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

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A Cu^{2+}-specific colorimetric reversible fluorescent receptor HL was designed and synthesized which showed naked eye observable colour change from colourless to pink on addition of aqueous buffer (pH 7.4) solution of 30 ppb Cu^{2+}. Short response time (≤ 5 sec) and low detection limit (nearly 3 ppb) make HL suitable as a reliable "dip-in" open eye sensor for Cu^{2+}. Bio-imaging application in live brine shrimp *Artemia* enabled HL to detect Cu^{2+} at as low as 10 ppb exposure.

Copper, the third most essential trace element, plays an important role in the functioning of intracellular mechanisms in all forms of life.[1-5] Misregulation in the cellular homeostasis of copper triggers many neurodegenerative diseases, which include Alzheimer's and Parkinson's diseases, prion diseases, amyotrophic lateral sclerosis, and many more.[6-8] Excessive to Cu^{2+}, on the other hand, can have adverse effects on human health, aquatic lives and environment. Consequently, the U.S. Environmental Protection Agency (EPA) has set the permissible level of copper in drinking water at 20 µM.[9] Development of reliable small molecule-based non-toxic signalling systems for detection of Cu^{2+} in living species is therefore of paramount importance.[10-36] Among all designed Cu^{2+}-only sensors, colorimetric and fluorimetric ionophores have attracted special attention.[10-36] Traditionally, fast electron transfer resulting in fluorescence quenching, or "turn-off", was used to detect Cu^{2+}.[10-21] However, over the last decade fluorescence "turn-on" has emerged as a superior technique for sensitive, selective, and accurate detection of Cu^{2+} in solution.[22-36] Unfortunately, most of the reported Cu^{2+} specific artificial molecular probes behave irreversibly, and with a relatively sluggish response affecting practical detection of subcellular Cu^{2+}.[36] Instant sensing, re-usability, and low detection limit are desirable attributes of Cu^{2+} bio-sensors. This motivated us to search for an "off-on-off" sensor instead of an "on-off-on" sensor possessing rapid reaction time. Although, there are examples of sensors exhibiting reversible responses,[33,37-39] these suffer from some limitations, such as presence of two different fluorophores, presence of a second metal ions (e.g. Cd^{2+}, ratiometric), low yield of synthesis, long response time, quenching phenomenon, and lack of toxicity- and *in-situ* bio-sensing data.[33,37-39]

Light microscopy is an attractive technique to work/view live and stained samples.[40] For real-time environmental testing outside of the laboratory, colorimetric sensors should meet the requirements of practical imaging of living systems in a low cost *in-situ* manner. To this end, we designed and synthesized a new chemosensor **HL** (Scheme 1) which showed naked eye observable colour change from colourless to pink in presence of 30 ppb Cu^{2+} at physiological pH conditions. As a reversible receptor, **HL**, was found to possess high sensitivity, low detection limit (nearly 3 ppb), and a rapid response time (≤ 5 sec). These attributes make it suitable for practical *in-situ* bio-imaging application in gram negative bacteria *Escherichia (E.) coli* as well as in higher group living organisms such as marine crustacean, *Artemia*, popularly known as brine shrimp. In the present study, we sought to explore *Artemia* as a tool for studying the increased bio-accumulation of Cu^{2+} in the gastro intestinal tract of the shrimp using colorimetric sensor-based simple and robust light microscope.

Synthesis of **HL** was accomplished by coupling of Rhodamine 6G hydrazide with 3-trimethylethylenediaminomethyl-5-bromosalicyldehyde (hereafter denoted as 3-NN-5-BrSali) in CH_3OH (Scheme 1). **HL** was obtained in 82 % yield. The Rhodamine 6G hydrazide fluoroprobe was prepared following known procedure[41] while the 3-NN-5-BrSali metal chelating unit was obtained through a modification of the literature method.[42] The molecular structure and bulk purity of the compound **HL** was confirmed by elemental analysis, ^1H NMR, ESI-MS and, finally, unambiguous structural

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† Electronic Supplementary Information (ESI) available: Synthesis, characterization data, spectroscopic data, toxicity and bio-imaging results.
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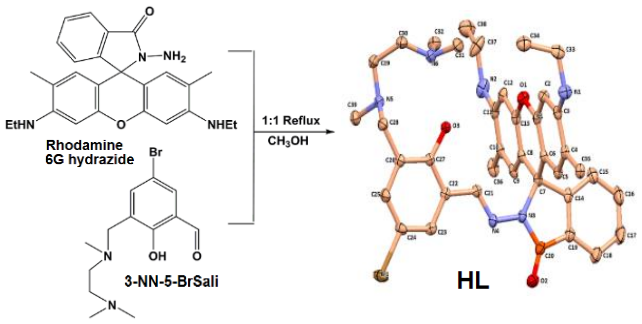

Scheme 1 Synthetic strategy for the preparation of **HL**.

confirmation was established through single crystal XRD data[43] (see the ESI†).

The absorption spectrum of 10 μM **HL,** taken in 1:1 (v/v) CH_3CN/HEPES buffer (50 mM) at pH 7.4 showed a weak absorption, with λ_{max} at 525 nm but to the naked eye the solution appeared to be nearly colourless. The effect of different metal ions (Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Ba^{2+}, and Cr^{3+}) on the absorption and emission of **HL** was investigated next. Cell abundant K^+, Ca^{2+}, Na^+, and Mg^{2+} (10 μM) gave insignificant changes in the absorption spectrum. Most of the other metal ions also did not show any response whereas Cu^{2+} gave a pronounced increase in absorbance (Fig. 1a) with formation of a strong pink colour which was easily discernible by the naked eye (Figs. 1b and S1†). Fluorescence (FL) emission of 10 μM **HL,** without and with addition of 10 μM concentrations of various ions was monitored at 553 nm. The excitation wavelength was 510 nm. Except in the case of Cu^{2+}, the FL was very weak in all other cases. With Cu^{2+}, the FL intensity was enhanced ca. 25 fold (Fig. 2). This is likely on account of the increased absorbance at the excitation wavelength upon addition of Cu^{2+}. Additionally, non-radiative pathways of decay may be inhibited. The quantum yield, Φ_f, was estimated to be 0.2.

The above observations can be attributed to the strong coordination of the ONN donor sites from 3-NN-5-BrSali moiety with Cu^{2+}. The binding stoichiometry was determined to be 1:1 by Job's plot (Fig. S2†) using the changes in absorption at 525 nm as well as emission

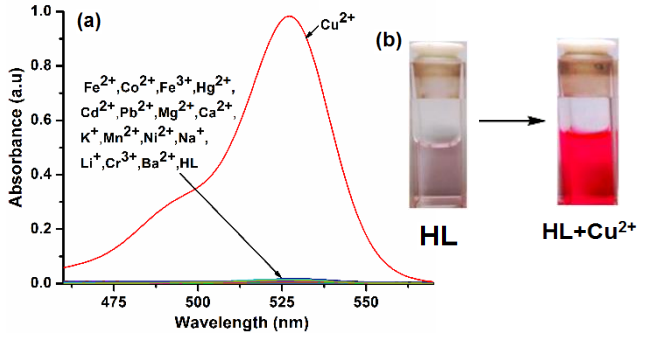

Fig. 1 (a) UV-vis spectra of **HL** (10 μM) upon addition of 1 equiv of different metal ions, (b) colour change of 10 μM **HL** in the absence and presence of 10 μM Cu^{2+}.

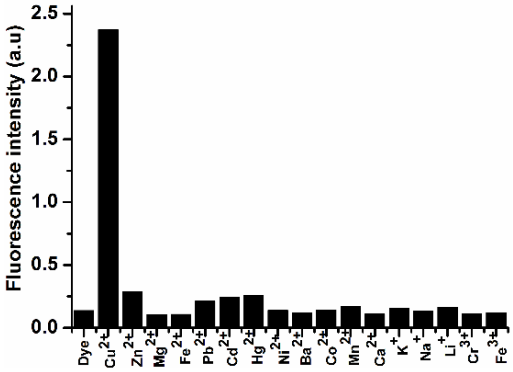

Fig. 2 Fluorescence intensity of **HL** (10 μM) upon addition of 1 equiv of different metal ions.

at 553 nm, as a function of Cu^{2+} concentration. Molecular ion peaks at m/z 787.76 and 788.75 corresponding to the adduct [HL – H^+ + Cu^{2+}]$^+$ in the ESI-MS (Fig. S3†) provided further direct evidence for 1:1 complex formation between **HL** and Cu^{2+}. The association constant (Ka) for **CuL** was calculated from spectrophotometric titrations (Fig. 3 and Fig. S4†) performed in CH_3CN/HEPES buffer at 25 °C and were found to be 0.44 x 10^5 M^{-1} based on UV-vis results (Fig. S5†), and 0.3 x 10^5 M^{-1} from FL titration data (Fig. S6†). The linearity of the Benesi–Hildebrand plot[44] further confirmed the stoichiometry of "**CuL**" (Figs. S5† and S6†). The ^{1}H NMR titration experiments of **HL** with Cu^{2+} in CD_3CN showed that the imine proton (H_{10}) and aromatic protons (6-8 ppm) became broad with increase of [Cu^{2+}], and exactly at 1:1 (**HL**:Cu^{2+}), the peaks virtually disappeared (Fig. S7†), providing corroborative evidence in support of 1:1 association stoichiometry in **CuL**. Final confirmation regarding stoichiometry was established from the synthesis and characterization of **CuL** in the solid state (refer to ESI†). The pH dependence of the FL responses of **HL** to Cu^{2+} were performed in the absence and presence of 5 equiv of Cu^{2+} in 1:1 CH_3CN/H_2O. In the pH range 5-9, fluorescence intensity reached a plateau and thus all spectral analyses were performed in HEPES buffer solution (pH=7.4) prepared in 1:1 CH_3CN/H_2O.

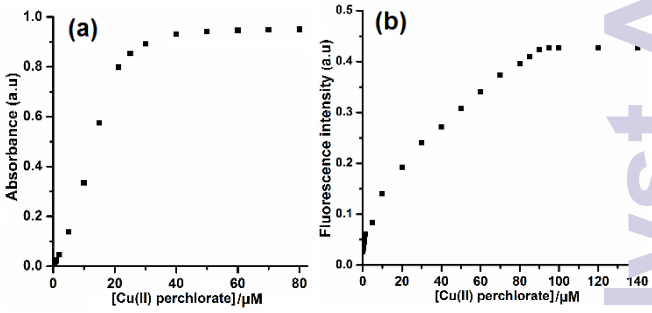

Fig. 3 Plots of absorbance (a) and fluorescence (b) intensity maxima as a function of Cu^{2+} concentration.

Selective response of **HL** towards Cu^{2+} remained unchanged even in the presence of a wide range of competitive metal ions such as transition/heavy metal ions or alkali/alkaline metal ions (5 equiv) (Fig. 4) illustrating the potential of **HL** as an extremely specific Cu^{2+}

sensor. Different counter anions with various sizes and shapes such as acetate, perchlorate, nitrate, chloride, bromide, and sulfate also had no influence on the spectral changes seen with Cu^{2+} (Fig. S8†). Similar naked eye observation of colour change was seen irrespective of the nature of the Cu(II) salts. It was further concluded from studies of **HL** with copper in three different oxidation states (Cu^0, Cu^+ and Cu^{2+}), that the selectivity is specifically towards Cu^{2+} (Fig. S9†).

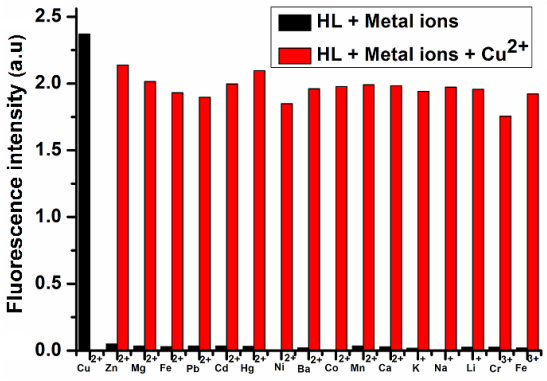

Fig. 4 Fluorescence response of equimolar (10 μM) **HL** and Cu^{2+} in presence of 5 equiv of other cations in HEPES buffer.

The limit of detection (LOD) was calculated from spectral titration data (Fig. 3). Results from fig. S11† (refer to ESI†) indicated the analytical detection limit to be ~ 3 ppb. As can be further seen from Fig. 5a, a colour change of **HL** to pink was easily discernible by the naked eye upon addition of only 30 ppb of Cu^{2+}, i.e., the naked eye detection limit is ≤30 ppb. It is further noted that the response time was short (≤ 5 sec), as evident from absorption spectral change as a function of time (Fig. 5b).

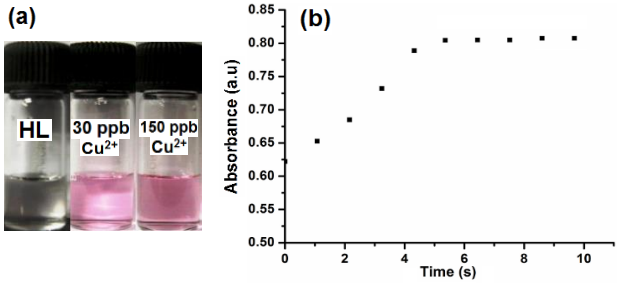

Fig. 5 (a) Naked eye detection of change in colour of HL upon addition of Cu^{2+}, (b) Time response plot of **HL** at 525 nm in presence of 30 ppb Cu^{2+}.

To ascertain the reusability of **HL**, the reversibility of its binding to Cu^{2+} was studied by adding 1 equiv of $EDTA^{2-}$ to a mixture of **HL** and Cu^{2+}. This led to bleaching of the pink colour of the solution as well as inhibition of the emission at 553 nm (Figs. 6a & 6b). The characteristic pink colour as well as FL intensity were recovered upon further addition of Cu^{2+} to the colourless solution (Figs. 6a & 6b). For real-time and instrument free instant detection of Cu^{2+}, the "dip-in" experiment was also carried out with a test-strip made out of filter paper. The colour of the strip changed from colourless to pink when a dried strip initially soaked in **HL** solution was immersed into a Cu^{2+} solutions (100 and 1000 ppb, Fig. S12†).

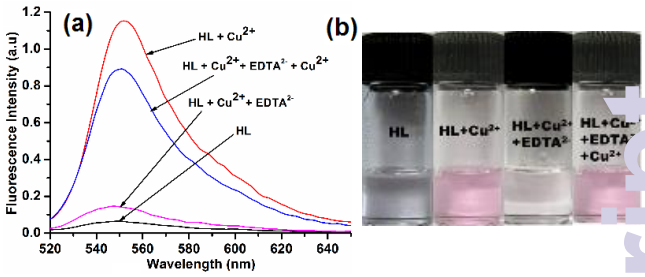

Fig. 6 (a) Reversible titration response of **HL** for Cu^{2+} (30 ppb), (b) visual illustration of the reversibility behaviour of **HL** with 30 ppb of Cu^{2+}.

Potential bio-imaging applicability, cell permeability and toxic nature of the receptor were studied first by *in-situ* cellular uptake ability of *E. coli* cells. Viewing through light microscope before and after the addition of Cu^{2+} to these cells revealed that, post Cu^{2+} and ... treatment, the colour of the bacterial cells changed to light-brow... (Fig. S13†). Based on these findings at hand we next hypothesized that **HL** could sense/visualize Cu^{2+} in higher group of invertebr... Brine shrimp *Artemia* is widely used as a model marine micro-crustacean due to its small body size, short life span, and capa... to bio-accumulate different heavy metal ions.[45,46] Generally, in crustaceans, metal ions are bio-accumulated mostly in the g... intestinal (GI) tract[47-49] as it is highly exposed to the aqua... environment due to uptake of large quantity of sea water to maintain osmotic balance. Different concentrations of Cu^{2+} (84, 64, 42, 9 pp...) were prepared in 10 mL of aged sea water and approximately 100 *Artemia* one day after hatch were added in each tube and incubated for 20 minutes at 25 °C and then sequentially exposed to **HL** (2 equ...) under identical conditions, washed with HEPES buffer and observed through a light microscope (Olympus DP72 U-TVO 63XC). Light microscopic images (Fig. 7) confirmed that the receptor **HL** could indeed be used as a colorimetric bio-sensor for Cu^{2+}. Intrinsic

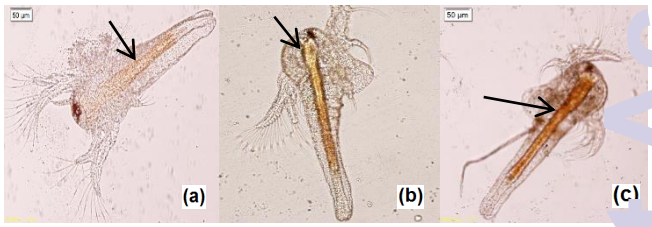

Fig. 7 Light microscopy images of live *Artemia*: (a) control, one d... after hatch, (b) exposed to 20 ppb **HL** solution, and (c) exposed to 1... ppb Cu^{2+} followed by addition of 20 ppb **HL** solution. Arrows indicat... the position of GI-tract.

bio-accumulation nature of *Artemia* enabled **HL** to detect Cu^{2+} at as low as 10 ppb. *Artemia* bath challenge study with 20 μM **HL** showed no mortality of the cysts after 24 h co-culture (Tables S2†, see the ESI†). Similar experiment with *E. coli* also showed no reduction in CFU/mL counts with time (24 h, Tables S3†, see the ESI†) which further demonstrate that **HL** can be used as a nontoxic colorim... staining agent.

In conclusion, we have developed a new colorimetric and fluorescent bio-sensor **HL**, bearing a rhodamine 6G unit as a fluorophore linked to a salicyldehyde derived tridentate organic moiety as a metal binding pocket/ion recognition unit (optical sensor) in a single molecule which binds one equivalent Cu^{2+} very selectively. The key finding with the integrated fluoroionophore **HL** is its unique ability to show naked eye detectable colour change from colourless to pink in presence of as low as 30 ppb of Cu^{2+}. The response time was found to be ≤ 5 sec. Detection limit could be improved further to 10 ppb through bio-accumulation of Cu^{2+} by *Artemia*. This combined approach of enhanced accumulation of metal ions followed by colorimetric metal ion-selective sensor-based detection may find important application in monitoring heavy metal induced toxicity/pollution in aquatic (marine and terrestrial) environments. In this study HL was developed to detect and measure Cu^{2+} concentration quickly in aquatic environments at very low concentration. In general, during water sampling, the samples are collected in an appropriate water sampler and analysed in-field. Therefore, addition of acetonitrile into the natural water sample would have no impact in the analysis or to the environment. Finally, the sensor exhibited reversible as well as reusable behaviour.

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Table of Contents

Synopsis: A Cu^{2+}-specific reversible chemosensor capable to show naked eye detectable instant change in colour in presence of as low as 30 ppb of Cu^{2+}. Utilizing brine shrimp *Artemia*, the LOD could be improved further to 10 ppb through bio-accumulation of Cu^{2+}.

Graphic: