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Highly Fluorescent and Specific Molecular Probing of (Homo)Cysteine or Superoxide: Biothiol Detection Confirmed in Living Neuronal Cells

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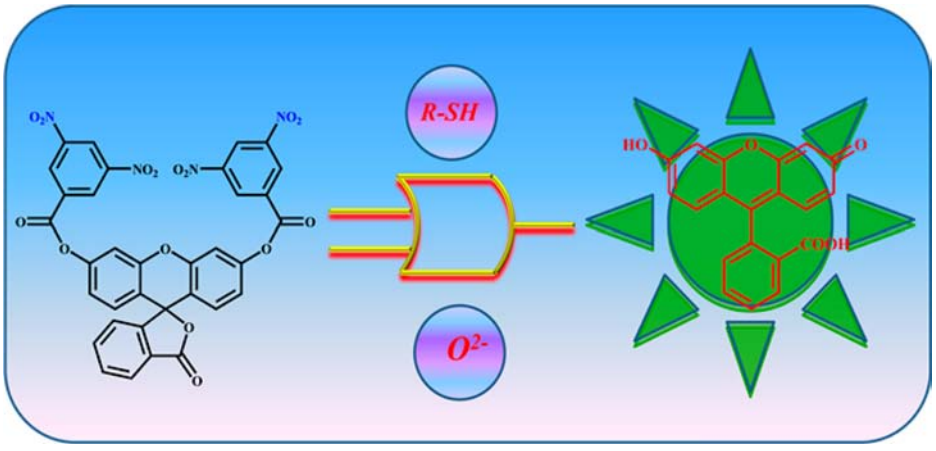
ABSTRACT



Chemodosimetric action in detection of cysteine and homocysteine (310- and 290-fold) and superoxide inputs (336-fold increase) gives significant fluorescence intensity increases. Detection limits of 2.13 × 10^{-5} M, 1.37 × 10^{-5} M, and 2.71 × 10^{-5} M, respectively, are biorelevant and are consistent with "OR" logic gating, demonstrated in intracellular biothiol detection in neuronal cells by way of novel fluorescein derivatization. As per our knowledge, this is the first example of a novel fluorescent probe based on the nucleophilic substitution reaction of biothiols and superoxide through ester cleavage.

Selective and sensitive detection of biothiols such as cysteine (*Cys*), homocysteine (*Hcy*) and glutathione (*GSH*) has became an important issue because of the vital role of these analytes in maintaining various biological processes.[1] *Cys* and *Hcy* are very important small biomolecules, essential for growth of cells and tissues in living organisms. Among other considerations, the imbalance in the levels of these biothiols are thought to lead to many serious disease pathways. A decreased level of *Cys* can cause retardation in growth, hair pigmentation, edema, lethargy, liver damage, muscle and fat loss, skin lesions and weakness.[2] Increased levels of *Hcy* in blood plasma may lead to Alzheimer's disease and cardiovascular disease.[3] *GSH* deficiency will lead to

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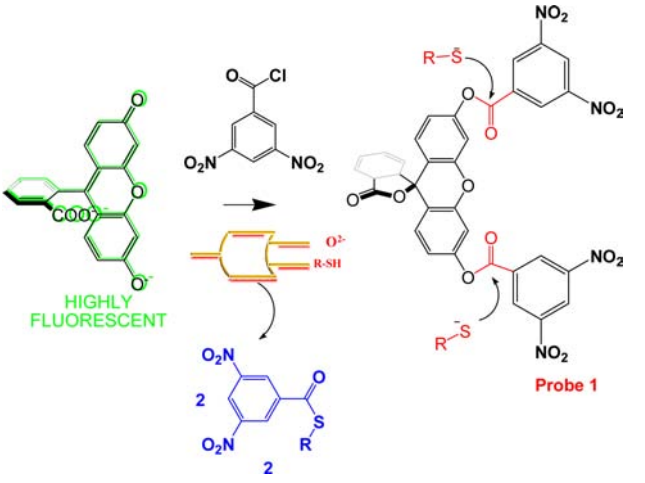
oxidative stress, which is considered among the possible causes of Alzheimer's disease.[4] Thus, the selective detection of biothiols is a very important issue. For the detection of biothiols, previously reported chemodosimeters,[5] based on disulfide cleavage,[6] cyclization of aldehydes,[7] nucleophilic additions,[8] ligand reactions[9] and Michael additions,[10] have been reported, among other modes.

Reactive oxygen species (ROS) are thought to play a decisive role in neurodegenerative disease disorders (Alzheimer's and Parkinson's disease) and in environmental chemistry.[11] Reactive oxygen species (ROS) include superoxide, hypochlorite, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite. Superoxide (O_2^-) has a short half-life; real-time detection is an important task. O_2^- is involved in a range of physiological processes in living organisms, such as aging, muscle fatigue, ischemia–reperfusion and inflammation.[12,13] The detection of superoxide with high selectivity and sensitivity continues to be a significant challenge.

Selectivity and sensitivity are currently important advantages of chemodosimeters over chemosensors; in recent times, a lot of research is being conducted in the development of new chemodosimeters. Recently, in our research group, we have developed a novel rechargeable *meso*–aryl BODIPY-based chemodosimeter for the selective detection of cysteine over other biothiols (Hcy and GSH).[14] Herein, we report a novel fluorescein-based probe for selective detection of biothiols and superoxide with a dramatic change in fluorescence intensity.

We designed our probe via the concept of simple benzoyl protection of the hydroxyl groups of fluorescein (Scheme 1). With this in mind, we started our synthesis from fluorescein and commercially available 3,5-dinitrobenzoyl chloride in a facile one-step reaction with good yield and purity. The probe was first characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (ESI). We obtained selective sensing for biothiols and superoxide through a nucleophilic addition–elimination reaction at the benzoyl position (Scheme 1) releasing unsubstituted fluorescein as the final product.

Scheme 1. Synthesis of Probe **1** and Mechanism



Spectroscopic properties of probe **1** were determined under physiological conditions (20 mM HEPES, pH 7.4). First, the probe was dissolved in DMSO and then subsequently diluted in 80% 10 mM HEPES, pH 7.2. The detection properties of our new probes can be assessed via UV–vis absorption and emission spectroscopy.

The presence of the benzoyl group encouraged us to check probe reactivity with different amino acids. When testing the probe with sulfur-containing amino acids (L-Cys, Hcy, *N*-acetyl-L-Cys, Met, GSH) and 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 (~ 10 equiv), changes in the color of the probe solution were found with *Cys* and *Hcy* only. In these assays, we used 3 mL of probe solution (4×10^{-6} M, buffered H_2O/DMSO 80:20; pH 7.2; 10 mM HEPES) and incubated with

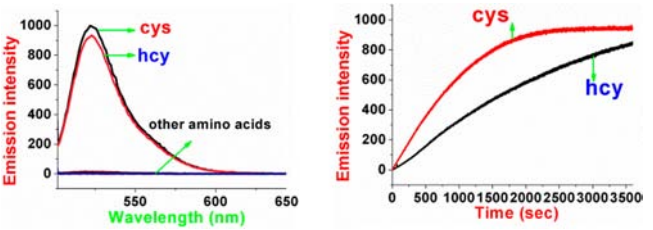


Figure 1. (Left) Emission spectra of probe **1** (4.0×10^{-6} M, buffered H_2O/DMSO 80:20; pH 7.2; 10 mM HEPES) with amino acids L-Cys, Hcy, *N*-acetyl-L-Cys, Met, GSH, Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, Val) (3.3×10^{-3} M in water) incubated for 20 min at RT. (Right) Time-dependent emission spectra of probe **1** (4.0×10^{-6} M, buffered H_2O: DMSO 80:20; pH 7.2; 10 mM HEPES) with *Cys* and *Hcy* (~10 equiv in water).

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10 equiv of amino acid. These two analytes also gave dramatic increases in fluorescence intensity: 310- and 290-fold, respectively (Figure 1). Also when testing with other competing amino acids under analogous conditions, no changes in emission intensity were found, suggesting no participation of other amino acids in ester hydrolysis (Figures S5, S6, Supporting Information).

Since superoxide is well-known as a "super nucleophile,"[15] the probe was tested with superoxide: the same response as that for biothiols was found via ester cleavage (Figure 2). When other reactive oxygen species were assayed, there was no response; also, there was no interference or competition of other ROS with superoxide activity (Figure S7, Supporting Information). As per the above studies, we obtained two inputs for this sensing as biothiols and superoxide; when implicated, the logic gate was found to be an "OR" gate, as shown in Figure 3.

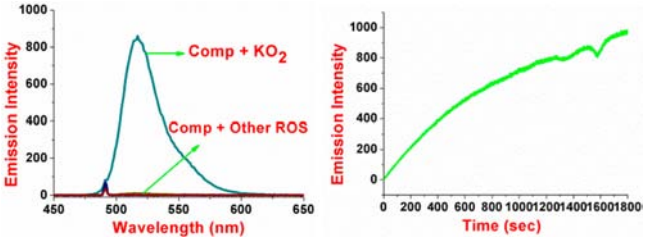

Figure 2. (Left) Emission spectra of probe **1** (4.0×10^{-6} M, buffered H_2O/DMSO 80:20; pH 7.2; 10 mM HEPES) with (ROS) KO_2, H_2O_2, NaOCl, ^{t}BuOOH, •OH, •O^{t}Bu, *m*−CPBA. (3.3×10^{-3} M in water) incubated for 20 min at RT. λ_{exci} = 490 nm, slit width Ex, Em = 1.5. (Right) Time-dependent emission spectra of probe **1** (2.0×10^{-6} M, (buffered H_2O/DMSO 80:20; pH 7.2; 10 mM HEPES) with KO_2 (~10 equiv in water).

From the above emission studies, two input logic gate "OR" systems can be represented. Here, the probe has been established as a molecular system that exhibits combinational logic gate properties based on two biologically-important inputs: biothiols and superoxide. The optical output completes the OR logic gate. We plot the "truth table" for the response of these inputs in which output "1" is fluorescence enhancement (@ 522 nm) and "0" is no change in emission intensity (Figure 3). Because of the selectivity, analytes give no intermediate signals between a strong signal or no signal.

To support the proposed sensing mechanism, we performed a small scale reaction of probe 1 with 2 equiv. of *L*−*Cys*. *L*−*Cys* (3.0 mg) was added to a solution of the probe 1 (20 mg) in DMSO: D_2O (9:1), triethyl amine (100 μL) and was left for 45 min at RT. After this time reaction mixture was filtered, and the filtrate was subjected to ESI mass spectrometry. The filtrate is seen as a fluorescein, resulting in fluorescence intensity increase (Figure S4, Supporting Information).

To evaluate the practicality of Probe **1** as a real biological sensor for intracellular biothiol detection, we performed an

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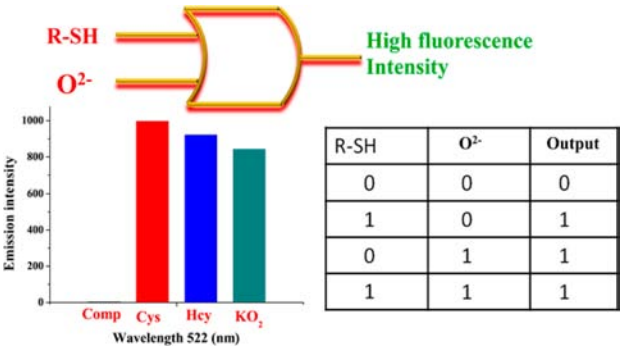

R-SH	O^{2-}	Output
0	0	0
1	0	1
0	1	1
1	1	1

Figure 3. "OR" logic gating with biothiols and superoxide along with truth table for the substrate and probe **1**.

assay of probe **1** with SH−SY5Y cells (Figure 4). Cells were first treated with probe **1** (10 μM) for 30 min before being washed with PBS buffer. Then, incubation with Mitotracker Red (100 nM) was performed for 10 min, followed by washing before imaging (probe **1**, λ_{exci} = 490 nm; Mitotracker, λ_{exci} = 590). For the "NEM experiment," cells were pretreated with *N*-ethylmaleimide (NEM) (1.0 mM) for 30 min; this consumes all the cysteine residues in the cells. Washing with PBS buffer preceded incubation with probe **1** (10 uM) for 30 min. Cells were washed, incubated with Mitotracker Red (100 nM) for 10 min, and washed again before imaging was acquired. Mitotracker Red confirmed cell viability and intracellular fluorescence of Probe **1**.

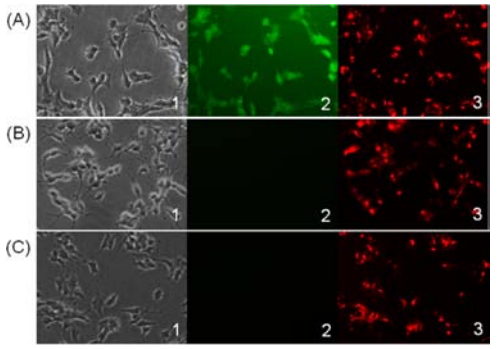

Figure 4. Fluorescence microscopic images of living SH−SY5Y cells. (A) SH−SY5Y cells were incubated with probe **1** (10 μM) for 30 min and incubated with Mitotracker Red (100 nM) for 10 min. (B) SH−SY5Y cells were pre−incubated with NEM (1.0 mM) for 30 min, incubated with probe-**1** (10 μM) for 30 min, and then incubated with Mitotracker Red (100 nM) for 10 min. (C) SH−SY5Y cells were incubated with vehicle (DMSO) for 30 min. 1, bright-field images; 2, fluorescence images (490 nm); 3, fluorescence images (590 nm) [Scale bar length = 10 μm]. Excitation laser source is Mercury lamp.

As a result of these SH−SY5Y cell assays, the probe was found to be cell permeable and selective for the detection of intracellular cysteine which was also supported by the "NEM" experiments. The Mitotracker Red experiment

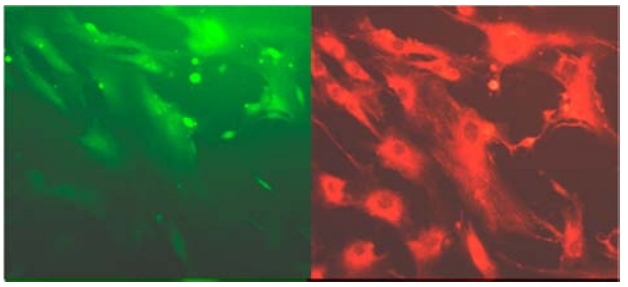

Figure 5. Magnified images of SH−SY5Y cells incubated with probe **1** (left) and Mitotracker Red (right). Scale bar 10 μm.

showed the permeability of probe inside the cells along with entry into the mitochondria (Figure 5) to allow for neurological detection of cysteine.

In conclusion, a novel fluorescein-based probe has been developed that acts as a fluorescent chemodosimeter for the detection of biologically important biothiols (cysteine and homocysteine: 310- and, 290-fold, respectively), while at the same time serving as a superoxide input (336-fold increases). Fluorescence intensity and "turn-on" responses at 522 nm is consistent with liberation of free fluorescein and can be interpreted as novel "OR" logic gating with biothiols and superoxide as two inputs. Also, this probe is useful in detecting intracellular biothiols. To our knowledge, this is the first probe for biothiols and superoxide detection through ester hydrolysis. In the future, variations of this probe may be used for site-specific drug delivery.

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Supporting Information Available. Methods, experimental procedures, additional spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.