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ARTICLE TYPE

Extremely Selective and Fluorescent Detection of Cysteine or Superoxide: with Aliphatic Ester Hydrolysis

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A novel fluorescence probe modality demonstrated with fluorescein affords a highly selective aqueous–based detection of cysteine over other biothiols, e.g. homocysteine, with a limit of detection of 11.3 *μM*.

Low molecular weight bio-thiols are essential molecules in nature and include Cysteine (Cys), homoCysteine (hcy) and glutathione (GSH). These amino acids are involved in many physiological processes such as protein synthesis, metabolism, detoxification, signal transduction and gene regulation. Also, they are involved in regulating redox states and in composing the structure of proteins.[1] Thus, these biothiols, by their nature, have adopted roles for normal health, but are also involved in the status of numerous different diseases. The structures of *Cys* and *Hcy* are very similar (differing by one methylene group); it is a difficult task to develop probes that possess selectivity enough to discriminate biothiols (especially between *Cys* and *Hcy*). An imbalance of *Cys* concentration is thought to relate to the cause of certain diseases; it may lead to neurotoxicity[2] and deficiency, causes hair depigmentation, creates edema, slows growth, lethargy, promotes liver damage, muscle weakness, skin lesions as well as muscle and fat loss.[3] An elevated level of *Hcy* in human plasma is related to incidence of Alzheimer's disease and cardiovascular disease; cellular *GSH* deficiency leads to oxidative stress which is suspected to be a main cause of certain neurodegenerative disorders. Cancers and AIDS are also related to biothiol concentration.[4,5] Fluorescent molecular sensors for the detection of biothiols have great inherent advantages in their simplicity, potential selectivity and sensitivity in which the possibility of discerning intracellular and inter-compartmental detection allows for a great advantage over other methods of detection. Among the various types of fluorescent molecular sensors are chemodosimeters (based on the reaction of analyte with probe) which are more selective and sensitive in the detection of biothiols than chemosensors.

There are a number of probes that detect both *Cys* and *Hcy;*[6] but excellent *selective* detection especially between *Cys* and *Hcy* is rare because of the structural similarities between analytes.[7] Recently, in our research group, we have developed a novel rechargeable *meso*–aryl BODIPY–based chemodosimeter for the selective detection of Cys over other biothiols (*Hcy* and *GSH*)[8a]. In a continuation of our interest in detecting biothiols, we have developed a novel route to detect biothiols *via* ester hydrolysis[8b] as well as selective detection of Cys, confirmed by chloropropioanate ester hydrolysis.[8c]

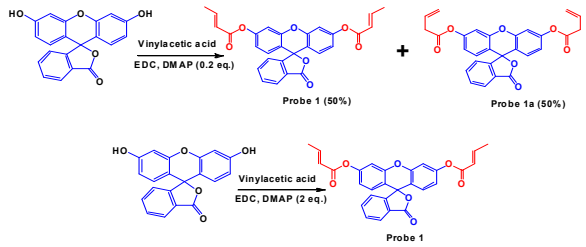
Reactive oxygen species (ROS) play a key role in neurodegenerative disease disorders (Alzheimer's and Parkinson's disease), and in environmental chemistry.[9] Recently, there have been many published trials about how to detect and understand ROS *in vivo/vitro*.[10] Reactive oxygen species (ROS) include superoxide, hypochlorite, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite. Among these ROS, detection of superoxide is an important issue because of its short life time. In an earlier report we demonstrated that superoxide can be detected by ester hydrolysis in which an aromatic ester bearing electron withdrawing groups was used. Here, we have shown the same strategy of detection can be used with aliphatic esters too.

Herein, we report a Fluorescein-based probe for the selective detection of *Cys* over other sulphur–containing amino acids**.** We designed our probe by a simple esterification reaction of Fluorescein and vinyl acetic acid. We began our synthesis from Fluorescein and the commercially available vinyl–acetic acid by a simple single esterification step. In one facile step involving very convenient reaction conditions, probe **1,** bearing *two* ester moieties, was obtained. Firstly, when we used 0.2 equiv of DMAP we obtained a mixture of probe **1** and **1a** in 1:1 ratio (Fig. S5). So to obtain probe **1** exclusively, we used 2 equiv of DMAP (Scheme **1**). Here, probe **1** was obtained by an isomerization of a vinylic group in making the more stable unsaturated α,β group. This probe has been previously synthesized from butanoic acid.[11] We characterized the probe first by ^{1}H and then by ^{13}C NMR spectroscopy; confirmation was also shown by mass spectrometry (ESI). Importantly, the thermodynamic product (**1**) goes against the initial notion of controlling intermediate ring size in our probe. With a vinylic group such as that found in **1a**, the carbonyl of the ester group by the amino group of the covalently–bound Cys would give rise to an important difference based on intermediate ring size. In the case of Cys, the formation of an 8-membered ring would be expected (Fig. S8); an unfavorable 9-membered ring would be expected for homoCysteine. However, we now have simply a steric methyl group which appears to play a part in allowing for respective intermediate ring size formation that are 7-(Cys) and 8-membered (Hcy). Despite the smaller ring size, the steering of Cys / Hys selectivity based on this sterical methyl group appears to be effective. We obtained revealing that

selective sensing for *Cys* over *Hcy* and *GSH* through a nucleophilic addition–elimination reaction at the vinylic position was possible giving Fluorescein as the highly fluorescent product molecule as shown mechanistically below (Fig. 1). The mechanism behind this fluorescence sensing is supposed to be internal conversion (IC) as reported previously.[12]

Spectroscopic properties of probe **1** were determined under physiological conditions (buffered H_2O:DMSO 80:20; pH 7.4 PBS). Firstly, the probe was dissolved in DMSO and then subsequently diluted in 80 % PBS buffer, pH 7.4. The detection properties of our new probes can be accessed via UV–vis absorption and emission spectroscopy.

The presence of the vinyl group allowed us to explore the probe with different amino acids. The probe was first tested with sulfur–containing amino acids (*L*–Cys, Hcy, *N*–acetyl–*L*–*Cys*, *Met*, *GSH*), as well as with non–sulfur–containing amino acids (*Ala*, *Arg*, *Asn*, *Asp*, *Gln*, *Glu*, *Gly*, *His*, *Ile*, *Leu*, *Lys*, *Phe*, *Pro*, *Ser*, *Thr*, *Trp*, *Tyr*, *Val*) as aqueous solutions on the order of ~10 equiv. Here, we used 3 mL of 4.0×10^{-6} M, 1X PBS buffer, pH 7.4 probe solution and incubated with 10 equiv amino acid; after 30 min, significant changes in the color of the probe solution was found with *Cys*.



Scheme **1**: Synthesis of probe **1**.

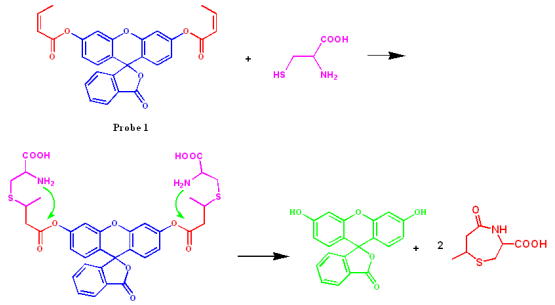


Figure 1. Proposed *Cys* probing mechanism.

When assessed quantitatively with analyte *via* emission studies, a dramatic fluorescence intensity increase was found (Fig. 2). A dramatic enhancement in fluorescence intensity was quantified for *Cys (1390–fold)*, compared to the starting probe. A steady increase in fluorescence intensity was observed when the concentration of *Cys* was increased (Fig. 3a). The detection limit was estimated to be *11.3 μM*, indicating the utility of the probe for detection of Cys in biological systems (Fig. S7). Also, when a time–dependent study was performed in which the probe was exposed to *Cys* for over 1 h, a steady increase in fluorescence intensity was formed (Fig. 3b). To verify the selectivity and practical utility of the probe, we performed a competition study of *Cys* solution in the presence of other thiol–containing species: *Hcy*, *N*–acetyl–*L*–*Cys*, *Met*, and *GSH*; interestingly, there was no interference in fluorescence intensity (Fig. S6).

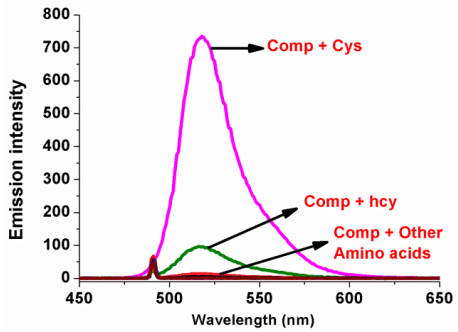


Figure 2. Emission spectra of probe **1** (4.0×10^{-6} M, buffered H_2O: DMSO 80:20; pH 7.4 PBS) with amino acids *L*–*Cys*, *Hcy*, *N*–acetyl–*L*–*Cys*, *Met*, *GSH*, *Ala*, *Arg*, *Asn*, *Asp*, *Gln*, *Glu*, *Gly*, *His*, *Ile*, *Leu*, *Lys*, *Phe*, *Pro*, *Ser*, *Thr*, *Trp*, *Tyr*, *Val*) (66 μM in water) incubated for 20 min at RT. Slit width for Ex. & Em. = 1.5 nm.

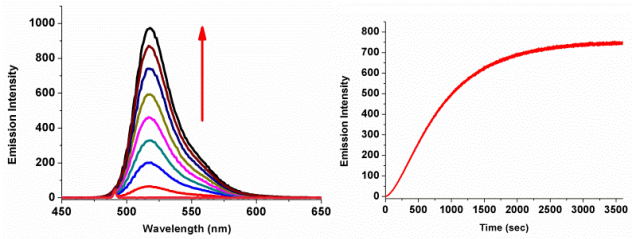


Figure 3. Emission spectra of probe **1** (4.0×10^{-6} M, buffered H_2O: DMSO 80:20; pH 7.4 PBS) with increasing concentrations of *L*–*Cys* (33.3 – 266 *μM* in water) and incubated for 20 min at RT. (*right*) Time–dependent emission spectra of probe **1** (2.0×10^{-6} M, buffered H_2O: DMSO 80:20; pH 7.4 PBS) with Cys (200 μM in water, λ_{exci} = 490 nm) slit width Ex. & Em. = 1.5 nm

In order to achieve sensing in more biologically-relevant aqueous conditions, we performed studies in different solvent combinations like 90:10 H_2O:DMSO, 90:10 H_2O:EtOH, 80:20 H_2O:EtOH, 80:20 H_2O:MeCN and 90:10 H_2O:MeCN (Fig. S9). As a result, we found 80:20 H_2O:DMSO is currently the best combination to balance time and fluorescence intensity requirements, as DMSO is promoting this reaction. To support the proposed mechanism, a separate reaction was carried out with probe **1** (1.0 equiv) and *Cys* (2.5 equiv) in 30 mL of a MeOH: H_2O (90: 10, v/v) solution. The reaction mixture was stirred at RT for 1 h. Then, Et_3N (100 *μL*) was added and the solution was stirred for 1 h. The solvent was then removed, and the crude product was checked directly with ESI–MS which revealed a value corresponding to Fluorescein as a major product. Finally, the product was confirmed by ^{1}H–NMR spectroscopy. The main product was Fluorescein which results in a high emission intensity (520 nm).

To further evaluate the utility of the probe as a sensor for intracellular biothiol detection, SH–SY5Y cell assays were

performed. The probe was found to be cell–permeable and selective for the detection of intracellular Cys; this was further supported through the NEM experimental technique. Also, the NEM experiment demonstrated there is no competition of superoxide over Cys in living neuronal cells as NEM is not a scavenger for superoxide.

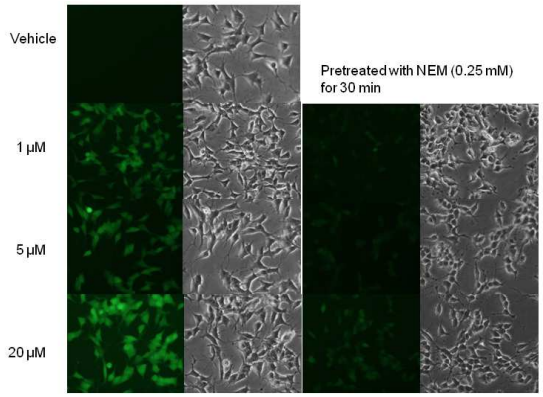


Figure 4. Fluorescence microscope images of living SH–SY5Y cells with vehicle (DMSO) and probe (20 μM) with corresponding bright field and fluorescence images *(left)* and cells pretreated with NEM (*right*) [Scale bar 10 μM].

Finally, the probe with different ROS: There was only a response to superoxide (Fig. 5); 770-*fold* over other ROS. Superoxide is a strong nucleophile[13] which takes part in hydrolysing esters and changes the fluorescence intensity of organic molecules. When manually increasing the concentration of superoxide, there was an increase in the fluorescence intensity; rises in fluorescence intensity also increased with experimental time (Fig. 5). Also, there was no interference or competition involving other ROS with superoxide activity (Fig. S11). From all the sensing data we can confidently construct an "OR" logic gating pattern with Cys and superoxide.

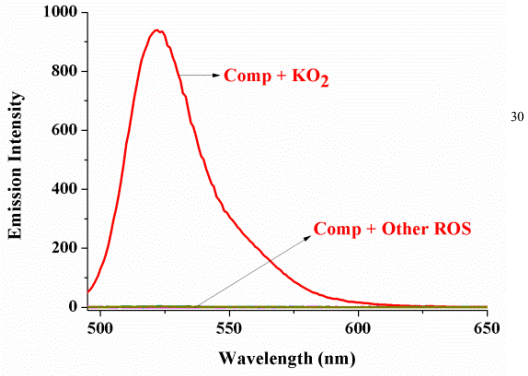


Figure 5. Emission spectra of probe **1** (4.0×10^{-6} M, H_2O: DMSO 80:20) with (ROS) KO_2, H_2O_2, NaOCl, ^{t}BuOOH, •OH, •OtBu, (66 μM in water) incubated for 30 min at RT incubated for 20 min at RT. λ_{exci} = 490 nm. Slit width for Ex. & Em. = 1.5 nm.

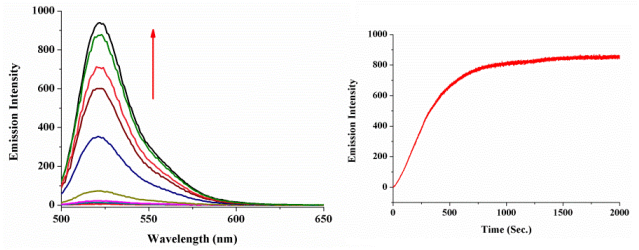


Figure 6. Emission spectra of probe **1** (4.0×10^{-6} M, H_2O: DMSO 80:20) with increasing concentration of KO_2 (33.3 – 266 μM in water) incubated for 30 min at RT. (*right*) Time–dependent emission spectra of probe **1** (2.0×10^{-6} M, H_2O: DMSO 80:20) were formed with KO_2 (200 μM in water) with λ_{exci} = 490 nm (slit width Ex. & Em. = 1.5 nm).

In conclusion, a novel fluorescence probe modality demonstrated with Fluorescein affords a highly selective sensor platform for Cys over other relevant and closely–related biothiols. Simple esterification of Fluorescein with vinylacetic acid affords a ratiometric, fluorescent and selective probe for the detection of Cys over other thiol–containing amino acids with a detection limit of 11.3 μM and a ~1390–fold increase in fluorescence intensity within 20 minutes. This sensing pattern was also confirmed in living neuronal cells. Also, the probe can be used as sensor for selective detection of superoxide over other ROS. As per our knowledge, this is first such probe for the selective detection of Cys and superoxide with *"OR"* logic gate.

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† Electronic Supplementary Information (ESI) available: Experimental details, in–line spectral data, MS spectra, See DOI: 10.1039/b000000x/

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