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A new fluorescent 'turn on' probe for rapid detection of biothiols

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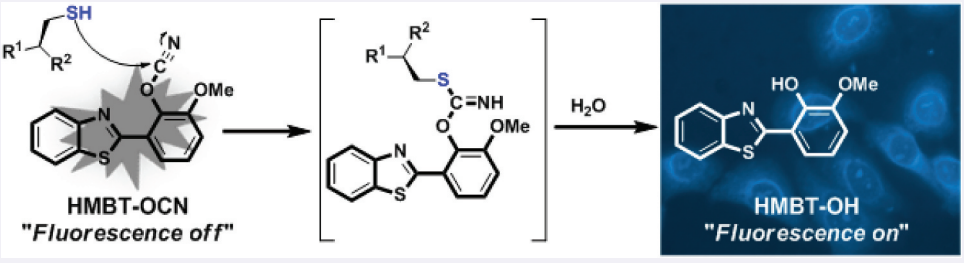
ABSTRACT

We designed and synthesised a novel molecular probe exhibiting high selectivity and sensitivity towards reactive sulphur species (RSS) over other amino acids and biologically relevant species, as well as scrutinised its spectroscopic behaviours under physiological conditions and in living milieu. We used an electrophilic cyanate group as a masking agent to block the excited state intramolecular proton transfer process of 2-(2-cyanato-3-methoxyphenyl)benzo[d]thiazole (**HMBT-OCN**), which readily hydrolyses to the highly fluorescent structure, 2-(2′-Hydroxy-3′-methoxyphenyl) benzothiazole (**HMBT-OH**), in the presence of reactive sulphur species.



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Introduction

Reactive sulphur species (RSS) such as cysteine (Cys), homocysteine (Hcy), glutathione (GSH), as well as hydrogen sulphide (H_2S) play fundamental roles in biological systems [1–3]. Glutathione, for example, as the body's master antioxidant, helps to maintain biological redox homoeostasis and cell growth [4]. Cysteine, a semi-essential sulphur-bearing amino acid, is extensively involved in protein synthesis [5]. Homocysteine is the by-product of protein metabolism, a well-known risk factor for heart disease [6]. H_2S, the smallest RSS, is an important gaseous signalling molecule (i.e. gaseotransmitter) in the nervous, inflammatory, and cardiovascular systems produced endogenously from cysteine and homocysteine via a range of enzymatic pathways [7]. Since abnormal RSS levels in the living system are associated an array of disease states, for a better understanding of how they contribute to both physiology and pathology, it is pivotal to assess levels of RSS in their biological environments, which could aid the early diagnosis of some diseases.

Recent related research has focused on the development of fluorescence assays for probing and tracking RSS in biological systems. Numerous types of RSS-responsive fluorescent probes based on different detection strategies have appeared in scientific literature [8–12], and remarkably, most operate via specific chemical reactions that exploit the reactive nature of RSS [13–20].

The general strategy of designing an RSS-specific fluorescent probe relies on masking a fluorescent dye, mostly with an ester-based reactive group, which upon reacting with RSS splits away to deliver the emissive form of the dye [21–30]. For the efficient discrimination of RSS over other biological reactive species such as reactive nitrogen (RNS) and oxygen species (ROS), the masking groups are necessarily highly specific to reactive sulphur. However, a dramatic weakness of ester-based recognition groups is their cross-sensitivity to enzyme-mediated hydrolysis, which interferes

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considerably in biological systems' detection of sulphur species [31]. Given the demand for alternative recognition motifs with improved analyte selectivity and sensitivity, response time, and biocompatibility, we sought alternative sulphur specific-reactive groups that can be easily integrated with the probe structure.

Herein, we described a novel approach to recognising cellular sulphur species by constructing a pre-fluorescent dye modified with an electrophilic cyanate as recognition motif. The probe was highly specific to sulphur species, responded very rapidly (<1 min), and could be efficiently used for molecular imaging of RSS. We built the probe structure upon an excited state intramolecular proton transfer (ESIPT)-based dye, which uses an electrophilic cyanate as the RSS reactive unit. We chose 2-(2′-hydroxyphenyl)benzothiazole (**HMBT-OH**) as the signal reporter dye not only for its desirable photophysical features, but also its high biocompatibility in living cell imaging applications [32–38].

An electrophilic cyanate group (**RO**–**CN**) is assumedly a reactive nitrile that readily reacts with sulphur species to form thiocarbamate, which, in aqueous environments, rapidly hydrolyses to corresponding hydroxyl compound [39–41]. Accordingly, we integrated a cyanate group to a fluorescent dye and generated a non-fluorescent molecular probe. We anticipated that the oxygen-nitrile bond of the non-fluorescent dye would cleave in the presence of reactive sulphur species in order to deliver the free hydroxyl derivative of the dye, which would in turn induce a turn-on type fluorescence response.

As Scheme 1 illustrates, we synthesised the probe, **HMBT-OCN**, in a good yield by simply treating **HMBT-OH** with commercially available cyanogen bromide in the presence of triethylamine at −10°C. We clearly confirmed the structure of the probe molecule (**HMBT-OCN**) by using ^{1}H, ^{13}C NMR, and elemental analysis, as detailed in the Supporting Information.

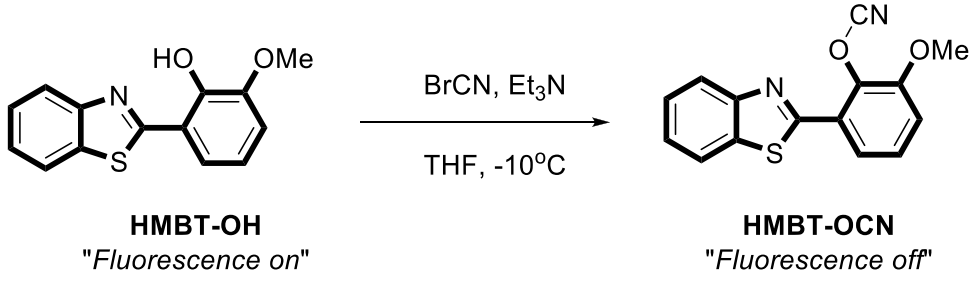
HMBT-OH is a well-known small fluorescent molecule with an emission band of 400–500 nm based on an excited state intramolecular proton transfer (ESIPT) mechanism. However, masking the hydroxyl group of **HMBT-OH** molecule inhibits the intramolecular proton transfer and makes the molecule non-emissive ('*off mode*'). In our design, we used an electrophilic cyanate group as a masking agent to block the ESIPT-based emission of the dye molecule, which was expected to split away in the presence of sulphur species.

Results and discussion

We commenced our investigation to release the masking agent from the probe molecule by screening a wide range of RSS and other possible reactive species such as ROS, RNS, and biologically relevant metal ions in aqueous solution at physiological conditions by measuring changes in absorbance and fluorescence behaviours. As Figure 1(b) shows, owing to the blocked ESIPT mechanism, the probe molecule had no fluorescence emission ('off mode') under ultraviolet irradiation (λ_{ex} = 300 nm). However, the fluorescence emission intensity of the dye centred at 420 nm improved dramatically after addition of 10.0 equivalents of glutathione due to the cleavage of the O–CN bond.

To optimise sensing conditions, we carefully scrutinised all variables, including time, pH, and cetrimonium bromide (CTAB) concentration. To the best of our knowledge, adding CTAB to aqueous sensing media dramatically increases the sensitivity of probe molecule by creating a micellar system; by contrast, without CTAB, the reactivity of the probe to thiols diminishes severely (Fig. S2 in the Supporting Information). In the presence of 3.0 mM of CTAB, the probe molecule exhibited a remarkable sensitivity to RSS in aqueous media. Moreover, **HMBT-OCN** was extremely resistant to pH changes and had no fluorescence-related signal in the absence of GSH at extreme pH values (Figure 2(b)). After careful examination, the optimum sensing conditions determined to be 20 μM of **HMBT-OCN** and 3.0 mM of CTAB in a phosphate buffer (0.1 M at pH 7.0).

We carefully examined the fluorescence-sensing behaviour of **HMBT-OCN** with the addition of GSH at optimum sensing conditions (20 μM of **HMBT-OCN** + 3.0 mM CTAB in 0.1 M of phosphate buffer, pH = 7.0)



Scheme 1. Synthesis of **HMBT-OCN.**

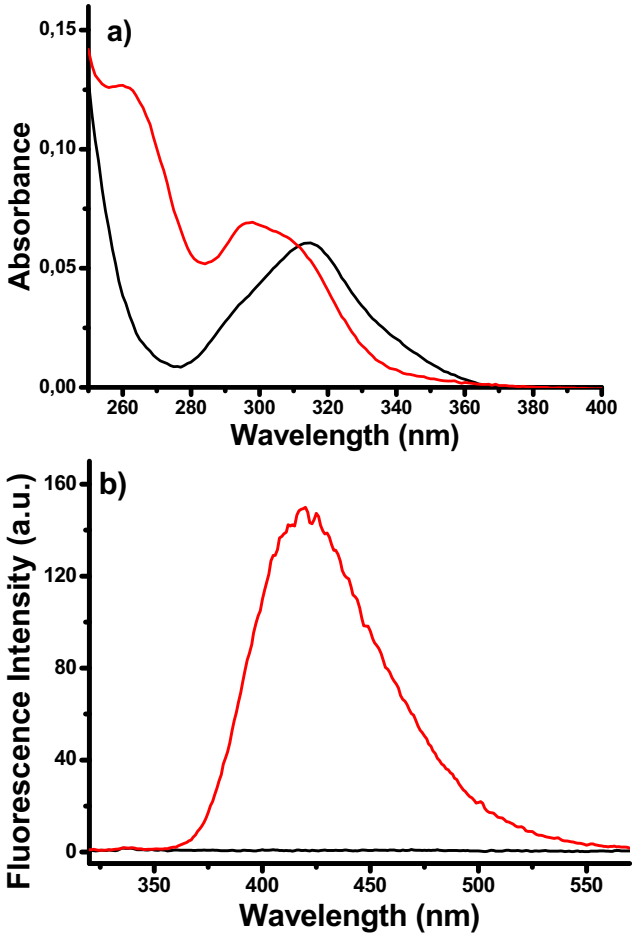


Figure 1. (a) Absorption and (b) Emission spectra of **HMBT-OCN** (20 μM) (black line) and **HMBT-OCN** (20 μM) + GSH (10.0 equiv.) (red line) in 0.1 M phosphate buffer, pH 7.0 (3.0 mM CTAB) (λ_{ex} = 300 nm, at 25 °C).

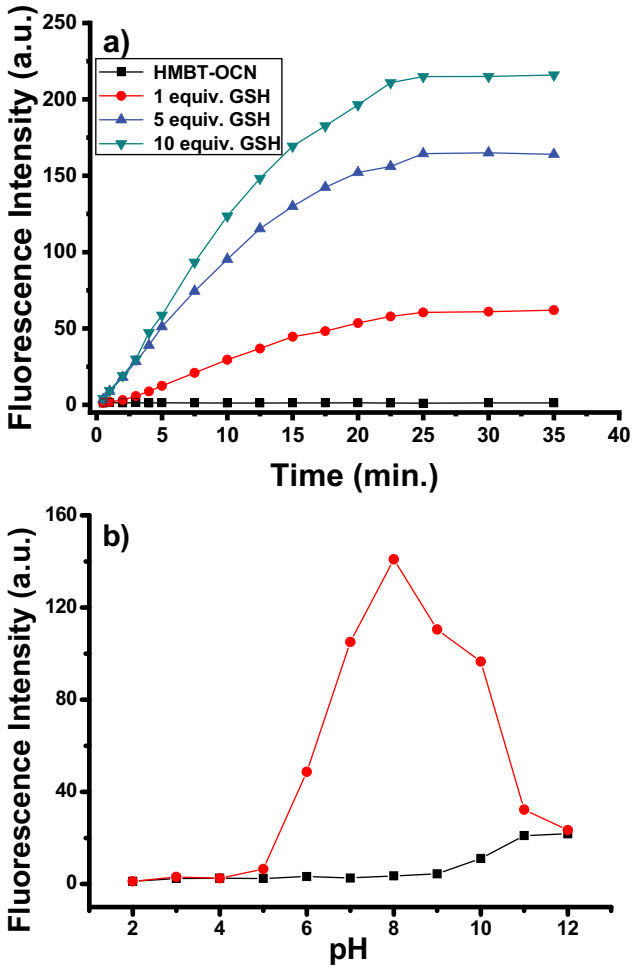


Figure 2. (a) Time profile of **HMBT-OCN** (20 μM) in the presence of GSH (1.0(●), 5.0(▲), and 10.0(▼) equiv.) (b) Effect of pH in the presence of GSH (10.0 equiv.). (λ_{ex}: 300 nm, λ_{em}: 420 nm at 25 °C).

upon excitation at 300 nm. As expected, the masked dye molecule, **HMBT-OCN**, had no fluorescence emission due to the blocked ESIPT process in the initial state. However, adding GSH (10.0 equiv.) produced a 'turn on' response in fluorescence spectra centred at 420 nm within a very short visible response time (<1 min) (Figure 2(a)), which resulted from the regeneration of the ESIPT mechanism (Figure 1(b)). As Figure 1(a) shows, **HMBT-OCN** also enables to detect GSH by tracking changes in the ultraviolet-Vis spectra. The characteristic absorbance band of **HMBT-OCN** at 315 nm disappeared with the addition of GSH, and a new emission band formed at 297 nm, with an 18 nm blue shift belonging to the unmasked dye molecule (**HMBT-OH**).

Under optimised conditions, **HMBT-OCN** gave similar responses to other sulphur derivatives including cysteine/homocysteine and H_2S as well (Figure 4). Due to the similar sensing performance of probe towards each other RSS of interest we selected glutathione (GSH) as a model target RSS for further experiments.

To understand the boundaries of sensing strategy, we performed a fluorescence titration experiment by adding increasing amounts of GSH upon excitation at 300 nm. The fluorescence emission intensity of **HMBT-OCN** at 420 nm increased linearly with the consistent addition of GSH over a wide range of concentrations (2–400 μM). Peak saturation occurred with the addition of 10.0 equivalents of GSH with a 210-fold enhancement factor (Figure 3). Ultimately, we determined the minimum amount of detectable GSH as being 550 nM based on S/N = 3 (Fig. S1 in the Supporting Information).

We further interrogated the selectivity of **HMBT-OCN** to the addition of other potentially reactive species – ROS, RNS, and anions – in biological processes (Figure 4 (a)). With the addition of an excess of the reactive species (50.0 equiv.) including F^-, Cl^-, Br^-, I^-, AcO^-, NO_2^-, SCN^-,

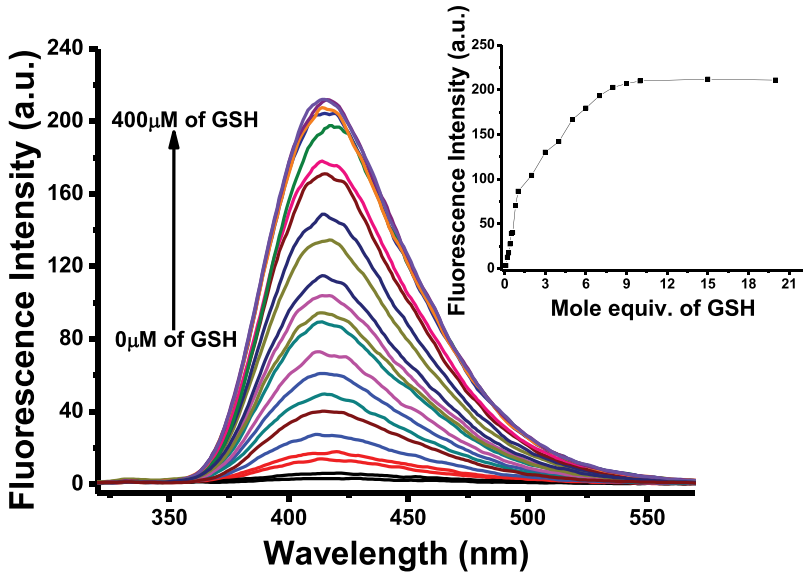


Figure 3. Fluorescence spectra of **HMBT-OCN** (20 μM) in the presence of increasing amount of GSH (0–400 μM) 0.1 M phosphate buffer, pH = 7.0 (3.0 mM CTAB). Inset: Calibration curve. (λ_{ex} = 300 nm at 25 °C).

CN^-, $H_2PO_4^-$, SO_4^{2-}, SO_3^{2-}, $S_2O_3^-$, $S_2O_5^-$, CO_3^{2-}, ClO^-, and H_2O_2, the probe molecule did not display any spectroscopic response.

Notably, the probe was silent in the presence of naturally occurring amino acids bearing acidic, basic, and neutral side chains (Figure 4(b)). Also, no positive response was monitored upon addition of metal species (50.0 equiv.) including Na^+, K^+, Cr^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Hg^{2+}, Ag^+, Pd^{2+}, and Au^{3+}. To prove the sensitivity of the probe molecule, we cautiously surveyed the interference of other reactive species and naturally occurring amino acids. It is important to note that adding excess of anions and other biologically relevant species (50.0 equiv.) had a slight, if any, effect on the fluorescence response of **HMBT-OCN** to the addition of GSH (10.0 equiv.) (Fig. S4 in the Supporting Information). Moreover, the presence of naturally occurring amino acids including alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, histidine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan, and valine did not influence the selectivity or sensitivity of the probe molecule (Fig. S5 in the Supporting Information). Such results clarified that the sensing strategy was highly selective and sensitive only with the addition of RSS.

Other studies have anticipated the proposed mechanism of the sensing event (Scheme 2) [39,40]. The carbon atom of the cyanate group is highly electrophilic and suspicious to the addition of any nucleophilic reagent. In the presence of a reactive sulphur species (e.g. GSH), **HMBT-OCN** promptly undergoes the nucleophilic addition reaction and forms a thiocarbamate intermediate, which easily hydrolyses the corresponding highly fluorescent-free **HMBT-OH** dye in aqueous media. To verify its agreement with the findings of other studies, we investigated the outcome of the reaction between **HMBT-OCN** and GSH and identified the major product as **HMBT-OH**.

Encouraged by the high selectivity and sensitivity profile of **HMBT-OCN**, we examined its performance in cell imaging applications. To that end, we pre-treated the human lung adenocarcinoma cells (A549) with 2 mM *N*-methylmaleimide (*N*MM), a sulphur-trapping agent, for 30 min to eliminate physiological reactive sulphur species present inside the cells. Then, we successively treated the cells with **HMBT-OCN** and CTAB and incubated them for 30 min after each addition. We took fluorescence and bright-field images of cells before and after the addition of GSH (200 μM). As Figure 5(a) illustrates, the pre-treated A549 cells displayed no significant fluorescence emission before the addition of GSH. By contrast, an obvious blue fluorescence appeared in cells with GSH treatment. Thus, from the outcomes of the experiments, we concluded that **HMBT-OCN** is cell permeable and enables the detection of exogenous GSH in cells. In addition, cells treated with **HMBT-OCN** were intact and showed healthy spread and adherent morphology during and later the labelling process with **HMBT-OCN**, indicating an absence of cytotoxic effects.

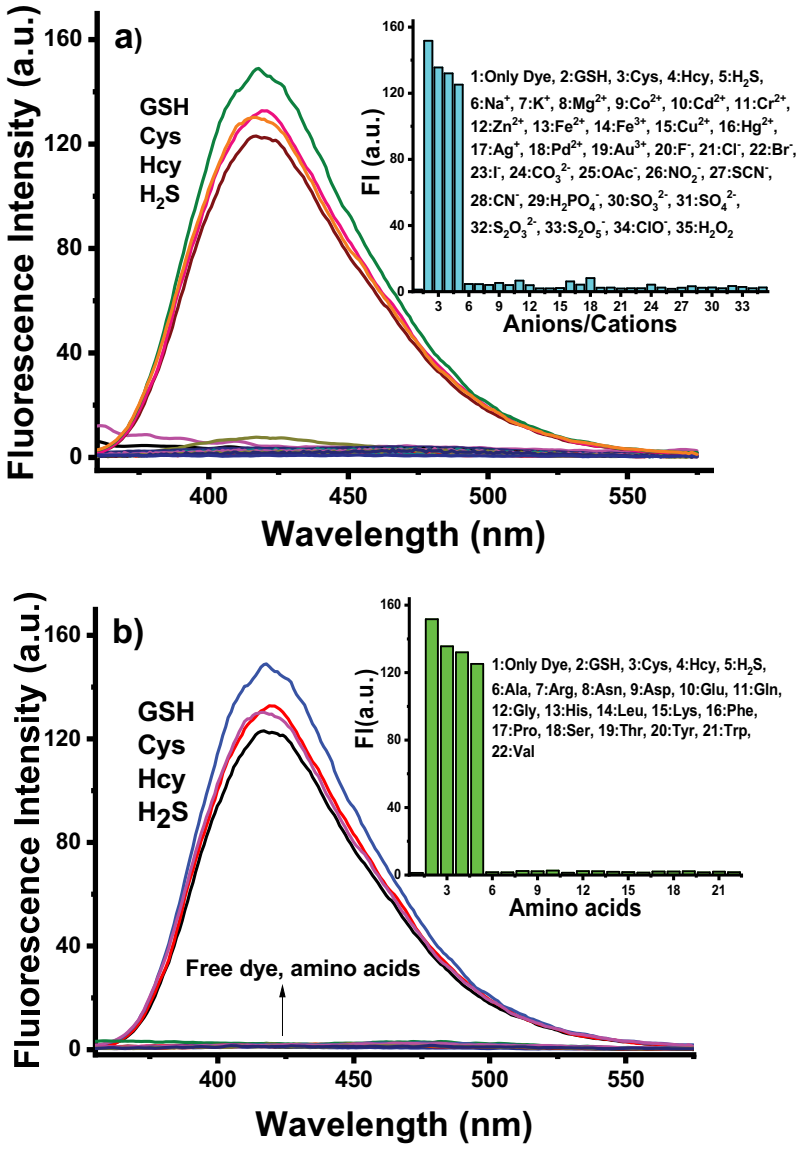
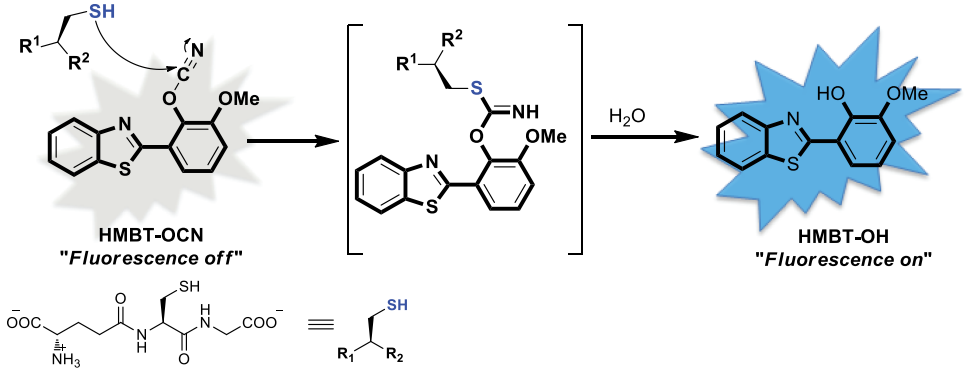


Figure 4. Fluorescence spectra of **HMBT-OCN** (20 μM) + GSH, Cys, Hcy or H_2S (200 μM, 10.0 equiv.) or (a) other ROS/RNS and anions/cations (b) amino acids (1000 μM, 50.0 equiv.) in 0.1 M phosphate buffer, pH 7.0 (3.0 mM CTAB) (λ_{ex}: 300 nm, at 25 °C). Inset: Bar graph notation.



Scheme 2. Proposed sensing mechanism of **HMBT-OCN.**

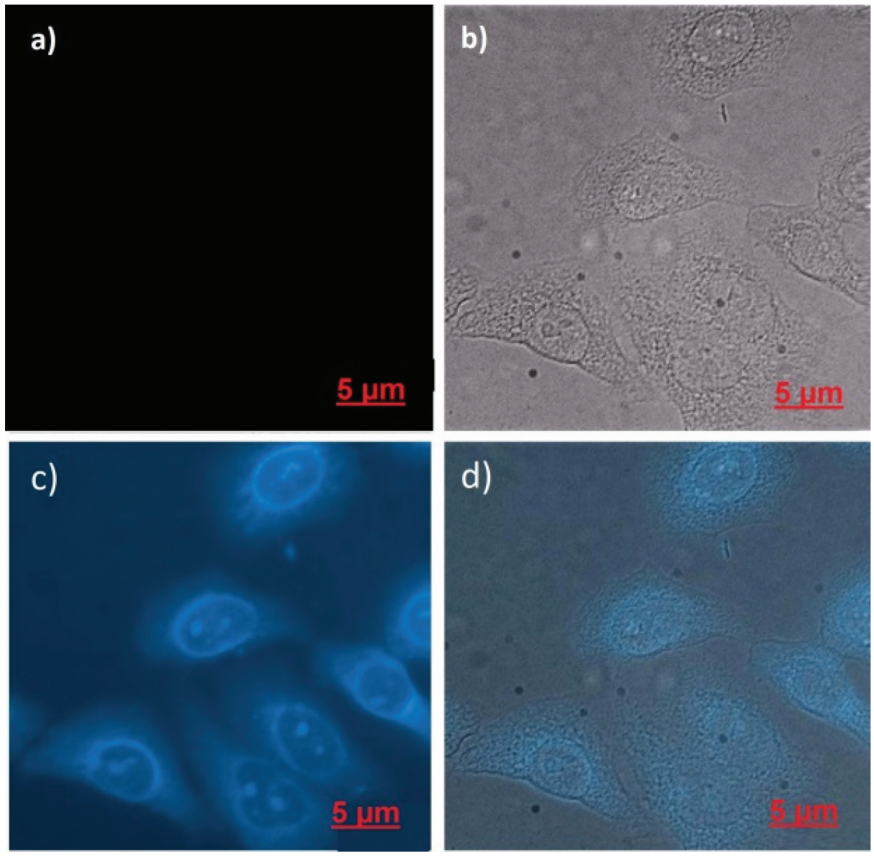


Figure 5. Images of A549 cells: (a) Cells pretreated with 2 mM *N*MM for 30 min and then incubated with **HMBT-OCN** (20 µM) in (b) brightfield image of cells (c) cells pretreated with 2 mM *N*MM then incubated with **HMBT-OCN** (20 µM) and GSH (200 µM). (d) merged images of frame (b) and (c). Scale bar represent 5 µm.

Conclusion

In sum, we demonstrated the design and synthesis of a novel molecular probe, **HMBT-OCN**, which selectively and sensitively detects reactive sulphur species (RSS) over other amino acids and biologically relevant species. The probe molecule exhibited outstanding features such as a low detection limit (550 nM), a high turn-on response (210-fold), and a short visible response time (< 1 min) upon the addition of GSH. Studies on improving the selectivity of the probe towards a specific RSS is under investigation.

Acknowledgments

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Disclosure statement

The authors declare no conflict of interest.

Experimental section

All reagents were purchased from commercial suppliers (Aldrich and Merck) and used without further purification. ^{1}H NMR and ^{13}C NMR were measured on a Varian VNMRJ 400 nuclear magnetic resonance spectrometer. Elemental analysis experiment was performed with LECO CHNS-932 elemental analysis apparatus. UV absorption spectra were obtained on Shimadzu UV-2550 Spectrophotometer. Fluorescence emission spectra were obtained using Varian Cary Eclipse Fluorescence spectrophotometer. Cell imaging was performed with Zeiss Axio fluorescence microscope. pH was recorded by HI-8014 instrument (HANNA). All measurements were conducted at least in triplicate.

Synthesis and characterization of probe (HMBT-OCN) molecule

2-(2′-Hydroxy-3′-methoxyphenyl)benzothiazole (**HMBT-OH**) was synthesized by using a literature procedure [32].

A solution of cyanogen bromide (130 µL, 0.4 mmol) in anhydrous tetrahydrofuran (1 mL) was cooled to −10°C (dry ice/acetone bath). Then, a solution of **HMBT-OH** (104 mg, 0.4 mmol) and triethylamine (58 µL, 0.4 mmol) in anhydrous

tetrahydrofuran (1 mL) was added dropwise with magnetic stirring under argon atmosphere. A white precipitate of triethylammonium bromide salt was observed. The mixture was allowed to stir for 1 h. The solution was separated from the salt by filtration and concentrated in vacuo and purified by column chromatography (Hexane:EtOAc 4:1) to afford the compound **HMBT-OCN** as a white solid (84.6 mg, % 75 isolated yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.53-7.44 (m, 3H), 7.32-7.27 (m, 3H), 7.24-7.20 (m, 1H), 3.90 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 157.2, 146.8, 146.1, 143.1, 130.3, 128.5, 127.7, 127.2, 124.3, 124.1, 123.8, 118.5, 118.1, 116.1, 56.3. Anal. cald. for $C_{15}H_{10}N_2O_2S$: C, 63.81; H, 3.57; N, 9,92; S, 11.36 found; C, 62,75; H, 4.11; N, 8.11; S, 9.65.

Supplementary information file containing the details of stock solution preparations, absorbance/fluorescence measurements, cell culture/imaging experiments, and characterization data for probe is available at the journal website at.

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