Electronic Supplementary Information (ESI)

Naked Eye Reversible Instant Sensing of Cu^{2+} and Its *In-Situ* Imaging in Live Brine Shrimp *Artemia*

Ratish R. Nair,[a] M. Raju,[a] Neha M. Patel,[b] Ishan H. Raval,[b] E. Suresh,[ac] Soumya Haldar*[bc] and Pabitra B. Chatterjee*[ac]

[a]Analytical Discipline and Centralized Instrument Facility (AD&CIF), CSIR-CSMCRI, G. B. Marg, Bhavnagar-364002, Gujarat, India.

[b]Marine Biotechnology and Ecology Discipline, CSIR-CSMCRI, G. B. Marg, Bhavnagar-364002, Gujarat, India.

[c]Academy of Scientific and Innovative Research (AcSIR), CSIR-CSMCRI, G. B. Marg, Bhavnagar-364002, Gujarat, India.

Contents   Page No.

1. Materials and physical measurements

Rhodamine 6G, 5-bromosalicaldehyde, paraformaldehyde and N,N,N'-trimethylethylenediamine, perchlorate salts of different metals, tetrabutylammonium- and sodium salts of anions, HEPES, PBS and other analytical reagents were purchased from sigma Aldrich and were used without further purification. All microbiological media (Nutrient Agar, Nutrient Broth, Luria Bartani (LB) Broth) were purchased from HiMedia (India). *Artemia* cyst were purchased from Tetra (Japan). All the other chemicals are reagent grade and commercially available. These were used without any further purification. The melting point of the compound **HL** was measured using a Mettler-Toledo FP-62 instrument. FT-IR spectra were recorded as KBr pellets using a Perkin-Elmer Spectra GX 2000 spectrometer, while for ^{1}H NMR spectra (500 MHz), a Bruker Avance II 500 FT-NMR spectrometers using SiMe4 (δ_0) as internal reference.was used. The UV-Vis spectra were recorded using Cary 500 scan UV-vis-NIR spectrophotometer & Shimadzu 2700 UV-vis-NIR spectrophotometer. The fluorescence spectra were recorded using Edinburgh instruments Xe900 (μF 920H) spectrometer. The ESI-MS in positive ion mode were measured on a Micromass Q-ToF micro^{TM} whereas microanalyses (for C, H, and N) were performed on an Elementar Vario MICRO CUBE analyser. pH of the solutions were measured using Thermo Scientific Orion Versastar Advanced Electrochemistry meter and the same was also employed to measure molar conductivity (Λ_M) for a 1 mM solution of the complexes **CuL** in acetonitrile at 298 K. The quantum yield was calculated following literature.[1] Analysis of copper in **CuL** was carried out in triplicates with ICP-OES instrument from Parkin Elmer, model 2000 dv. 1300 W RF power under argon gas flow (Nebulizer, 0.86 L/min; Auxillary, 1.2 L/min; Plasma, 15 L/min) with a 0.65 L/min sample uptake rate. The measuring mode was axial for Cu with the wavelength 327.393 nm. Standard reference solutions purchased from Merck were used for the

calibration of the ICP-OES instrument. The images of the *Escherichia coli* cells and live brine shrimp *Artemia* were viewed through optical microscope (Olympus DP72 U-TVO 63XC).

2. **Single crystal X-ray Crystallography**

Diffraction quality crystal of **HL** (block, pale yellow, 0.34 × 0.32 × 0.28 mm^3) were obtained from acetonitrile solvents by slow evaporation at room temperature. Intensity data for the compound **HL** was measured employing a Bruker SMART APEX CCD diffractometer equipped with a monochromatized Mo K$_\alpha$ radiation (λ = 0.71073 Å) source at 150 (2) K. No crystal decay was observed during the data collection. Accurate cell parameters and orientation matrices were determined from setting angles of 9844 reflections in the ranges 2.22 ° ≤ θ ≤ 27.77 °. The intensity data were corrected for empirical absorption. The absorption corrections based on multi-scan using the *SADABS* software were applied.[2] SAINT software packages were used for data integration and reduction.[3] The structure was solved by direct methods using SHELXTL[4] and refined on F^2 by a full-matrix least-squares procedure with anisotropic displacement parameters for all the non-hydrogen atoms based on all data minimizing wR = $[\Sigma \ [w((F_0^2 - F_c^2)^2]/\Sigma \ w \ (F_0^2)^2]^{1/2}$, $R = \Sigma ||F_0| - |F_c||/\Sigma|F_0|$, and $S = [\Sigma[w(F_0^2 - F_c^2)^2]/(n - p)]^{1/2}$. SHELXL-97 was used for both structure solutions and structure refinements.[5] A summary of the relevant crystallographic data and the final refinement details are given in Table S1. The hydrogen atoms were calculated and isotropically fixed in the final refinement [d(C-H) = 0.95 Å, with the isotropic thermal parameter of U_{iso}(H) = 1.2 U_{iso}(C)]. Crystallographic diagrams were drawn using the ORTEP-3 software package at 40 % probability level.[6]

3. Synthesis of HL

To a solution of rhodamine 6g hydrazide (428 mg, 1 mmol) in methanol was added mannich ligand 3-rimethylethylenediaminomethyl-5-bromosalicyldehyde (3-NN-5-BrSali) (315 mg, 1 mmol). The mixture was refluxed for about 5 h. It was then cooled and filtered. The residue so obtained was washed thrice with ethanol (3 x 10 mL) and finally, dried in vacuo over P_4O_{10}. The yellow crystalline product was obtained by further recrystallization from acetonitrile solution. Yield: 82 %. MP: 223 °C. Important FT-IR bands (KBr pellet, cm^{-1}) 3423, 2967, 2860, 1720, 1620, 1517,1450, 1300, 1267, 1217, 1014, 742.^{1}H NMR (CD$_3$CN, 500 MHz): δ 8.61 (s, 1H), 7.92 (d, J = 7.5 Hz, 1H), 7.56 (s, 2H), 7.54 (m, 2H), 7.11 (s, 1H), 6.98 (d, J = 7.5 Hz, 1H), 6.35 (s, 2H), 6.30 (s, 2H), 3.33 (s, 2H), 3.18 (m, 4H), 2.52 (m, 5H), 2.27 (s, 7H), 2.11 (s, 4H), 1.85(s, 6H), 1.25 (t, J = 14 Hz, 6H). Anal. Calcd for $C_{39}H_{45}N_6BrO_3$: C, 64.55; H, 6.25; N, 11.58. Found: C, 63.15; H, 6.35; N, 11.69 %. ESI-MS (+ive, m/z): 725.73 & 727.73 [M + H$^+$].
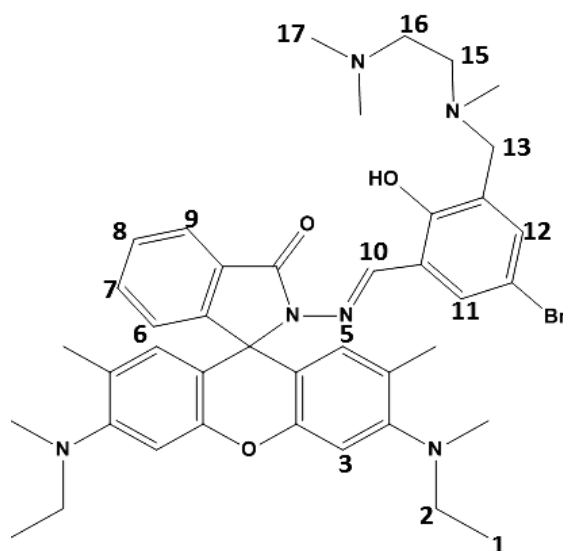
HL

4. Procedure for the synthesis of CuL
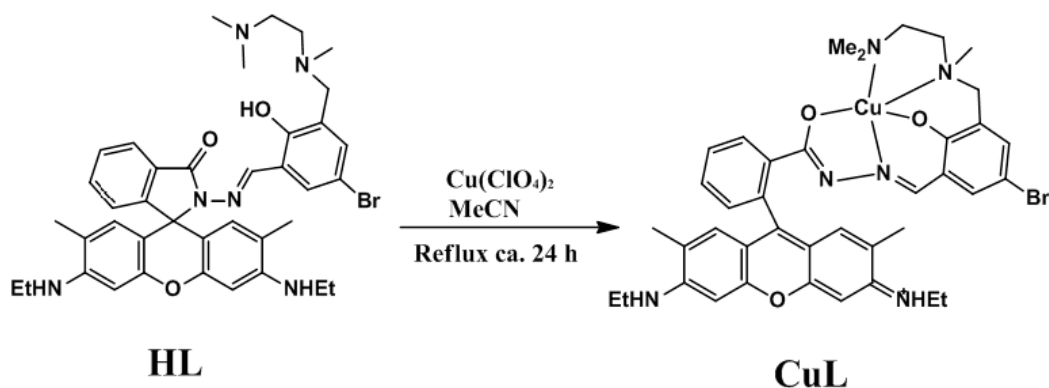
To a solution of **HL** (725 mg, 1 mmol) in acetonitrile was added Cu(ClO$_4$)$_2$.6H$_2$O (370 mg, 1 mmol) and then was stirred under reflux for about 2 h. The reaction mixture was allowed to cool and the precipitate obtained was filtered yielding a reddish-pink coloured compound. Yield: 62 %. Selected FT-IR bands (KBr pellet, cm^{-1}) 3376, 2972, 2870, 1608, 1501, 1365, 1307, 619. Anal. Calcd for C$_{39}$H$_{44}$N$_6$BrCuO$_3$: C, 59.42; H, 5.63; N, 10.66. Found: C, 58.97; H, 5.60; N, 10.55 %. ESI-MS (+ive, m/z): 787.20 & 788.27 (M + H$^+$). The composition and purity of this compound was further established by ICP-OES analysis of copper which confirm one equivalent of the metal in 1 equiv of **CuL**. Furthermore, the compound is electrically nonconducting in CH$_3$CN solution.

5. General procedures for absorbance and fluorescence experiments

The absorbance and fluorescence experiments were carried out in an aqueous mixture of 1:1 acetonitrile:water in HEPES (50 mM) buffer at pH 7.4. 100 mL stock solutions (20 μM) of perchlorate salts of Cu^{2+}, Fe^{2+}, Cr^{2+}, Pb^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}, Na$^+$, Ca^{2+}, K$^+$, Hg^{2+}, Ni^{2+}, Li$^+$, Ba^{2+}, and Cd^{2+} were prepared in 1:1 acetonitrile : H$_2$O. Final test solutions were prepared by taking 1 mL of the above metal stock solution and 1 mL of 20 μM **HL** solution to further yield 10 μM of each component. In case of fluorescence experiments, the resolution was set at 1

nm. The excitation value was given at 510 nm and the emission spectrum was collected at 553 nm.

6. General procedure for UV-vis titration

Stock solutions for UV-vis titrations were prepared in 1:1 ACN:H$_2$O in the presence of 50 mM HEPES buffer at pH 7.4. The stock concentration of HL were kept constant at 20 µM and the stocks of copper perchlorate were prepared by increasing its concentrations from 0.01 µM to 100 µM in 1:1 acetonitrile:water. Binding constant, Ka, was calculated following Benesii-Hildebrand equation (shown below).[7]

$$1/(A-A_o) = [1/\{K_a(A_{max}-A_o)\, [M^{x+}]_n\}] + [1/(A_{max}-A_o)]$$

A$_0$ is the absorbance of **HL** in the absence of metal salt, A is the absorbance recorded in the presence of metal salt, A$_{max}$ is the maximum absorbance obtained upon addition of metal ion and K_a is the association constant. The association constant (K_a) could be determined from the slope of the straight line obtained by plotting of $1/(A-A_0)$ against $1/[Cu^{2+}]$ (Fig. S4) and was found to be 0.44 x 10^5 M^{-1}.

7. General procedure of fluorescence titration

The solutions were prepared similarly as were for UV-vis titration. The binding constant for **HL** was determined following modified Benesii-Hildebrand equation, i.e. $1/(I-I_0) = [1/\{K_a(I_{max}-I_0)\, [M^{x+}]_n\}] + [1/(I_{max}-I_0)]$, where I$_0$ is the emission intensity of **HL** in absence of copper salt, I is the emission intensity of **HL** in presence of different concentrations of copper salts and I$_{max}$ is the emission intensity of **HL** when the complete saturation is reached. From the plot of $1/(I-I_0)$ against $1/[Cu^{2+}]$ for **HL** (Fig. S5), the value of K_a was found to be 0.3 x 10^5 M^{-1}.

8. General procedure of NMR titration

^1H NMR titrations were carried out by preparing the solutions in neat acetonitrile. The concentration of **HL** was kept at 2 mM and the concentration of copper salts were changed in an increasing order from 0.5 to 4 mM.

9. Determination of detection limit from UV-vis experiments

The limit of detection (LOD) of **HL** for Cu^{2+} was determined by plotting $[(A-A_0) / (A_\alpha-A_0)]$ against $Log[Cu^{2+}]$ (Fig. S9). The LOD value was found to be 0.9 ppb i.e. **HL** can detect Cu^{2+} at this very low concentration by UV-vis spectrophotometer.

10. Determination of LOD from fluorescence titrations

Plotting $[(F-F_0) / (F_\alpha-F_0)]$ against $Log[Cu^{2+}]$ generates Fig. S10. The LOD value calculated from this correlation was found to be 0.7 ppb for Cu^{2+}. Thus, fluorometrically, **HL** can detect Cu^{2+} even at less than 1 ppb concentration.

11. General procedure for preparing test strips

The test strips were prepared from filter papers and were immersed into the DMSO solution of the receptor **HL**. After drying, the test strips coated with **HL** were immersed into different concentrations of copper salts solutions.

12. *Artemia* culture and imaging

Artemia cysts were encapsulated in visible light (20 lux) at vigorous aeration. As pollutants, different concentrations of Cu^{2+} (9, 42, 64, and 84 ppb) in 10 mL of acetonitrile-water (1:1) HEPES buffer (pH 7.4) were used. *Artemia* were exposed to these Cu^{2+} solutions first for 20

minutes at 25 °C. Free Cu^{2+} which were present in the solution was removed through centrifugation (repeated twice to avoid any background colour). *Artemia* were exposed to **HL** (1:2, Cu^{2+}:**HL**) taken in a 1:1 (v/v) HEPES buffer (pH 7.4) (acetonitrile-water). Approximately 100 numbers of *Artemia* were added in each tube. After completion of different exposures (mentioned above) individual *Artemia* were mounted on a glass slide and monitored through a light microscope. Tubes containing just **HL** were also used to get control image. Additionally, toxic effects of **HL** and Cu^{2+} were also evaluated in a bio-assay against *Artemia*.

13. Bacterial culture and staining

Single colony of *Escherichia coli* was inoculated in Luria Bartani (LB) broth and allowed to grow at 37 °C with constant overnight shaking (120 rpm) for making the homogeneous suspension. The cultured cells were harvested after centrifugation at 4000 rpm for 10 min. Next, harvested cells were washed with phosphate buffer saline (PBS). Subsequently, the cells were first expressed to different concentration of Cu^{2+} (9, 42, 64, and 84 ppb) in acetonitrile-water (1:1) HEPES buffer (pH 7.4) for 30 min at 25 °C. Images were obtained following the above mentioned procedure for used *Artemia*. Bacteria were observed in a light microscope. For control study bacterial cells images were also recorded in the absence of metal ion.

14. Toxicity study of HL towards *Artemia*

To explore the non-toxic nature of **HL**, *Artemia* cysts (700 mg in 500 mL autoclaved sea water) were allowed to hatch overnight with vigorous aeration under visible light. Soon after the complete hatching, approximately 50 *Artemia* were added in each tube (in 10 mL acetonitrile-water (1:1) HEPES buffer (pH 7.4)). Two sets of tubes (in triplicate) were prepared. *Artemia* were allowed to grow overnight (24 h) in the absence (control) and presence of **HL**

(20 µM) and in the next day mortality were counted to check the toxicity of **HL** towards *Artemia*.

15. Toxicity testing of the sensor HL on *E. coli*

E. coli culture (18-24 h old) was inoculated in Nutrient broth with 20 µM of **HL** and incubated at 37 °C for 18-24 h. Culture was serially diluted and plated on nutrient agar. The colonies formed were counted after 18-24 h incubation at 37 °C. Viable microbial count was calculated as follows: cfu/mL = (cfu/plate) x dilution factor

where cfu is colony forming unit.

16. Quantum yield calculations

For fluorescence quantum yield calculation an optically identical solution of rhodamine 6G (Φf = 0.95 in acetonitrile) was used as standard at an excitation wavelength of 510 nm and the quantum yield was calculated using the following equation.

$$\Phi_{unk} = \Phi_{std} \times \frac{(F_{unk}/A_{unk})}{(F_{std}/A_{std})} \left(\frac{\eta_{unk}}{\eta_{std}}\right)^2$$

Φ_{unk} and Φ_{std} are the radiative quantum yields of the sample and standard, respectively. F_{unk} and F_{std} are the integrated emission intensities of the corrected spectra for the sample and standard, respectively. A_{unk} and A_{std} are the absorbances of the sample and standard at the excitation wavelength, respectively and η_{unk} and η_{std} are the indices of refraction of the sample and standard solutions, respectively.

Table S1. Summary of the Crystallographic Data for the sensor **HL**

Composition	C41 H48 Br N7 O3
Formula wt.	766.77
Crystal system	Triclinic
Space group	P-1
Crystal color	Pale yellow
a (Å)	11.7286(8)
b(Å)	15.6534(10)
c(Å)	22.6519(15)
α(º)	93.3110(10)
β(º)	99.2660(10)
γ (º)	107.5000(10)
V(Å^3)	3889.5(4)
Density (Mg/m^3) / Z	1.309/4
2 ϑ_{max} [deg]	52.00
Temp (K)	150(2)
μ mm^{-1}	1.104
Reflections collected/unique	15107/11941
R(int)	0.0235
Data/restraints/parameters	15107/0/963
$R1(F_0)$, $wR2(F_0)$ ($I \geq 2\ \sigma(I)$)	0.0463, 0.1255
$R1(F_0^2)$, $wR2(F_0^2)$ (all data)	0.0611, 0.1359
F(000)/GOF on F^2	1608/1.051
CCDC No.	1056727

Table S2. Toxicity results of **HL** on *Artemia*

No. of *Artemia*	control	Sensor, HL (20 µM)
50	50	50
50	50	50
50	50	50

No mortality was observed in any of the tubes. This data indicates that **HL** has no impact on the toxicity of *Artemia*.

Table S3. Table showing toxicity of **HL** on *E. coli* (cfu/mL)

Concentration (µM)	Control (in absence of HL)	Sensor, HL (20 µM)
Count of *E. coli*	3.8×10^{12}	3.5×10^{12}

Fig. S1 Naked eye detection of Cu^{2+} in visible light. Conditions: **HL** (10 µM) in CH_3CN-water (1:1 v/v, 50 mM HEPES buffer, pH 7.4) and metal ions (10 µM).

Fig. S2 Job's plot for **HL** monitored at 525 nm (**a**, absorption) and at 553 nm (**b**, emission) in presence of different concentrations of Cu^{2+}.
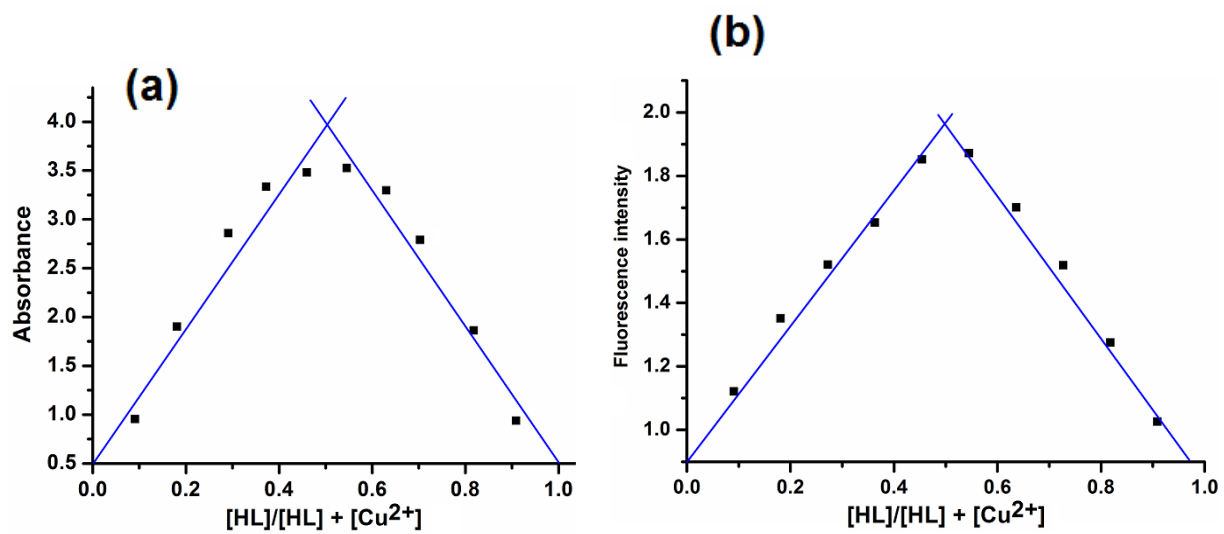
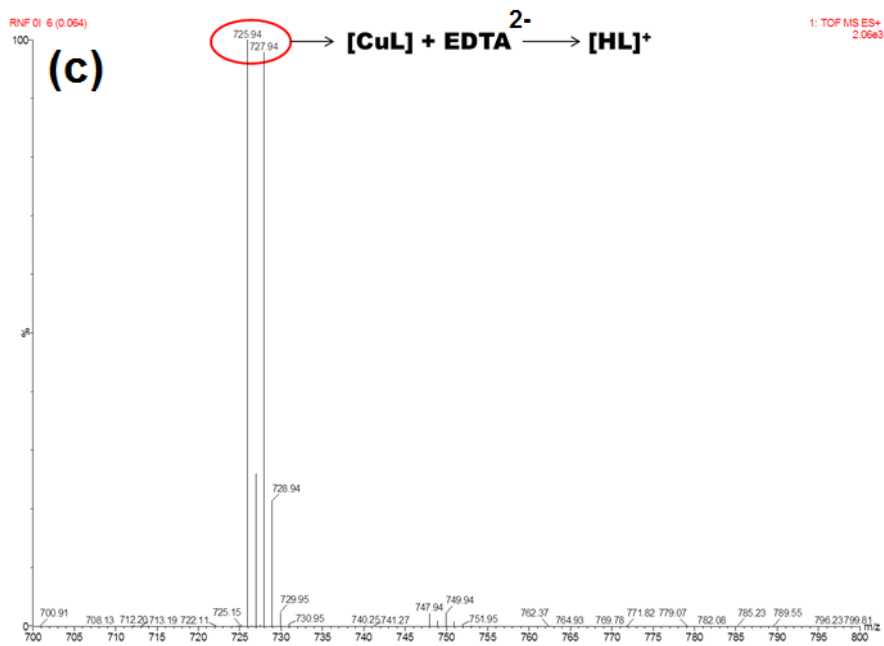**Fig. S3 ESI-MS spectra of HL (a), CuL (b) and regeneration of HL+ (c).**

$[CuL] + EDTA^{2-} \longrightarrow [HL]^{+}$
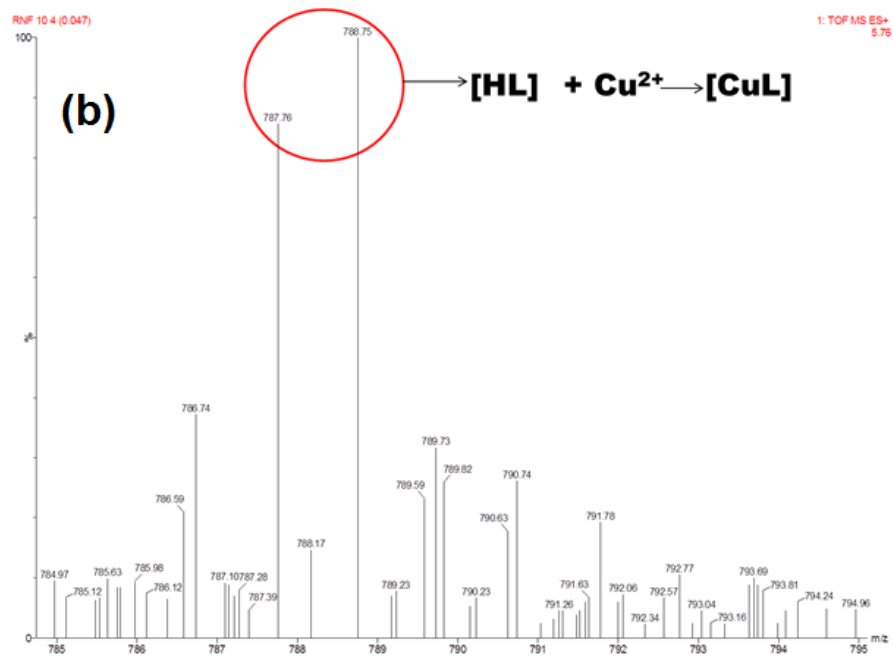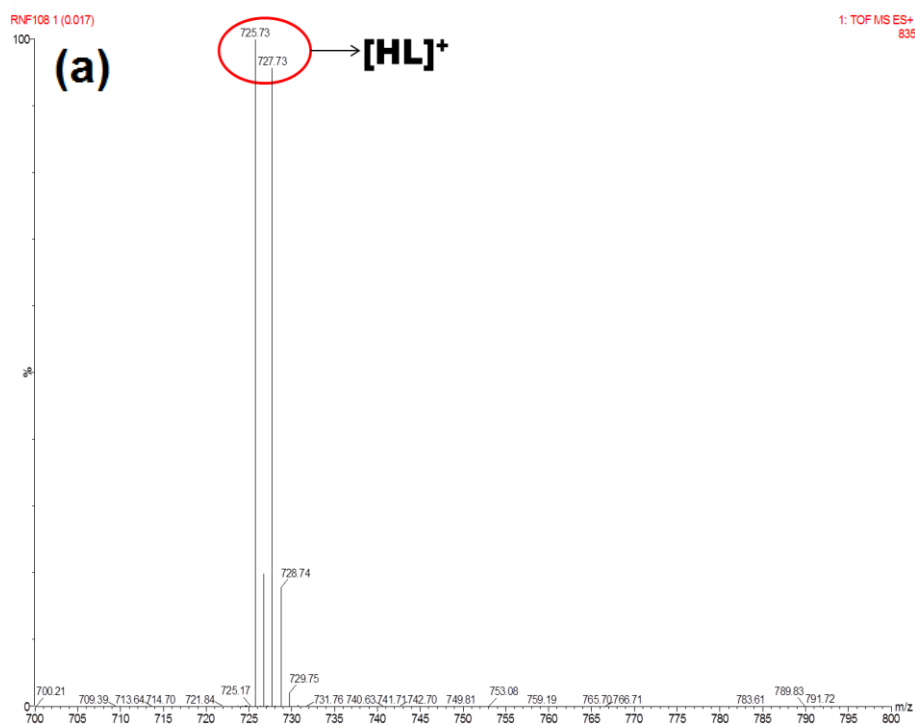
Fig. S4 (a) Absorption and (b) fluorescence titration profiles of HL (10 µM) upon addition of increasing concentration of Cu^{2+} (0 (0 eq)-40 (4 eq) µM in (a) and 0-100 µM in (b)) in HEPES buffer solution.
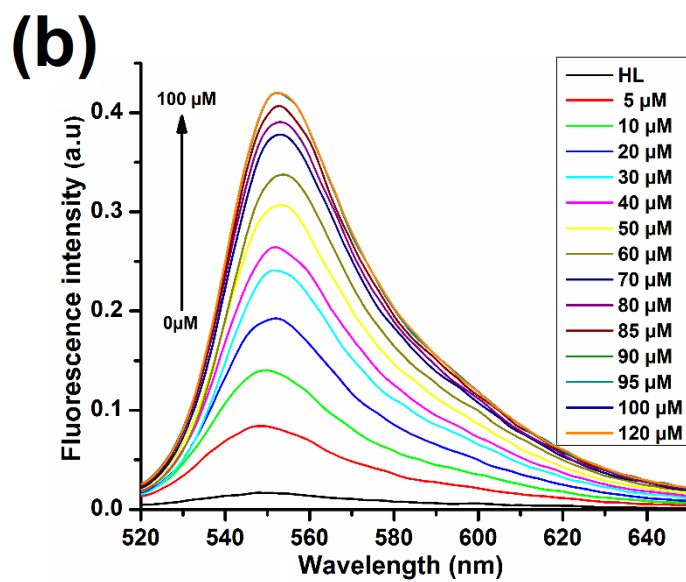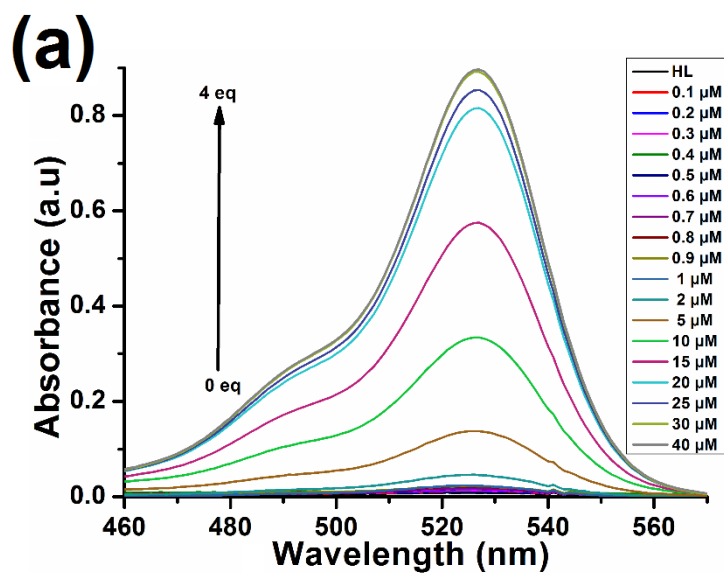
Fig. S5 Benesii-Hildebrand plot from absorption titration data of **HL** (10 µM) in presence of copper salt.
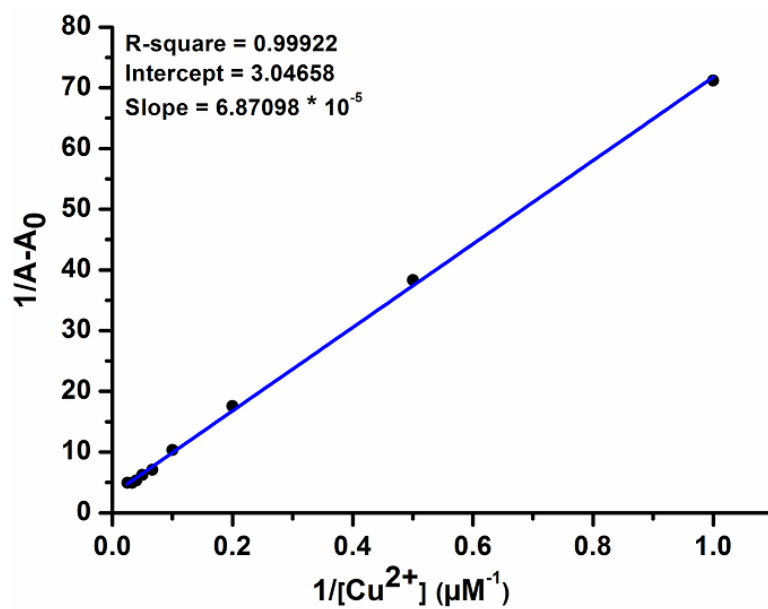
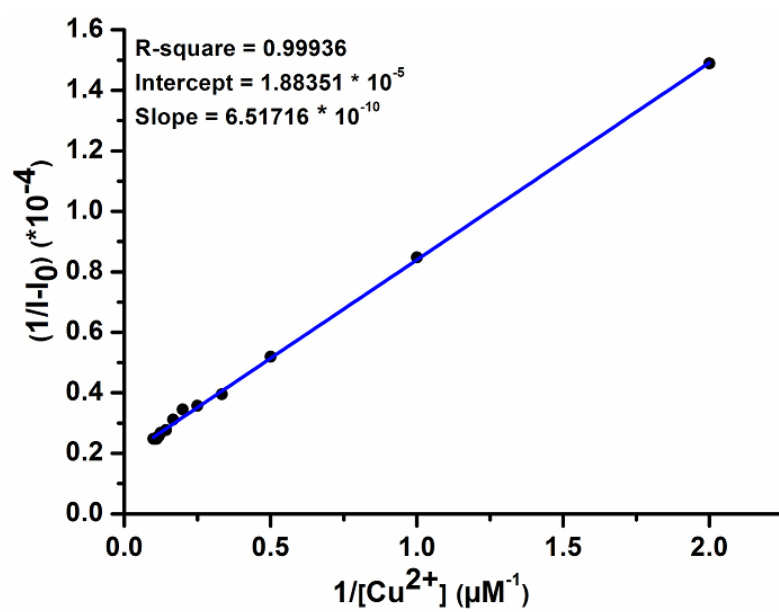**Fig. S6** Benesii-Hildebrand plot from fluorescence titration data of **HL** (10 µM) in presence of copper salt.

R-square = 0.99936
Intercept = $1.88351 * 10^{-5}$
Slope = $6.51716 * 10^{-10}$

Y-axis: $(1/I\text{-}I_0) (*10^{-4})$

X-axis: $1/[Cu^{2+}] (\mu M^{-1})$

Fig. S7 ^{1}H NMR spectral stack of **HL** (2 mM) with various equivalents of copper salts.
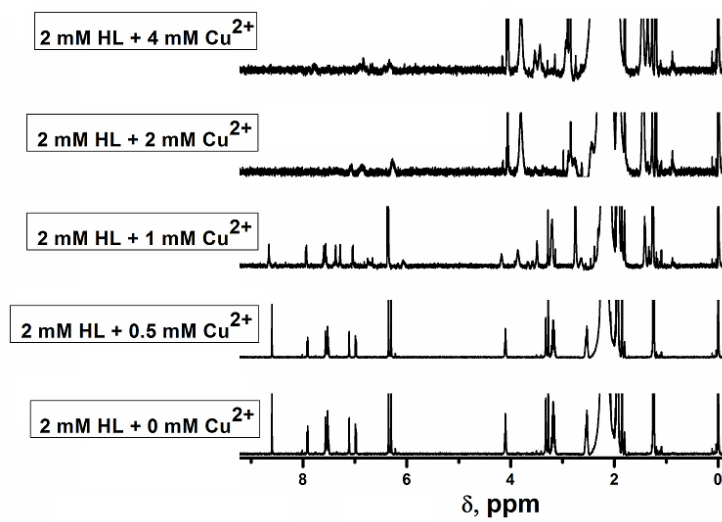
Fig. S8 Fluorescence spectra of **HL** (10 µM) in CH₃CN-Water (1:1 v/v, HEPES, pH 7.4) solution in the presence of different copper salts (10 µM) at 553 nm.
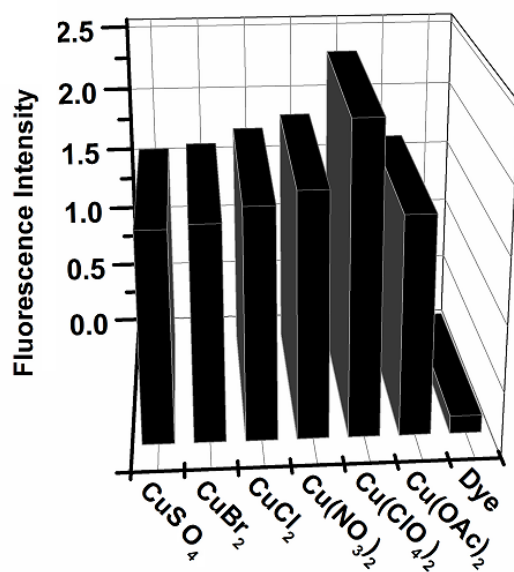
Fig. S9 Fluorescence response of **HL** (10 µM) at 553 nm in presence of copper (1 equiv) at different oxidation states.
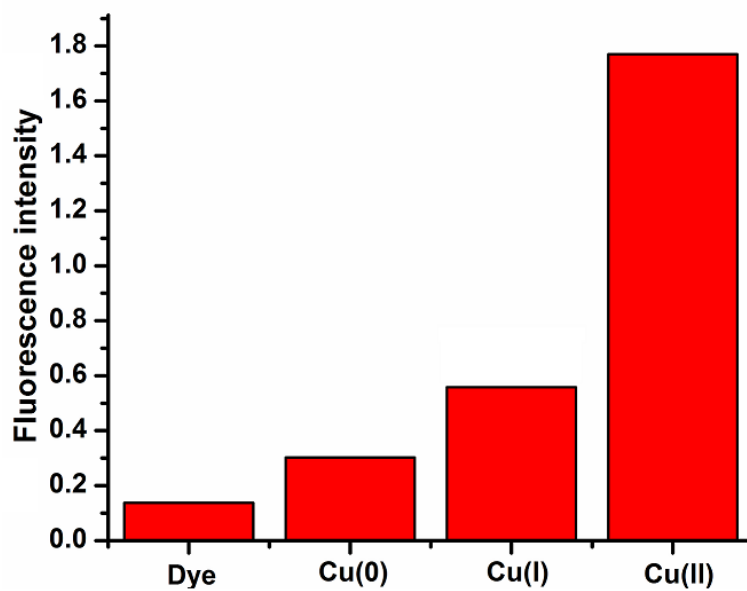
Fig. S10 LOD determination from absorbance titration data of **HL** (10 µM) in presence of copper(II) perchlorate salt.
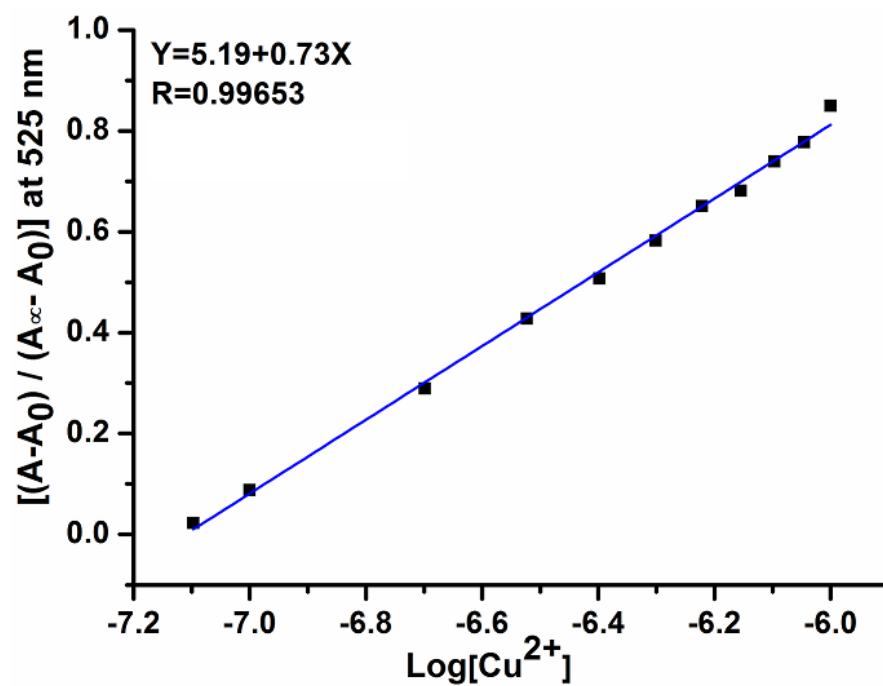

Y=5.19+0.73X
R=0.99653

Fig. S11 LOD determination plot from fluorescence titration data of **HL** (10 µM) in presence of copper salts.

Y=4.46+0.619X
R=0.99063

[(F-F$_0$) / (F$_\infty$- F$_0$)] at 553 nm
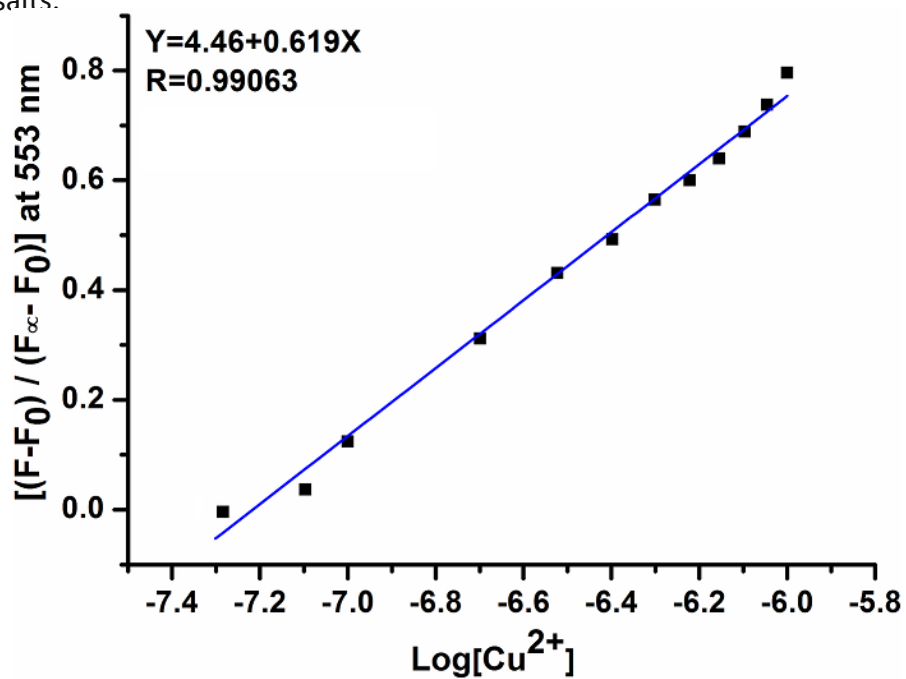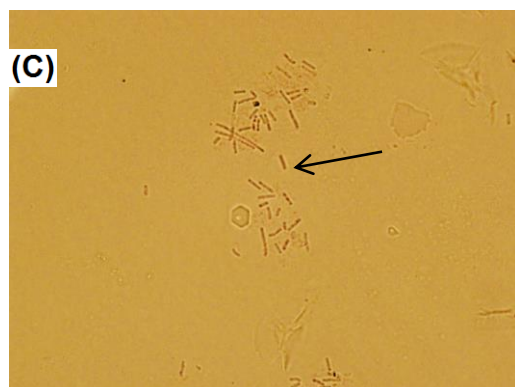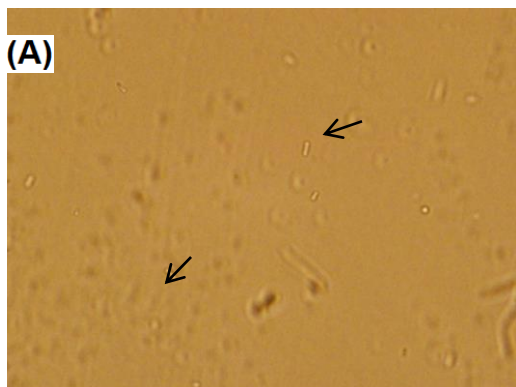
Log[Cu^{2+}]

Fig. S12 Colour change observed in test strips of **HL** at various concentrations of Cu^{2+} in water: From left to right. 0 ppb, 1000 ppb, 100ppb.

Fig. S13 Optical microscope images of (A) control cells of *E. Coli*, (B) cells exposed to **HL** (20 ppb), and (C) cells exposed to an aqueous solution of Cu^{2+} (10 ppb) for 30 min and then to **HL** (2 equiv, ACN-H_2O HEPES buffer, pH 7.4; 1:1 (v/v)).

References

(1) Q. Meng, Y. Shi, C. Wang, H. Jia, X. Gao, R. Zhang, Y. Wang and Z. Zhang, *Org. Biomol. Chem*., 2015, **13**, 2918.

(2) G. M. Sheldrick, *SADABS, Empirical Absorption Correction Program*, University of Göttingen, Göttingen, Germany, 1997.

(3) G. M. Sheldrick, *SAINT 5.1 ed*., Siemens Industrial Automation Inc., Madison, WI, 1995.

(4) G. M. Sheldrick, *SHELXTL* Reference Manual: Version 5.1, Bruker AXS, Madison,WI, 1997.

(5) G. M. Sheldrick, *SHELXL -97*: Program for Crystal Structure Refinement, University of Göttingen, Göttingen, Germany, 1997.

(6) L. J. Farrugia, *ORTEP-3* for WINDOWS; University of Glasgow: Glasgow, Scotland, 1997.

(7) S. Goswami, K. Aich, S. Das, A. K. Das, A. Manna and S. Halder, *Analyst*, 2013, **138**, 1903.