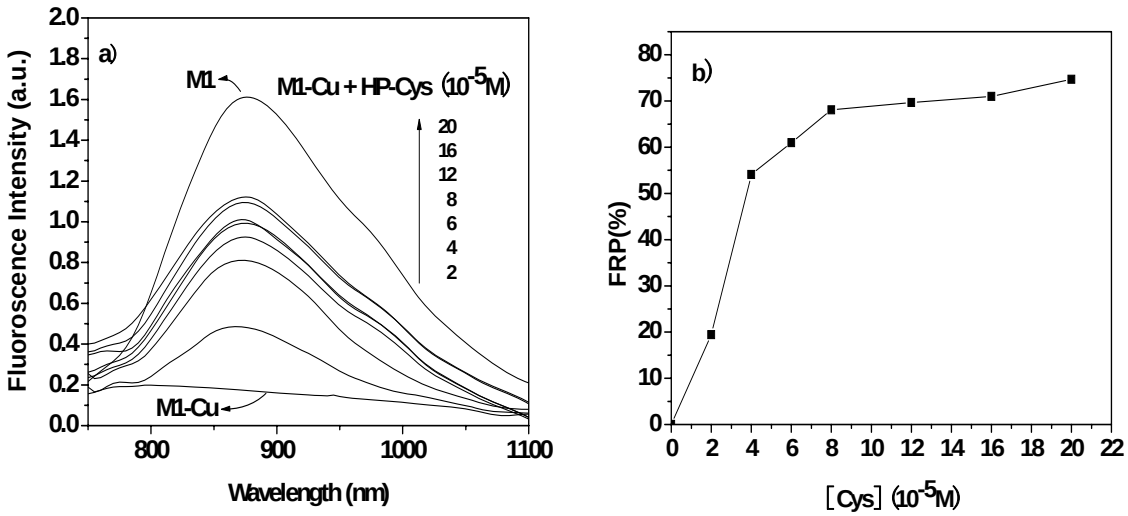


Figure S4.4. a) Changes in the fluorescence emission spectra of **M1**, **M1-Cu** and fluorescence titration of **M1-Cu** with increasing amounts of human plasma with Cys. The concentration of **M1** is fixed at 2 x 10^{-5}M. Excitation is at 670nm. b) FRP versus the concentration of human plasma with Cys.

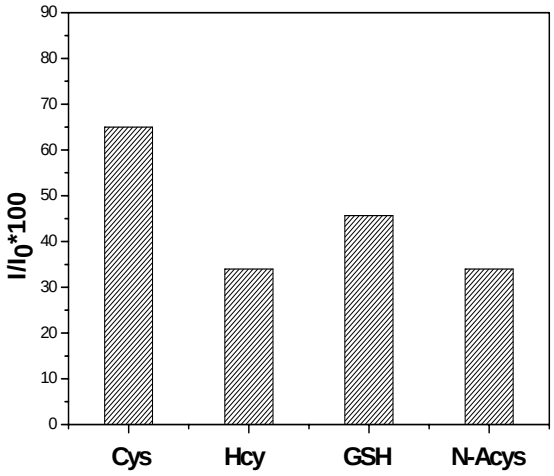


Figure S4.5. Fluorescence emission response of **M1-Cu** towards various bioactive thiols. Concentration of **M1** is 2 x 10^{-5} M; concentration of various bioactive thiols is 4 x 10^{-5} M.

5. Detection limit and response time measurements

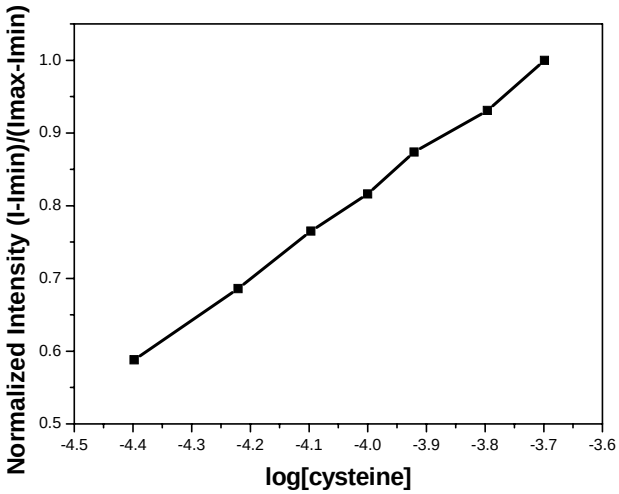


Figure S5.1 Fluorescence intensity of **M1-Cu** versus Cys concentration.

Y = A + B * X

Parameter	Value	Error		
A	3.16624	0.04127		
B	0.58665	0.01025		
R	SD	N	P	
0.99924	0.00609	7	<0.0001	For Cys: DL = 4.07×10^{-6}M

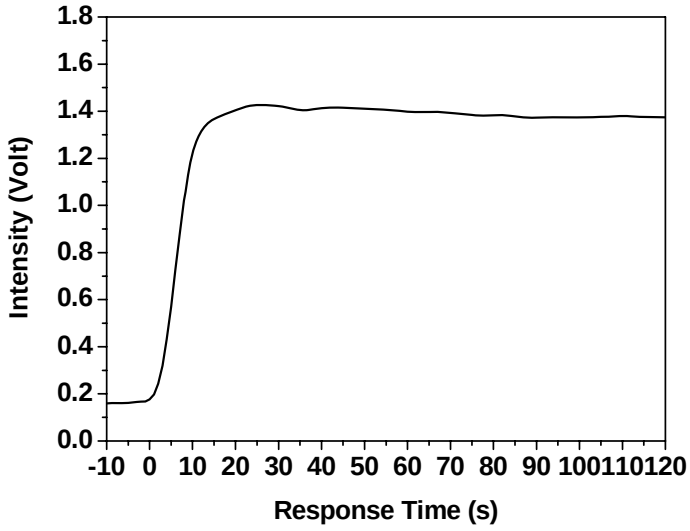


Figure* S*5.2 Response time measurement for a step change in Cys concentration from zero to 20 x 10^{-5} M.

6. Binding study

Determination of Cu^{2+}-amino acid binding constant. For the determination of binding strengths of the various Cu^{2+}-amino acid adducts, a series of **M1-Cu** complex solutions at a fixed concentration were mixed with the amino acid solutions at various concentrations. Absorption of the resultant mixtures at 680 nm was measured. Fluorescence intensity of the resultant mixtures at 875 nm was measured. All measurements were carried out in DMF: Methanol = 99:1 (v/v).

Binding constants of Cu^{2+} with amino acids using the Benesi-Hilderbrand method. Binding constants, K_B, were estimated from the ratio between the *y*-intercept and the slope of the linear line of best fit. The 1:2 donor-acceptor interactions were analyzed according to the Benesi-Hildebrand equation for spectrofluorometric titration (eq.1.6).

$$\frac{I_0}{I-I_0}=\left(\frac{c}{d-c}\right)^2\left(\frac{1}{K_B[substrate]^2}+1\right) \qquad \text{(eq. 1.6)}$$

Where I_O and I are luminescence intensity of **M1-Cu** at 875 nm, in the absence and presence of amino acid, respectively; c, d are constants. [substrate] is the concentration of the titrants (various amino acids). Binding constant calculation for representative amino acids were shown in Figure S6.

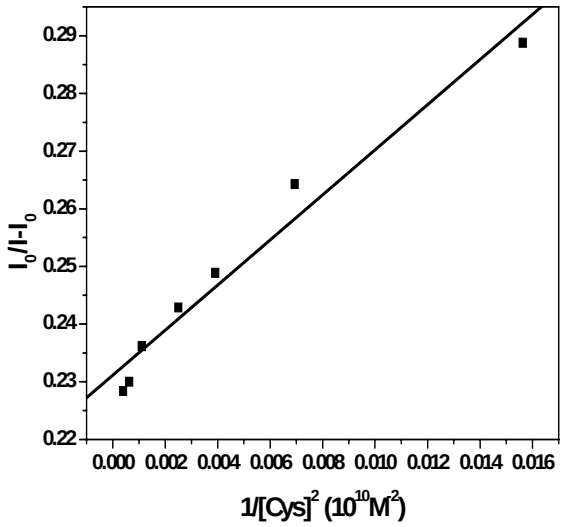


Figure S6. Benesi-Hilderbrand plot of **M1-Cu** with Cys.

Y = A + B × X

Parameter	Value	Error	
A	0.23112	0.00214	
B	3.90977	0.31887	
R	SD	N	P
0.98377	0.00425	7	<0.0001

The binding constant of **M1-Cu** with Cys in DMF/MeOH is

$K_B = A/B \times 10^{10} = 0.231/3.909 \times 10^{10} = 5.91 \times 10^8\ (M^{-2})$

Table S6.1. Binding constants of Cys and some amino acids with the copper (II) cation.

Compound	Binding Constant
M1	4.44×10^{8} (M^{-2})
Cys	5.91×10^{8} (M^{-2})
His	5.54×10^{7} (M^{-2})
Met	2.63×10^{7} (M^{-2})
Gly	8.01×10^{5} (M^{-2})

Chart S6.1 Structures of α-amino acids used in competitive study of fluorescence turn-on with **M1-Cu** complex.

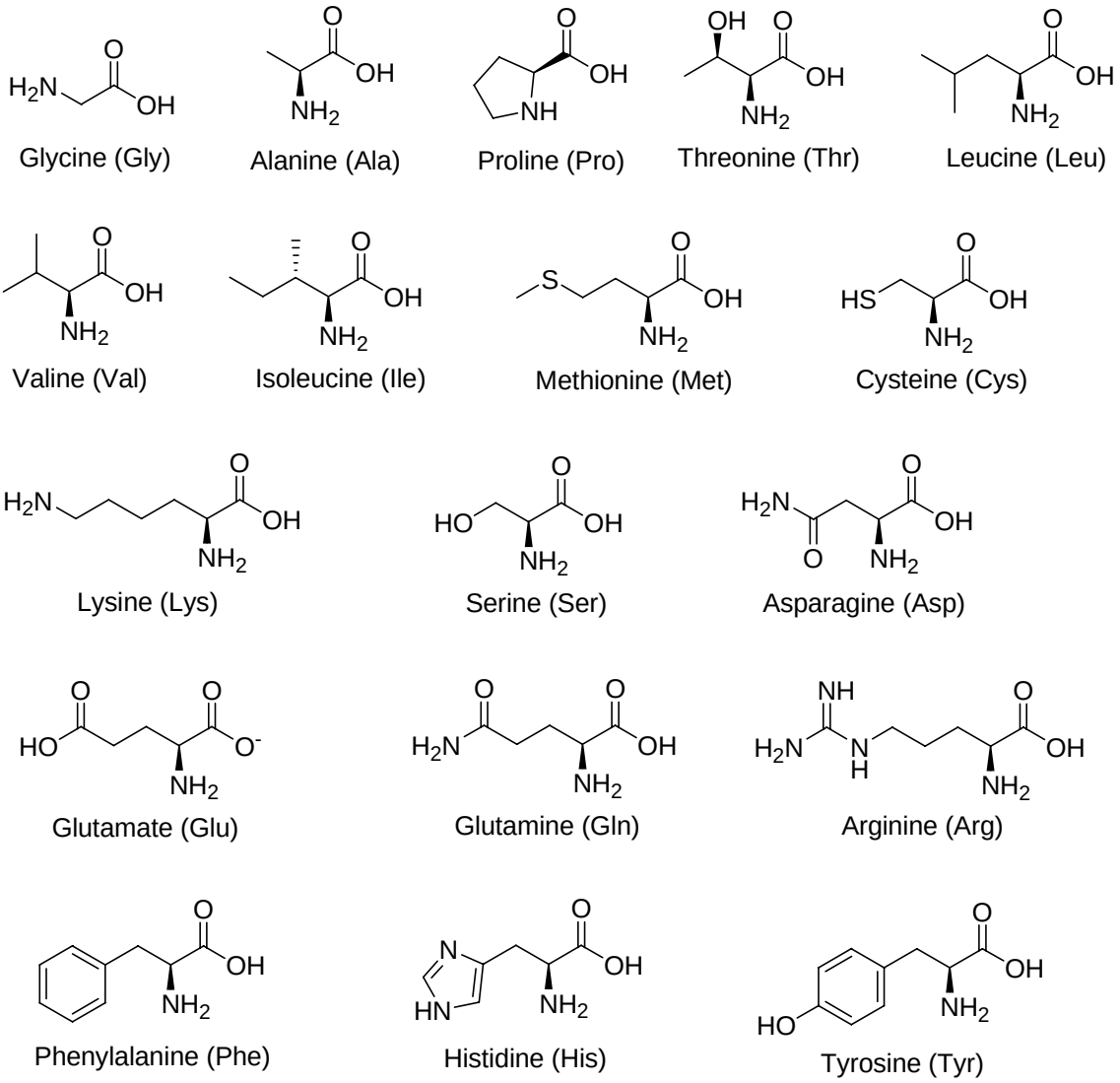
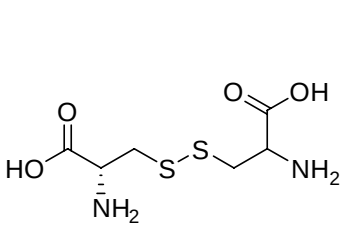
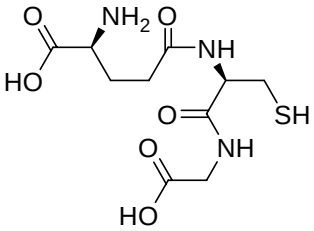


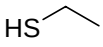
Chart S6.2. Structures of selected competitive chemicals and bioactive thiols



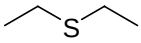
L-Cystine



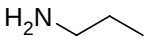
L-Glutathione



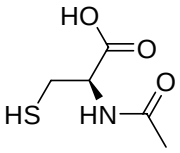
Ethanthiol



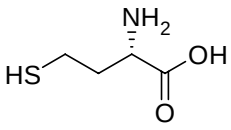
Ethyl Sulfide



Propylamine



N-Acetylcysteine



Homocysteine

References:

(S1) R. C. Benson, H. A. Kues, *J. Chem. Eng. Data*, 1977, **22**, 379.

(S2) J. N. Demas, G. Crosby, *A. J. Phys. Chem.* 1971, 7**5**, 991.