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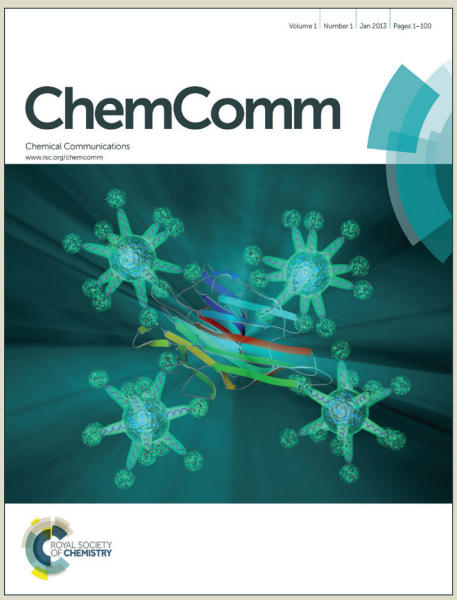

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ARTICLE TYPE

A novel fluorescence probe for estimation of Cysteine/Histidine in human blood plasma and recognition of endogenous Cysteine in live Hct116 cells

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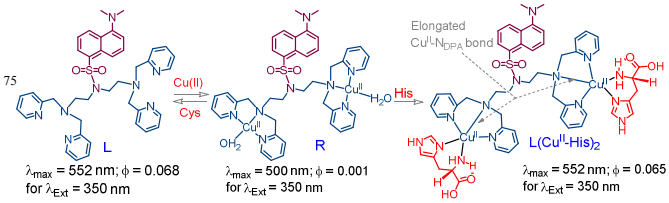
A new Cu(II)-complex is used a as a "*Turn-On*" luminescence probe for specific detection of endogenous Cys in live Hct116 cells and Cys present in human blood plasma without any interference from other amino acids, especially GSH and Hcy. Difference in the mechanistic pathway for Cys and His recognition is discussed.

High sensitivity and spatiotemporal resolution are two key issues that have made molecular probes with *fluorescence on* response an efficient tool for detection and visualization of minute amounts of the desired analytes(s) in live cells and tissues. Accordingly, numbers of such molecular probes are reported for vital biothiols, like cysteine (Cys) and histidine (His); as these play vital roles in many critical bioactivities.[1] Among these, Cys acts as a intracellular redox buffer that influence detoxification and critical metabolic functions.[1] Deficiency in Cys causes oxidative damage, haematopoiesis, psoriasis, leucocyte loss and metabolic disorders. Higher level of Cys in the human blood plasma (HBP) is known to cause cardiovascular and Alzheimer's diseases.[2] His can act as a neurotransmitter in the central nervous system of mammals. Its deficiency could affect human growth factor and may cause the impaired nutritional state of patients with chronic kidney disease.[3] Higher level of His could cause metabolic disorders like histidinemia.[4] Estimation of these amino thiols in HBP is essential for understanding the role of those in the pathogenesis of vascular diseases and these have provoked researchers in developing molecular probes that could quantitatively delineate these biothiols from other amino acids[2,3b] However, examples of molecular probes capable of detecting Cys and His in biological fluids in presence of Hcy and GSH are rather limited.[5e,n] Molecular probes that are generally being used for detection of biothiols either in physiological condition or in biological fluids are mostly based on chemodosimetric reaction and such process often suffers from the relatively large incubation time ranging from 20 minutes to 1 hr or more.[5a-c,h] Alternate approach utilizes the higher affinity of Cu(II) towards S-donor nucleophiles for designing molecular probes with fluorescence on response.[5e,g] Such reactions mostly happen within millisecond time scale and thus, this methodology has an edge over the other for application in diagnostic. Examples of molecular probe that could specifically detect Cys, even in presence of Hcy and GSH, in pure physiological condition are scanty,[5a-c] while limited such probes are known for His.[5f,j] However, majority of such reagents relied on chemodosimetric detection and estimation of the total thiol content in HBP.[6] Use of certain fraction of organic solvents were described in most such reports for solubilising the reagent. In our attempt to develop a molecular probe for instantaneous and selective detection of endogenous Cys or Cys/His in HBP, we have synthesized a novel bis-Cu(II)-complex (**R**, Scheme 1) using a new bis-dipicolyl amine derivative (**L**). Lower pK_a value of the thiol group in the side chain in Cys (8.00), as compared to Hcy (8.87) and GSH (9.20), is utilized for its specific detection without any interference from these two commonly interfering biothiols at pH 7.4. At this pH, Cys exist predominantly in its thiolate form and thus acts as a better ligand for Cu(II) than other biothiols.[5d] Also, the well known affinity of His to form stable complex with Cu(II) is utilized in the present study for its recognition.[7] Thus, this reagent (**R**) could be utilized for specific and quantitative estimation of Cys and His in HBP as well as for specific imaging of endogenous Cys over Hcy and GSH in live Hct116 cells. Such example is rather uncommon in contemporary literature.

Analytical and spectroscopic data confirmed the desired purity for new ligand (**L**), reagent (**R**) and other intermediates.† All studies with **L** were performed in the aqueous HEPES buffer-acetonitrile (96: 4 (v/v); pH 7.4) medium, while studies for **R** were performed in pure aq.-HEPES buffer (pH 7.4) medium.



Scheme 1. Molecular structure for receptor **R** and its reactions with Cys and His with associated steady state fluorescence properties.

Uv-vis spectrum of **L** (Fig. 1a) showed two distinct bands at 256 and 329 nms. Band at 256 nm was attributed to a charge transfer (CT) transition involving N_{Amine} as donor and dansyl moiety as acceptor. The other band at 329 nm was ascribed to a dansyl-based CT transition.[8] These two bands for **R** appeared at 252 and 324 nm, respectively.† Observed blue shifts in **R** was ascribed to a less favoured CT process, as compared to those in **L**.

Fluorescence responses of **L** (20 μM) towards Cu^{2+} in aq.-HEPES buffer-CH_3CN (96:4 (v/v); pH 7.4, RT) medium are shown in Fig.1b. A strong emission band (Fig. 1a) at λ_{max} of 552 nm (Φ = 0.068 for λ_{Ext} of 350 nm and dansyl amide as a standard)[8] was observed. Hypsochromic shift ($\Delta\lambda_F$) of 52 nm in emission maxima was observed upon binding of **L** to Cu^{2+} with anticipated quenching of the dansyl-based fluorescence. This luminescence quenching was attributed to the binding to the Cu^{2+}-centre (d^9).[9,5e]

A very weak emission band with λ_{Max} of ~500 nm was assigned to a new CT-based process on formation of a bis-Cu(II) complex.

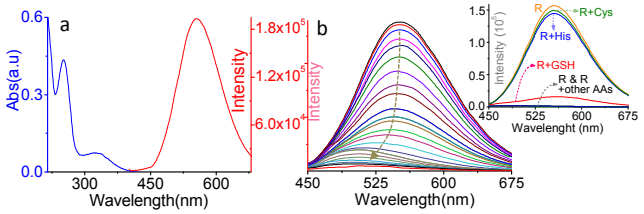


Fig. 1 (a) Uv-vis and luminescence (λ_{Ext} = 350 nm) spectra of **L** (20 μM) and (b) Emission spectra of **L** (20 μM) in presence of varying [$Cu(ClO_4)_2$] (0 to 4.6 x 10^{-5} M) (λ_{Ext} = 350 nm; slit width 3/3 nm); Inset: Luminescence spectra of **R** (20 μM) in the absence and presence of various amino acids (AAs); e.g. tryptophan (Trp), leucine (Leu), isoleucine (Ile), methionine (Met), threonine (Thr), tyrosine (Tyr), valine (Val), alanine (Ala), serine (Ser), glycine (Gly), cysteine (Cys), Glutathione (GSH), homocysteine (Hcy), proline (Pro) and arginine (Arg).

Systematic changes in emission spectra for **L** in presence of varying [Cu^{2+}] are shown in Fig. 1b. B-H plot revealed a 1: 2 binding stoichiometry and a binding constant of (4.2 ± 0.02) x10^8 M^{-2} for formation of **R** on interaction of **L** with Cu^{2+}(Scheme 1).† This binding stoichiometry was further confirmed from the results of the ESI-Ms studies.†

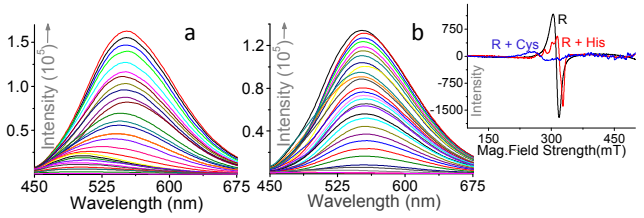


Fig. 2 Changes in luminescence spectra for **R** (20 μM) in presence of varying (a) [Cys] (0 - 2.8 x 10^{-3} M) and (b) [His] (0 - 3.2 x 10^{-3} M) using λ_{Ext} = 350 nm and slit width 3/3 nm; Inset: X-Band EPR spectrum of probe **R** (1.0 mM) in absence and presence of [Cys] (3.0 mM) and His (3.0 mM). All studies were performed in aq.-HEPES buffer (10 mM, pH 7.4) medium at 298K.

Luminescence spectra for **R** (20 μM) in the absence and presence of 200 mole equivalent of various AAs were recorded and are shown as an inset in Fig. 1. These studies clearly revealed that an increase in emission intensity was observed at 552 nm when Cys or His was added to the aq.-buffer solution of **R**. Other thio-amino acids, especially Hcy and GSH could not induce any change in emission spectral pattern. This result is significant, as Hcy or/and GSH generally interfere with the Cys detection.[5e,g,o]

Electronic spectra recorded for **R** in presence of Cys and His were little different, which signified a distinct difference in the nature of interaction between Cu(II)-centre in **R** and Cys or His.† In order to understand these, X-Band EPR spectra of **R** in absence and presence of Cys and His in aq. buffer medium were recorded (Inset in Fig.2). For **R**, solution spectra was isotropic with g_{av} = 2.099, while that in presence of His was having axial symmetry with $g_{||}$, $g_{\perp}$ and $A_{||}$ values as 2.230, 2.035 and 166.2x$10^{-4}$$cm^{-1}$, respectively.[10] Affinity of Cu(II) to form mixed ligand coordination complexes with His is well documented and this presumably supports the formation of L(Cu^{II}-His)$_2$ (Scheme 1).[11b] Interestingly, the EPR spectrum recorded for **R** in presence of Cys was significantly different. Cu^{2+} is known to be reduced to Cu^{+} by Cys with stoichiometric production of cystine.[11] Previous reports revealed that this Cu(I) forms coordination complex with the excess of Cys present in the reaction medium under the condition that is very similar to the present study.[11a] It is reported that O_2 (present in the reaction medium) could oxidize Cys-Cu(I) complex to cystine with simultaneous generation of Cu(0), which could undergo partial disproportionation to generate Cu(II) and Cu(I). This presumably accounts for the insignificantly small EPR signal (Fig. 2) for **R** treated with Cys.[11] The m/z signals in the ESI-Ms spectra were observed at 701.68 [(**R**-2Cu)+H^+] and 1235.66 [**R**+2His+ClO_4^-], respectively, when spectra for **R** were recorded in presence of excess Cys and His, respectively. These data supported the eventual demetalation reaction of **R** with Cys and complex formation reaction with His (Scheme1). All these confirmed that different reaction pathways for interaction of **R** with Cys and His that led to the *fluorescence on* response.

Systematic luminescence titrations were carried out for **R** (20 μM) with varying [Cys] (0 to 2.8 x 10^{-3} M) and [His] (0 to 3.2 x 10^{-3} M) in pure aq.-HEPES buffer medium (10 mM, pH 7.4). Increase in [Cys] or [His] caused a concomitant increase in emission intensity at ~552 nm (Fig. 2). Titration profiles revealed linear range of 90 - 2800 μM and 200 - 3200 μM, for Cys and His, respectively. As discussed above, the fluorescence changes shown in Fig. 2a for Cys involved several competing equilibrium and it was not possible to evaluate equilibrium constant for each individual process. However, B-H plot for emission titration with varying [His] (Fig. 2b) helped us to evaluate the binding constant ($K_{R.2His}$ = (1.4± 0.03) x 10^4 M^{-2}) for the formation of **L** (Cu^{II}-His)$_2$ (Scheme 1). The emission enhancement observed on binding of Cu(II) centres in **R** to His could be explained based on the much weaker and elongated Cu^{II}-N_{Amine} bond in L(Cu^{II}-His)$_2$ as compared to that in **R** (Scheme 1). Such an elongation of the metal-N_{Amine} bond with a consequential fluorescence enhancement are reported earlier for other Cu(II) complexes on binding to an anionic analyte.[12] This effectively nullifies the quenching influence induced by d^9 Cu(II)-centre.

Above discussions reveal that this reagent (**R**) could be utilized for quantitative estimation of total [Cys]+[His] present in a solution. Studies performed in presence of N-ethylmaleimide (NEM) enabled us to quantitatively evaluate the [His] in the solution mixture, as NEM reacted selectively with *cysteine* to afford thiomaleimides.† This methodology enabled us to selective detect Cys and His in ensemble of all other natural AAs in pure aq. buffer medium, including Hcy and GSH. To the best of our knowledge, such example is rare in the contemporary literature.

The respective concentration level of Cys and His in human plasma are typically 240-360 μM and 15-75μM.[6b,7] Detailed methodology, pre-treatment of the blood plasma sample and the generation of the calibration curve with linear fluorescence response range for estimation of unknown [Cys] and [His] in aq. buffer (pH 7.4) solution as well as in HBP sample (Fig. 3A) are discussed in details in supporting information.† The final concentration of the probe molecule **R** in each solution used for estimation of Cys and His in HBP samples was maintained at 20 μM. Please note that emission intensities at 552 nm for HBP samples treated with a standard solution of **R** gave the total amount (C_T = [Cys] + [His]) of these two AAs present in HBP sample. This solution was further treated with 1 mM of NEM for 30 min and the resulting solution was further treated with the solutions spiked with 200 μM and 300 μM of His as an internal standard. Corresponding emission intensities ($I_{HBP+200}$ and

$I_{HBP+300}$, respectively) were recorded for these two solutions. Average of two differences ($I_{HBP+200}$–I_{200}) and ($I_{HBP+300}$–I_{300}) and the calibration plot for His (†) led us to evaluate the actual [His] of (23 ± 2.1) μM (C_{His}) in HBP sample. Please note that I_{200} & I_{300} are emission intensities of the aq. HEPES buffer solution with 20 μM of **R** in presence of 200 and 300 μM of His, respectively. The difference, (C_T - C_{His}) yielded the actual [Cys] (239 ± 7.5 μM) in HBP sample. Thus the evaluated [Cys] and [His] in HBP were within the allowed limit for a healthy human being.[6b]

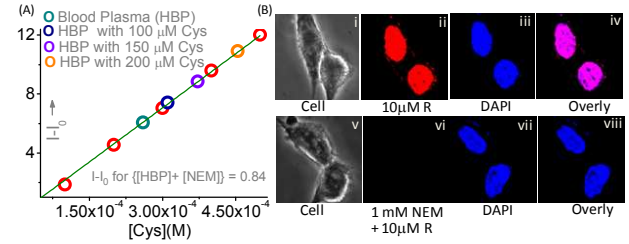


Fig. 3 (A) Plot of ΔI = (I-I_0) vs. [Cys], where I_0 and I are emission intensities of aq. HEPES buffer solution (pH = 7.4) of **R** at 552 nm (λ_{Ext} = 350 nm) in the absence and presence of known [Cys] (O) as well in blood plasma/blood plasma samples spiked with three different known [Cys]; (B) Confocal laser fluorescence microscopic images of Hct116 cells treated with 10 μM of **R** in HEPES buffer and various reagent mentioned in the Fig. 3 (B). Figs. iv & viii are overlay of the merged images and confirmed the intracellular fluorescence.

MTT assay revealed insignificant cytotoxicity this probe towards Hct116 cells with IC_{50} of 200 μM.† Further, to assess the possibility of using this reagent **R** as an imaging reagent for detection of intracellular Cys that could be present in live Hct116 cells, confocal laser microscopic studies were performed. Live Hct116 cells were incubated with **R** (10 μM) in aq.-HEPES buffer solution (pH 7.4) at 37°C and then washed thrice with phosphate buffer solution (PBS) to remove the surface adhered probe molecules. Control experiments were performed without exposing live Hct116 cells to the probe reagent solution; otherwise maintaining the identical experimental conditions. The bright-red fluorescence images were observed from Hct116 cells treated with **R**, while no such fluorescence images were observed from Hct116 cells used in control experiments (Fig. 3B). These results confirmed the cell membrane permeability of this reagent and it ability to react with the intracellular Cys to initiate the demetallation reaction (Scheme 1) and regenerate **L** with *fluorescence on* response. In order to confirm that this fluorescence on response was due to the reaction of **R** with intracellular Cys, live Hct116 cells were pre-incubated with 1mM of NEM for 30 min prior to the incubation with reagent **R** (10 μM) for a further period of 30 min. NEM is known to react specifically with Cys and would not interfere with the further detection of His. Insignificant fluorescence from cells of this control experiment (Fig. 3B(vi)) due to the interaction of the reagent **R** with endogenous His, which was lower than the lowest detection limit for His and thus could evade such detection. Thus, confocal images confirmed the ability of this reagent to detect intracellular Cys.

Conclusions

In brief, we have reported a unique reagent that is capable of detecting Cys and His in pure aq. buffer medium having pH 7.4 as well as suitable for quantitative estimation of His and Cys in HBL with fluorescence on response. This reagent is also cell membrane permeable and could detect the endogenous Cys present in live Hct116 cells. Two different mechanistic pathways are proposed for interaction of Cys and His with this reagent **R**.

Notes and references

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†Electronic Supplementary Information (ESI) available: [Synthesis, characterization details for **1**, **L** and **R**, other methodologies and sample preparation]. See DOI: 10.1039/b000000x/

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