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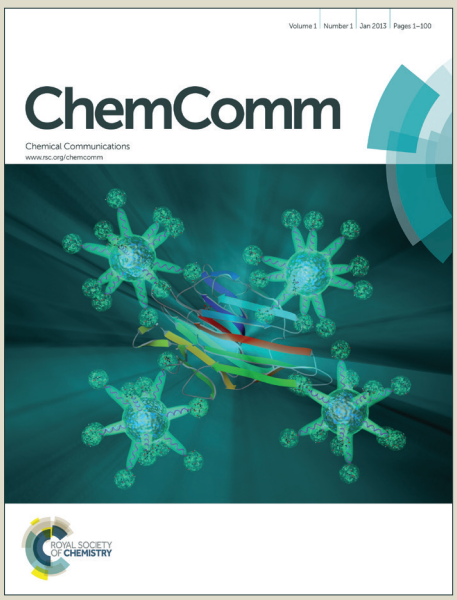

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Reagent for Specific Recognition of Cysteine in Aqueous Buffer and in Natural Milk: Imaging Studies, Enzymatic Reaction and Analysis of Whey Protein

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We report a new chemodosimetric probe (L) for specific recognition of cysteine (Cys) in aqueous buffer and in whey protein isolated from fresh cow's milk. Using this reagent we could develop a *luminescence*-based methodology for estimation of Cys released from a commercially available Cys-supplement drug by aminoacylase-1 in live cells.

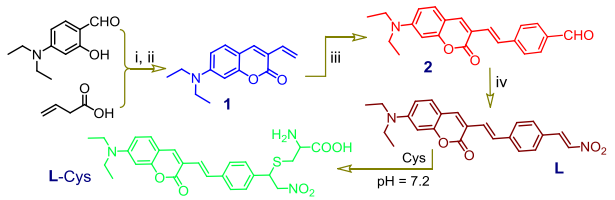
Post-translational modifications of specific amino acids play major roles in regulating protein function.[1] Major functional sites of proteins that typically respond to redox perturbations contain cysteine (Cys) residue.[1] Glutathione (GSH) plays a pivotal role in maintaining cellular antioxidant defence system.[1e,1f,2a] Abnormal levels of GSH is linked to many diseases such as HIV, aging, neurodegenerative diseases and cancer.[2a] Cys is one of the three main precursors that are required for GSH synthesis.[1b,e,f] Apart from this crucial role in human physiology, deficiency of Cys also has adverse influences on child growth, depigmentation of hair, edema, liver damage, skin lesions and weakness.[1c,d] Literature reports also suggest that free Cys is not ideally suited for human physiology for its toxicity and facile oxidation.[2b] Thus, appropriate and stable Cys-derivatives are prescribed as supplements, which participate in an enzymatic reaction to liberate Cys for maintaining its optimum concentration in human blood plasma (HBP). Whey protein (WP) concentrate is also used as an effective Cys supplement for GSH replenishment in its immune deficient state.[3a] Thus, specific detection and estimation of Cys and its derivatives have significance in clinical research and diagnostics. However, interference from Hcy (Homocysteine) and GSH makes it difficult owing to the similarities in structure and reactivity. Also, some of the reagents, which are commonly adopted for different chemodosimetric detection of Cys, also react with CN^-, an efficient nucleophile with relatively low hydration enthalpy.[3b,4] All these add to the challenge in designing a reagent for specific detection and estimation of Cys in biofluids. HPLC is the most conventional methodology that is being adopted for Cys estimation in biofluids. However, such a procedure involves intricate sample preparation process and more importantly requires a postcolumn derivatization technique.[4] Considering the complexity involved in such process, recent efforts are focused in designing fluorescence based molecular probes for selective estimation of Cys in biofluids as well as for use as an imaging reagent for detection of intracellular distribution of Cys.[1c,d] Strategies like chemodosimetric procedure or metal-Cys coordination has been usually adopted for specific recognition of Cys in aqueous medium.[5] Barring some reports,[6] examples for specific detection of Cys or its residues in presence of above referred competing biothiols are limited. Moreover, none of these reagents were utilized for developing an assay for enzyme like Aminoacylase-1 for *in-situ* generation of Cys from a prescribed drug N-acetyl cysteine (NAC), which is generally used for treating psychiatric and many other disorders[7] or for estimation of Cys/ Cystine residues in cow's milk whey.

Keeping these in mind, we have designed a chemodosimetric probe (**L**) for specific reaction with Cys in presence of all other amino acids (including Hcy and GSH), all common anions and cations (alkai, alkaline earth and transition metal ions that are common in human physiology) in physiologically relevant aq. medium. Using the specificity of this reagent **L** towards Cys, we could estimate Cys residues with free sulfhydryl functionality in whey protein as well as we could develop an efficient methodology for probing the release free Cys from NAC by Aminoacylase-1, an important enzyme for human physiology. Such example is scarce in the contemporary literature.

Kim and co-workers reported the role of pK_a in achieving the desired specificity to Cys (in presence of Hcy & GSH) for participating in Michael-type reactions.[6d] We have adopted this methodology for designing an efficient chemodosimetric probe (**L**, Scheme 1) for Cys. Choice of nitro olefin not only helped in achieving a favourable intramolecular charge transfer (ICT) transition, but also offered us the desired functionality for participating in the Michael-type reaction.[5j]

Synthesis (Scheme 1) and analytical/spectroscopic data for the new reagent **L** are provided in the supporting information. These data confirmed the proposed structure and the desired purity for **L**. Electronic spectrum of **L** (10 μM) in aq. HEPES buffer:CH_3CN (9:1, v/v; pH 7) showed a λ_{Max} of 468 nm in the visible region and this

was attributed to an ICT process with Me_2N- functionality as donor and -NO_2 as acceptor. Observed red shifts of this absorption band with increase in the solvent polarity (SI Fig. S13) confirmed the predominant ICT nature of this band.



i. DCC, DMAP, CH_2Cl_2, 0° C, 4h; ii. Cs_2CO_3,12h; iii. 4-bromobenzaldehyde, $Pd(OAc)_2$, PPh_3, NaOAc, DMF, 80°C, 16h; iv CH_3NO_2, NH_4OAc, 85°C, 3h.

Scheme 1: Methodology adopted for the synthesis of **L**.

Spectrum for **L** (10 μM) remained unchanged when it was recorded in presence of 100 equiv. of all common anions (X: F^-, Cl^-, $H_2PO_2^-$, CH_3COO^-, HSO_4^-, CN^-), cations (Na^+, Ca^{2+}, Mg^{2+}, $Fe^{2+/3+}$, Cu^{2+}, Cr^{3+}, Ni^{2+}, Zn^{2+}, Hg^{2+} †) and natural amino acids (AAs: Histidine (His), Leucine (Leu), Methionine (Met), Isoleucine (Ile), Phenyl alanine (Phe), Tryptophon (Trp), Tyrosine (Tyr), Valine (Val), Serine (Ser), Alanine (Ala), Arginine (Arg), Glycine (Gly), Aspartame (Asp), Glutamine (Gln), Proline (Pro), Aspartic acid (Asp), Glutamic (Glu) acid, Threonine (Thr), Lysine (Lys), Methionine (Met), GSH and Hcy), except for Cys.

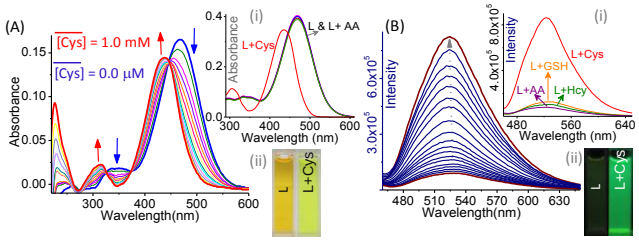


Fig. 1: (A) UV-Vis and (B) emission titration profile for **L** (10 μM) in the absence and presence of varying [Cys] (0-100 equiv.); Insets: (Ai) UV-Vis and (Bi) emission spectra of **L** in absence and presence of various AAs; Insets: snap shots of changes in (Aii) solution colour and (Bii) solution luminescence for **L** (10 μM) & **L** (10 μM) + Cys (100 μM) on irradiation with 365 nm UV lamp. All studies were performed in 10 mM aq. HEPES buffer:CH_3CN (9:1, v/v; pH 7) medium at 298 K.

For Cys, a distinct hypsochromic shift of ~ 30 nm (Fig 1A) was observed with a visually detectable change in solution colour from reddish brown to light green (Fig. 1A, Inset (i)). Systematic titration with **L** (10 μM) in presence of varying [Cys] (0-100 equiv.) in aq. HEPES-CH_3CN medium (9:1, v/v; pH 7) revealed a ratiometric response with gradual bleaching of the ICT band for **L** at 468 nm and a concomitant growth of a new absorption band with λ_{Max} at 438 nm (Fig 1A). Titration profile revealed three simultaneous isosbestic points at 325, 372 and 445 nm, which confirmed the presence of only two species in equilibrium. Inset (i) of Fig. 1B also revealed that the luminescence band maximum for reagent **L** (10 μM) appeared at ~ 520 nm on excitation at 445 nm (λ_{Max}^{ICT}). Spectral pattern essentially remain unchanged upon addition of 200 mole equiv. of all natural AAs (except Cys) and all other common anionic/cationic analytes. Emission titration profile clearly revealed that a switch *ON* luminescence response with λ_{Max}^{Ems} of 520 nm (Φ_f^L ~ 0.06 & Φ_f^{L+Cys} = 0.32; using fluorescein with Φ_f = 0.92 as standard) was observed for varying [Cys] (0-100 mole equiv.). Simultaneously, a visually detectable change in solution luminescence [Fig. 1B Inset (ii)] was observed.

In order to confirm the Michael addition reaction between **L** and Cys, ^{1}H NMR spectra were recorded for **L** and **L** + Cys in DMSO-d_6 (Fig. 2) after the solution mixture of **L** and Cys was allowed to equilibrate for 20 min at RT. The olefin peaks at 8.25 ppm (H_a) and 8.15 ppm (H_b) for **L** disappeared, while appearance of two new peaks was observed at 5.29 ppm and 4.77 ppm, respectively, for H_{a1}/H_{a2} and $H_{b'}$ protons in **L**-Cys (Fig. 2).

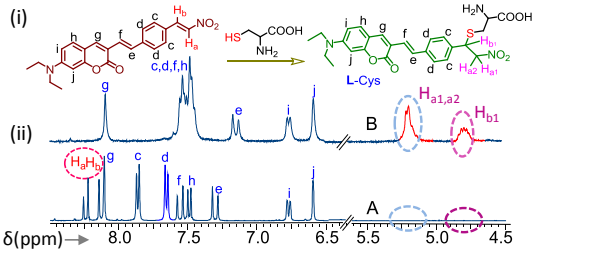


Fig. 2: (i) Proposed mode of binding between **L** and Cys; (ii) Partial ^{1}H NMR spectra of (A) **L** and (B) **L** + Cys in DMSO-d_6 medium.

Little upfield shifts were observed for aromatic protons and this was attributed to a reduced electron withdrawing influence of -NO_2 functionality in **L**-Cys due to a lesser extent of extended conjugation. Formation of the proposed **L**-Cys adduct was further confirmed from an isolated reaction product of **L** and Cys using HRMS [ESI-Ms] (Fig. SI S 18). Formation of **L**-Cys [Fig. 2(i)], is also expected to disfavour the ICT process and enhances the energy gap between the frontier orbitals (HOMO & LUMO), which agree well with the observed hypsochromic shift of ~ 30 nm in the UV-Vis spectra. Emission spectrum for **L**-Cys was distinctly different from that of **1** (Scheme 1 & Fig. SI S 15), which nullified the possibility for the observed emission from an analogous coumarin moiety. Presumably this enhanced HOMO-LUMO energy gap interrupted the non-radiative deactivation pathway of the ICT based excited state and accounted for the observed luminescence. Under identical condition, other AAs and common anionic/cationic analytes (*vide supra*) did not show any detectable change in the absorption and luminescence spectra. Examples of the molecular probes for specific recognition and estimation of Cys and its protein residues with free sulfhydryl$_{Cys}$ group in an ensemble of several other AAs are scanty and considering this, the result described in this article has significance.[1c] The switch *ON* luminescence response of the probe at 520 nm showed a good linear relationship with varying [Cys] (0 to 1000 μM; Fig. SI S 16). The lowest detection limit of Cys was determined as 23.65 nM based on signal to noise ratio of 3 at 520 nm (Fig. SI S 17).

Studies with Cys, Hcy and GSH (pK_a values of 8.22, 10.00 and 9.20, respectively)[8,6d] at different pH were performed to examine the influence of pH on the observed specificity of the reagent towards Cys (Fig. 3A). Figure 3A clearly demonstrates the higher sensitivity and selectivity of the reagent **L** towards Cys at pH 7. Interference from GSH became noteworthy only at pH ≥ 8. Due to the lowest pK_a value, the thiolate/thiol ratio was higher for Cys at pH 7.0, as compared to Hcy and GSH, which resulted in the higher reactivity and the desired specificity of **L** towards Cys.

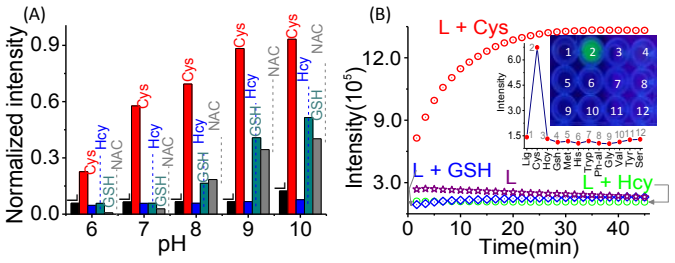
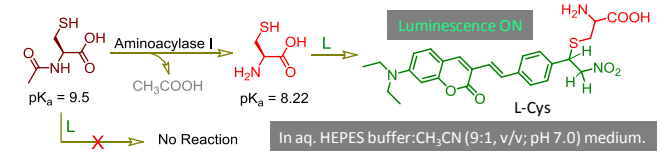


Fig. 3: (A) Changes in fluorescence response (λ_{Ext} = 445 nm) of **L** (10 μM) with Cys, Hcy, GSH and NAC at varying solution pH; (B) Time dependent fluorescence response of **L** (10 μM) with 200 mole equiv. of Cys, Hcy and GSH. All studies were performed in 10 mM aq. HEPES:CH_3CN (9:1, v/v; pH 7.0) with λ_{Ext} = 445 nm; Inset: (B) Micro plate fluorescence reading of **L** with different AAs and corresponding plots of relative changes in emission intensities. Inset: change in solution fluorescence on irradiation with 365 nm UV lamp in presence of different AA.

Fig. 3B further reveals that this chemodosimetric reaction takes approximately 25 minutes to complete. A comparison study (Fig. 3B) further revealed that the possibility of any interference from GSH and Hcy was minimal under the identical experimental conditions. The microplate reading experiment with 0.2 μM of **L** and respective AA (200 mole equiv.) in aq. HEPES:CH_3CN (9:1, v/v) solution further confirmed that only Cys could induce a detectable change in solution fluorescence (Fig. 3B Inset).



Scheme 2: Hydrolysis of NAC by aminoacylase-1 and subsequent reaction of in-situ generated Cys with **L**.

After confirming that this reagent could detect Cys in an essentially aq. buffer medium (aq. HEPES:CH_3CN; 9:1, v/v) at pH 7.0, we explored the possibility of using this reagent for monitoring the release of free Cys from NAC by aminoacylase-1, an important mammalian enzyme. NAC is widely used as a pro-drug and Cys supplement for the treatment of acetaminophen overdose, glutathione replenishment, HIV patients etc.[7] Aminoacylase-1 generates Cys inside the cells from NAC.[9a] Although NAC has a free sulfhydryl group, our control experiment with **L** and 200 mole equiv. of NAC revealed no change in the luminescence response of **L** (Fig.4(i)). This confirmed that NAC failed to participate in any reaction with **L** at pH 7.0 (Scheme 2). This was presumably due to the higher pK_a value of NAC (pK_a = 9.5)[9b], which did not favour the reaction with **L** at pH 7. This finding nullified the possibility of any interference from NAC in our studies on the release of Cys from NAC by aminoacylase-1 (Fig. 4 & Fig. SI 19). Aminoacylase-1 is a binuclear Zn(II)-dependent metallopeptidase, which hydrolyses acetylated cysteine.[10] Coordination to the Zn(II)-center(s) enhances the electrophilicity at the C-center of -$CONH_{NAC}$ functionality and lowers the activation barrier for N-C bond cleavage of the amide moiety in NAC and generation of Cys.[10] In the current study, we utilized the *luminescence ON* response for **L**-Cys formation due to a reaction between **L** and Cys, released by enzymatic action of aminoacylase-1 on NAC (Scheme 2). Methodologies adopted for this experiment are provided in the supporting information. In absence of any aminoacylase-1, no change in emission intensity was observed at 520 nm (Fig. 4(i)). However, analogous studies with definite concentration of aminoacylase-1 showed gradual increase in emission intensity at 520 nm with time (Fig. 4(i)) and confirmed a reaction between Cys (generated *in-situ*) and the reagent **L** for the formation of **L**-Cys. Increase in luminescence intensities with increase in [Aminoacylase-1] (0 to 2000 units) was also observed [Fig 4(ii)].

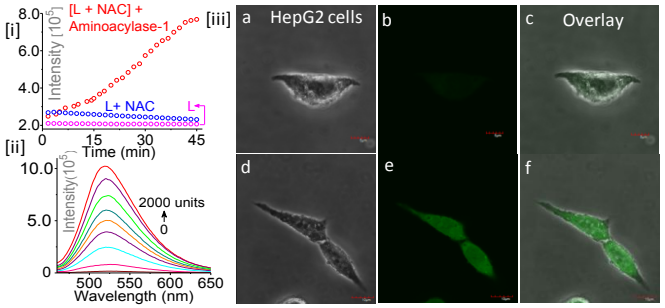


Fig. 4: (i) Fluorescence response of **L** (10 μM), **L** + NAC and [**L** + NAC (200 equiv.)] + aminoacylase-1 (1000 units) with time at 37°C; (ii) changes in luminescence spectra on incubation of [**L** + NAC (200 equiv.)] with varying [aminoacylase-1] (0 - 2000 units) for 45 minutes; (iii) confocal laser fluorescence microscopic (clfm) images of live HepG2 cells: (a) - (c): bright field, dark field and overlay images of cells treated first with 2 mM NEM and then with 10 μM of **L**, respectively; (d) – (f): analogous images for cells treated with 2 mM NEM, and then these cells were washed after incubation of 20 min—followed by exposure of these washed cells to 20 μM NAC. Comparison of Figs. (4b & 4e) or Figs. (4c & 4f) clearly establish the bright green luminescence for the formation of **L**-Cys due to a reaction between **L** and Cys, produced *in-situ* by reaction of NAC and intracellular aminoacylase-1. For emission studies, λ_{Ext} = 445 nm was used.

In-situ generation of Cys in the live HepG2 cells (Fig. 4) was demonstrated successfully by utilizing the facile reaction between Cys released from NAC by intracellular aminiacylase-1. Availability of the intracellular aminoacylase-1 was accounted for the choice of HepG2 cells. Comparison of the Figs. (4b & 4c) or (4c & 4f) clearly revealed that the reagent **L** could react with Cys, generated within these HepG2 cells from NAC by enzymatic reaction of aminoacylase-1. These experiments also confirmed the cell membrane permeability and usefulness of **L** as an imaging reagent. Further, MTT assay studies with live HepG2 cells confirmed the insignificant toxicity of the reagent.†

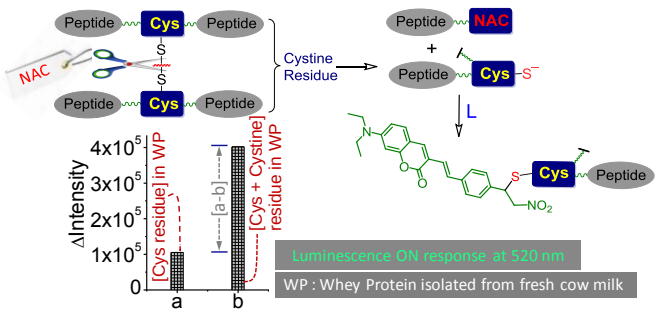


Fig. 5: (A) Scheme showing the cleavage of –S—S– bond in WP in presence of NAC; (B) Difference in the emission responses (ΔIntensity) for **L** (10 μM) in presence of WP (500μl) and WP (500μl) pre-treated treated with NAC (500 μl) at 37°C as compared to the emission response for free **L**. Studies were performed in 10mM aq.HEPES:CH_3CN (9:1, v/v at pH 7.0) using λ_{Ext} = 445 nm and λ_{Ems} = 520. For all measurements were performed after 45 minutes of incubation at 37°C.

Suitability of this reagent for specific recognition and estimation of Cys-residues present in natural milk proteins was also explored. For this, WP was isolated from cow's milk (detailed methodology is provided in supporting information). Three important constituents of natural cow's milk whey are α-lactalbumin, β-lactoglobulin and Bovine serum albumin. The ratio for Cys-residues present in monosulfide and disulfide forms are 2:23 and among these disulfides, few are not accessible to external reagents.[11a,b] Earlier literature reports also reveal that NAC can cleave disulfide bonds.[11c] This was utilized for estimation of Cys-residues that could be present in WP in monosulfide and disulfide forms (Fig. 5). Change in emission intensity observed at column-a (Fig. 5B) basically represents the enhancement in emission for the formation of L-Cys-P and this was attributed to Cys-residues with free sulfhydryl$_{Cys}$ group in native WP, exposed to the solvent. While the difference in column-b and column a was attributed to the Cystine-residue of the native WP present in the disulfide form and are accessible to reagent NAC and **L** under the experimental condition (Fig. 5). These results clearly revealed that fluorescent tags like **L** could be utilized for detection of Cys or Cystine residues present in WP and are exposed to the solvent and external reagents.[12]

In conclusion, we have demonstrated that a new chemodosimetric reagent (**L**) could be used for detection of free Cys as well as Cys-residues with free sulfhydryl$_{Cys}$ group present in protein structures. Visually detectable change in solution colour and fluorescence allowed a naked eye detection of Cys in presence of all natural AAs, common anions and cations at pH 7 in an essentially aq. buffer medium. This non toxic reagent could also be used for probing the enzymatic hydrolysis of NAC, a common prescribed drug used as Cys-supplement, in aq. buffer as well as within the live HepG2 cells. Considering the specificity, luminescence on response and cell membrane permeability, this reagent (**L**) has immense application potential as an imaging reagent and for developing an efficient reagent for monitoring an important reaction of aminiacylase-1.

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Notes and references

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†Electronic Supplementary Information (ESI) available: [Experimental procedures and additional data.]. See DOI: 10.1039/b000000x/.Invention disclosure application no. 1061/DEL/2015.

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