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Selective fluorescence swing from cysteine to glutathione by switchover from solid to *in situ* probe in 100% water and bio-imaging studies for living species

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Two novel non-toxic Schiff-base-Cu^{2+} complexes, acting as fluorescent probes, synthesized in solid (1) and generated *in situ* (2), exhibit unprecedented selectivity and rapid sensitivity for sensing cysteine (Cys) and gluthathione (GSH), respectively, in 100% aqueous medium in *off–on* mode, exploiting the “displacement approach”. Reason behind remarkable swing from Cys to GSH for switchover from **1** to **2** is well established by experimental as well as theoretical evidences. The solid complex (probe **1**), reluctant to interact with bulky tri-peptide GSH owing to large steric congestion around Cu(II) centers, selectively detects small amino acid Cys by exploiting prompt decomplexation followed by Schiff-base ligand (L_1) hydrolysis and subsequent fluorogenic 4-methyl-2,6-diformyl phenol (DFC) generation. On the contrary, for sterically less congested *in situ* complex (probe **2**), the generation of the formyl moiety by means of partial hydrolysis of L_1 during complexation, fails to sense Cys owing to formation of stable non-fluorescent cyclic thiazolidine derivative, but reacts with GSH to produce DFC with almost same selectivity and sensitivity similar to probe **1**. Ratiometric responses at physiological detection level and bio-imaging studies to monitor Cys and GSH individually are analytically tuned for the first time in multi-cellular domain using the nematode, *Caenorhabditis elegans*.

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1. Introduction

Cellular biothiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) often play decisive role in controlling the oxidative stress, balancing the biological redox homeostasis appropriately in living systems [1–3]. Despite their structural similarities, alterations in the levels of the specific biothiols are linked to certain diseases [4–7]. Cys is an essential constituent of GSH and its deficiency may lead to slow growth, skin lesions, liver damage, *etc.*, while higher level is responsible for Parkinsonism, Alzheimer's, rheumatoid arthritis, *etc.* [4]. An elevated Hcy level in human plasma is also a risk factor for cardiovascular diseases, birth defects, *etc.* [5]. GSH, the most abundant intracellular biothiol (1–10 mM), is involved in the xenobiotic metabolism, intracellular signal transduction and gene regulation [1–3], while the abnormal levels are correlated with cancer, cardiovascular and neurodegenerative diseases [6,7]. Accordingly, it is truly essential to monitor individual biothiol levels, particularly in living systems to study the relationship with the aforesaid diseases.

Among various strategies to detect biothiols, fluorescence based sensing is advantageous due to its simplicity, high sensitivity and suitability for intracellular detection [8–11]. However, intracellular detection of individual biothiols in presence of others is extremely difficult owing to their close resemblance in structure and reactivity. Moreover, interference due to high level of intercellular GSH often limits the precise detection of Cys and Hcy in biological systems [8–11]. Consequently, among a large number of biothiol sensors reported so far [8–26], only a few are capable of discriminating Cys, Hcy and GSH from one another and with exceptions [27–29], most of them remain associated with serious limitations, including the use of biotoxic organic/water mixed medium, poor biocompatibility and delayed response. Hence, there still remains a

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great challenge to construct a fully water soluble fluorescent probe to discriminate among individual biothiols and side by side offering bio-imaging facilities in living organism.

In continuation of our study to "displacement approach" by exploiting transition metal based cationic fluorescent probes [30], owing to their better aqueous solubility with rapid response for effective *in vivo* detection ability, herein, we adopt the strategy to detect biothiols like Cys and GSH individually, using Cu(II)-coordinated Schiff-base probes followed by specific biothiol-induced demetalization and subsequent hydrolytic cleavage of the Schiff-base to generate strong fluorescence (Scheme 1). Although, such Cu(II)-based displacement strategy is quite simple and straightforward, to the best of our knowledge, only a few have so far been reported as "biothiol sensor as a whole" without specific discrimination ability [31–33]. Moreover, the displacement based sensors were only crosschecked with different amino acids and biological metal ions without providing any information for other bio-relevant species like NAD, NADH, GSSG, H_2O_2, Taurine, ds-DNA, Glucose, *etc.* Based on synthetic strategy and hydrolytic behavior of the ligand, probably for the first time, we report here the complete reversal of sensing ability from Cys to GSH in "*turn off–on*" mode with high sensitivity by switching from solid to *in situ* probe in 100% aqueous medium as well as its bio-imaging application in multi-cellular organism, *Caenorhabditis elegans*, via "displacement approach".

2. Experimental

2.1. General procedure

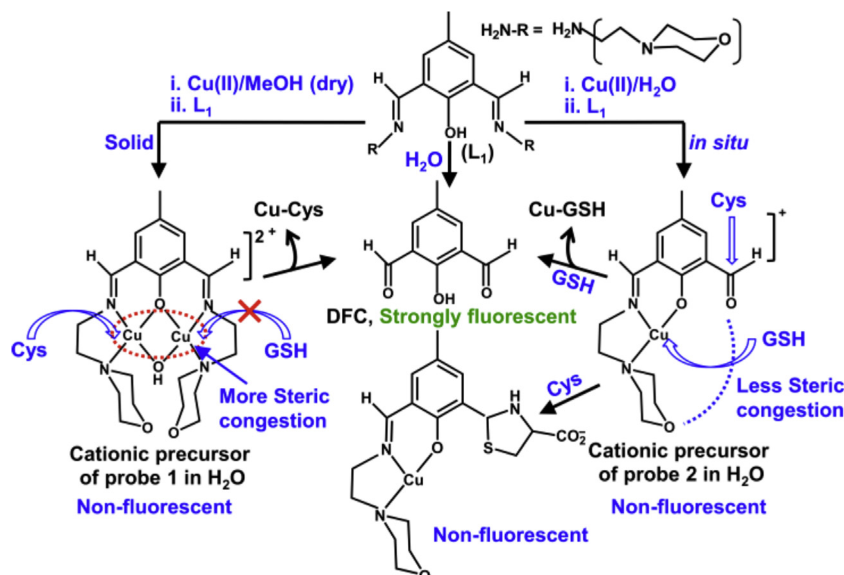
Unless otherwise mentioned, chemicals and solvents were purchased from Sigma-Aldrich Chemicals Private Limited and were used without further purification. Mili-Q Milipore®18.2 MΩ cm^{-1} water was used throughout all the experiments. Since the perchlorate salts are potentially explosive, only small amounts of the materials should be handled with care. The ESI-MS were recorded on a Waters Qtof Micro YA263 mass spectrometer in the positive mode. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and D_2O on a Bruker 300-MHz NMR Spectrophotometer using tetramethylsilane (δ = 0) as an internal standard. Elemental analyses were carried out using a Perkin-Elmer 240-elemental analyzer. IR spectra were recorded from KBr pellets with a Perkin-Elmer Spectrum-2 spectrophotometer and corresponding spectra in solution phases were carried out using Zn-Se ATR. EPR spectra were recorded using a JEOL JES-FA-200 instrument at liquid nitrogen temperature (77 K) in H_2O/MeCN 1:1 mixture. pH measurements were performed with Systronics digital pH meter (Model No. 335). The bio-imaging studies were carried out with a Leica DM-3000 Fluorescence Microscope (excitation ~480 nm) with a Leica DFC-450C Camera (Leica Fire Wire V1.39.0).

2.2. Synthesis of Schiff-base ligand (L_1)

4-Methyl-2,6-diformyl phenol (DFC) was synthesized according to published procedure starting from p-cresol [34]. For ligand (L_1) synthesis, to an anhydrous methanolic solution of DFC (0.328 g, 2 mmol), 4-(2-aminoethyl)-morpholine (0.521 g, 4 mmol) was added drop wise with constant stirring and 2 drops of AcOH were further added to it. The mixture was refluxed for 2 h and then filtered. The filtrate was then evaporated under reduced pressure to get the crude product as gel. It was purified by column chromatography followed by rotary evaporation to obtain the pure product and dried over P_2O_5 under vacuum; yield: 72.8% with respect to DFC. ESI-MS$^+$ for L_1 in anhydrous methanol: m/z Calcd for $[L_1 + H]^+$: 389.5179. Found: 389.2662. In water L_1 hydrolyzed to generate DFC. ESI-MS$^+$ for L_1 in water: m/z Calcd for $[DFC + H]^+$: 165.1521. Found: 165.1221. (See details of mass spectra in Supplementary content). Selected IR in cm^{-1} (Zn-Se ATR): 3400–3200 (br), 3046 (m), 2526 (w), 2040 (w), 1702 (s), 1449 (m), 1113 (m), 1031 (s). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.32 (s, 3H, $ArCH_3$), 2.56 (m, 4H, $-NCH_2$), 2.72 (t, 4H, $=NCH_2$, J = 6), 3.71–3.78 (m, morpholine ring $-CH_2$, 16H) 7.69 (s, 2 H, ArH), 8.35 (s, 2H, imine-H), 10.51 (s, 1H, ArOH) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): 20.20, 53.89, 55.89, 58.74, 66.91, 119.67, 124.13, 127.39, 131.79, 137.99, 163.49, 165.07, 189.54 ppm. In D_2O, L_1 is hydrolyzed to generate DFC and that was confirmed by the additional ^{1}H NMR peak generated at 9.96 ppm (s, 1H, –CHO) with abolition of phenolic–OH peak due to D exchange (See details NMR spectra in Supplementary content).

2.3. Synthesis of probe **1**

To an anhydrous methanolic solution of L_1 (0.776 g, 2 mmol), $Cu(ClO_4)_2 \cdot 6H_2O$ (0.741 g, 2 mmol) was added pinch wise with constant stirring for 2 h. After that 2 drops of water were added for



Scheme 1. Synthesis and proposed sensing mechanism for probes **1** and **2**.

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