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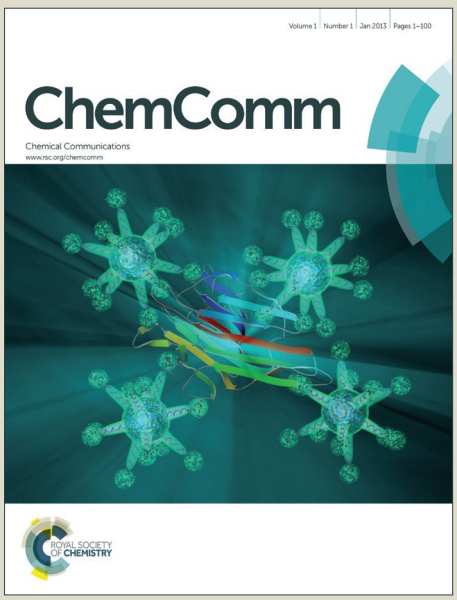

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DOI: 10.1039/C5CC07298K

Journal Name

COMMUNICATION

A simple excited-state intramolecular proton transfer probe based on a new strategy of thiol-azide reaction for the selective sensing of cysteine and glutathione

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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A simple azido-substituted fluorescence sensor AHBO showing selective turn-on response to cysteine (Cys) and glutathione (GSH) over homocysteine (Hcy), sulfide and other amino acids has been constructed, which is based on the mechanism of selective nucleophilic substitution-rearrangement reactions.

Intracellular thiols, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), play pivotal roles in biological processes.[1] For instance, GSH is the most abundant cellular thiol that maintains the reduced state of proteins and protecting the cells against reactive oxygen species (ROS), drugs or heavy metal ions.[2] Cys is involved in many important biological functions including protein synthesis, detoxification and metabolism.[3] Hcy is a risk factor for Alzheimer's, cardiovascular diseases and neutral tube defects.[4-5] Abnormal thiols concentration has been implicated with the development of many kinds of diseases. For example, elevated levels of Cys are considered to be linked with neurotoxicity, and its deficiency is involved in slow growth in children, hair depigmentation, loss of muscle and fat, skin lesion, liver damage and edema.[3] Glutathione deficiency is associated with various diseases such as leukocyte loss, cancer, AIDS and neurodegenerative diseases.[6] Therefore, the rapid, convenient, selective and sensitive detection of these biothiols is of great importance for clinical diagnosis.

Among various detection methods, fluorescence detection is more attractive as it offers several advantages including high sensitivity, simplicity of operation and non-invasiveness.[7-8] In the past decade, significant efforts have been devoted to the development of fluorescent sensors for biothiols.[9-25] The Peng group provided the first turn-on fluorescent chemodosimeter for discrimination of Cys over Hcy and GSH.[12] The Strongin group reported the first example of simultaneous fluorescent detection of Cys and Hcy based on the different relative rates in an intramolecular cyclization reaction.[13] Yang and his co-workers showed a BODIPY-based probe to produce a selective and ratiometric fluorescence change with GSH relative to Cys and Hcy.[18] The Yoon group developed a cyanine-based near infrared fluorescent probes that can selectively detect GSH in living cells.[22] Recently, Guo and co-workers showed a sensor for simultaneous fluorescence sensing of Cys and GSH from different emission channels.[20] These sensors are manly reaction-based and depend on the strong of nucleophilicity of the thiol group. The types of reaction include cyclization reaction, Michael addition, cleavage reaction, disulfide exchange reaction and others.[9-10,19] These developments greatly advanced the research on the fluorescent detection of biothiols. However, many of these sensors suffer from relatively low sensitivity, long response time and a complicated synthesis process.[19] In addition, considering that these biothiols have similar structures and reactivity but are associated with different diseases, the design of a highly selective detection system that discriminates them is still a significant challenge for clinic diagnosis.

Azido-substituted fluorescent dyes have been widely designed to selectively turn-on detect hydrogen sulphide because azide can be easily converted into amine upon treatment with hydrogen sulphide to lose its quenching property.[26-28] Herein, we present an azido-substituted hydroxyphenylbenzoxazole derivative **AHBO** (Scheme 1). In comparison with the reported azido-substituted fluorescent sensors for detection of hydrogen sulphide, **AHBO** shows no obvious fluorescence response to sulphide but selective turn-on fluorescence response to Cys and GSH. The exceptional and interesting response derived from thiol-azide reaction provides a new strategy for biothiols detection. To the best of our knowledge, **AHBO** is the first fluorescent sensor based on azide recognition for selective sensing of Cys and GSH without interference by sulphide.

Probe **AHBO** was prepared by the synthetic route outlined in Supporting Information. Briefly, 2-aminophenol was first readily reacted with 5-aminosalicylic acid under the catalysis of polyphosphoric acid (PPA) to form 2-(2'-hydroxy-4'-aminephenyl)benzoxazole (**1**). Then the amine group in the resulting compound **1** was further converted to diazo by common diazo-reaction in acid media in the presence of sodium nitrite and sodium azide, producing **AHBO** with 72%

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Electronic Supplementary Information (ESI) available: [detailed experimental procedures and additional figures]. See DOI: 10.1039/x0xx00000x

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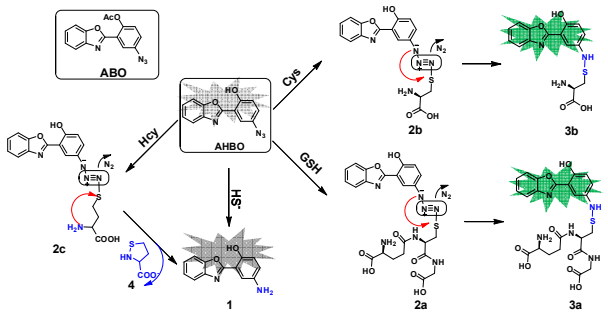
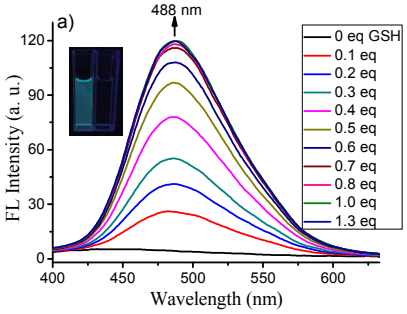
isolated yield. In addition, for comparison a referenced compound **ABO** was synthesized through the acetylation of **AHBO** with acetic anhydride. All these compounds were fully characterized by ^{1}H NMR, ^{13}C NMR and MS spectra.



Scheme 1 Chemical structures of **AHBO** and **ABO**, and the plausible reaction mechanism.

In CH_3CN/HEPES buffer (1:1, v/v, pH 7.4), **AHBO** exhibited two absorption peaks at 344 and 420 nm. Upon addition of GSH, the absorption intensity at 344 nm decreased, while the intensity at 420 nm increased with a well-defined isosbestic point at 365 nm (Fig. S1). Accordingly, upon excitation at 325 nm, the fluorescence intensity at 488 nm evidently increased upon successive addition of GSH (Fig. 1a). The fluorescence intensities at 488 nm linearly gradually increased with addition of GSH (0-7 μmol/L) (Fig. 1b). As high as 28-fold fluorescence enhancement at 488 nm was observed as 1 equivalent of GSH was added. The Job's plot revealed 1:1 stoichiometry between **AHBO** and GSH (Fig. S2). The detection limit was calculated to be 9.0×10^{-8} M. **AHBO** is a typical excited-stated intramolecular proton transfer (ESIPT) molecule, which should exhibit normal emission (N*) and tautomer emission (T*) (Scheme S1). If the hydroxyl was substituted, ESIPT molecules mainly show normal emission (N*).[29] As expected, the acetyl-substituted **ABO** reveals a main and shorter fluorescence band at 366 nm after addition of 2 equivalents of GSH (Fig. S4). From these results, we can propose that the 488 nm emission of **AHBO** treated with GSH should come from the tautomer emission. As far as we know, the product that **AHBO** reacts with GSH is the first small ESIPT molecule that only affords tautomer emission in polar aqueous media.[30] The fluorescence intensities of **AHBO** in the absence and presence of GSH are pH independent in the range of 7-8, demonstrating that AHBO can detect GSH in biological environment (Fig. S5).



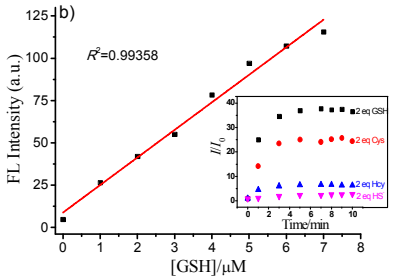


Fig. 1. a) The fluorescence spectra change of **AHBO** (10 μM) upon addition of GSH, λ_{ex}=325 nm. The inset shows the photo of **AHBO** in the presence and absence of 1 equivalent of GSH, which is excited by hand-held UV lamp (365 nm). b) The fluorescence intensity change of **AHBO** (10 μM) at 488 nm upon addition of GSH, indicative of good linear relationship. Inset: time dependent fluorescence intensity (488 nm) of **AHBO** (10 μM) in the presence of 2 equivalents of GSH, Cys, Hcy and sulphide, indicating that the reaction with GSH or Cys can be done within 10 min. All the solution is in CH_3CN/HEPES Buffer (1:1, v/v, pH 7.4).

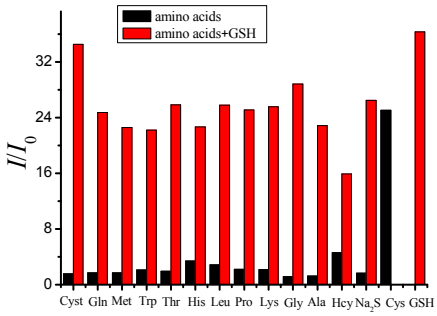


Fig. 2. The relative fluorescence intensity of **AHBO** (10 μM) at 488 nm upon addition of various amino acids (20 μM) in CH_3CN/HEPES Buffer (1:1, v/v, pH 7.4), λ_{ex}=325 nm (blank bar). Red bars represent the intensity with subsequent addition of GSH (20 μM).

The selectivity of **AHBO** to various amino acids and sulfide was further examined. As shown in Fig. 2, only GSH and Cys promote significant fluorescence intensity enhancement at 488 nm, whereas other amino acids cause no detectable spectra change. To explore the possible utility of **AHBO** as fluorescent sensor for GSH, competitive experiments were carried out in the presence of 2 equivalents of GSH and 2 equivalents of various other amino acids. **AHBO** can still turn-on fluorescence response to GSH without interference by other amino acids. It should be noted that azido-substituted fluorescent dyes universally showed good fluorescence response to sulfide.[24,26-28] But interestingly, **AHBO** affords no obvious fluoresce change even upon addition of 10 equivalents of sulfide and can efficiently detect GSH in the presence of sulfide (Fig. S7), which is possibly due to the unique molecular structure of **AHBO**. In addition, other possible reducing species in biological samples, such as Fe^{2+} and ascorbate showed no reactivity with **AHBO** (Fig. S7). These results suggested that **AHBO** shows excellent selectivity for GSH and Cys detection. The reaction times of **AHBO** to thiols were also studies. **AHBO** shows fast fluorescence response to GSH and Cys within 10 min no matter 2 or 10 equivalents of

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these thiols were added (Inset in Fig. 1b and Fig. S8). Interestingly, we found that no absorption spectra change of **AHBO** in the presence of sulfide was observed within 10 min (Fig. S8a and b). Although **AHBO** can react with sulfide to produce non-fluorescent **1**, the reaction rate is very slow, which efficiently avoids the interference of sulfide with detection of GSH or Cys.

To evaluate the possibility in biological system, **AHBO** was applied to detect GSH in human blood samples. In diluted (10%) deproteinized fetal bovine serum (FBS), turn-on fluorescence response of **AHBO** was obviously observed upon addition of GSH (Fig. S9). More importantly, the fluorescence intensity changes at 488 nm linearly depend on the added concentration of GSH, suggesting the potential application of **AHBO** in serum.

AHBO shows remarkable and selective fluorescence turn-on response to GSH and Cys over Hcy and sulfide. We were interested in achieving an understanding of the difference between these biothiols. **AHBO** showed no fluorescence and its fluorescence quantum yield (Φ) was very low (as small as 0.0007) because of the quenching property of azide. Upon treatment with sulfide, it is possible that **AHBO** would be transformed to **1** hanging with amino group. In fact, the product of **AHBO** that reacts with sulfide is undoubtedly confirmed to be **1** through TLC, NMR and MS spectra. However, like **AHBO**, **1** also has very low fluorescence quantum yield (0.002) due to the photo-induced electron transfer (PET) effect, which should be interpreted that **AHBO** showed no obvious fluorescence change upon addition of sulfide. Compared to Cys, Hcy has one extra methylene group, which makes Hcy to be more conducive to experience nucleophilic substitution-rearrangement (or S_NAr substitution) reaction. This property has been widely used to design fluorescent sensors for discrimination of Cys and Hcy.[14,18-19,21,25] Inspired by this, we propose the plausible reaction mechanism between **AHBO** and these thiols. As shown in Scheme 1, initial nucleophilic attack by sulfhydryl froup of thiols leads to the formation of an intermediate **2**. The intermediate **2** then release N_2 to provide the S-N product **3**. In the case of Hcy, the amino group then attacks the sulfur to form the non-fluorescent product **1**, and at the same time to yield a five-membered ring product **4**, which is confirmed by MS spectrum (Fig. S17). In the case of Cys and GSH, a similar intramolecular attack would lead to a strained four membered ring and a nine membered ring, respectively, which would be unstable and thus unfavorable. In fact, their final products should be the S-N compounds **3a** and **3b**. These compounds are fluorescent with the fluorescence quantum yields of 0.017 and 0.016, respectively. The S-N compounds **3a** and **3b** were also confirmed by MS spectra (Fig. S10-11). The corresponding peaks of **3a** and **3b** with exact molecular weight were also observed in LC-MS spectra (Fig. S15-16). Similar to GSH, the 1:1 stoichiometry between **AHBO** and Cys was obtained from Job's plot (Fig. S3). The ^{1}H NMR spectra titration of **AHBO** with GSH was studied (Fig. S13). Upon addition of GSH, no substitution on aromatic ring was observed, but up-field shifts for the aromatic protons of the benzoxazole core were found due to the displacement of azido by S-N bond. To further confirm the mechanism including substitution-rearrangement reaction, the control reaction of **AHBO** with N-acetylhomocysteine, a molecule that structurally similar to Hcy but lacks an amino group, was carried out. As expected, turn-on fluorescence response similar to that added with Cys was obtained because the intramolecular cyclic rearrangement reaction is inhibited (Fig. 3). Furthermore, **AHBO** showed identical fluorescence response to Cys and N-acetylcysteine. These results suggested that the selective fluorescence response of **AHBO** to GSH and Cys over Hcy should be attributed to the inhibition of cyclic transition states/intermediates reactions, which has been used to explain the mechanisms of other Cys- or Hcy-selective probes.

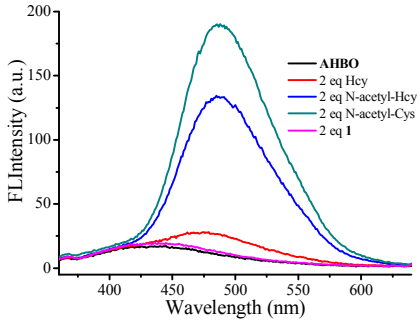

Fig. 3. The fluorescence spectra change of **AHBO** (10 μM) upon addition of 2 equivalents of Hcy, N-acetylhomocyeteine, N-acetylcyeteine and **1** in CH_3CN/HEPES Buffer (1:1, v/v, pH 7.4), λ_{ex}=325 nm.

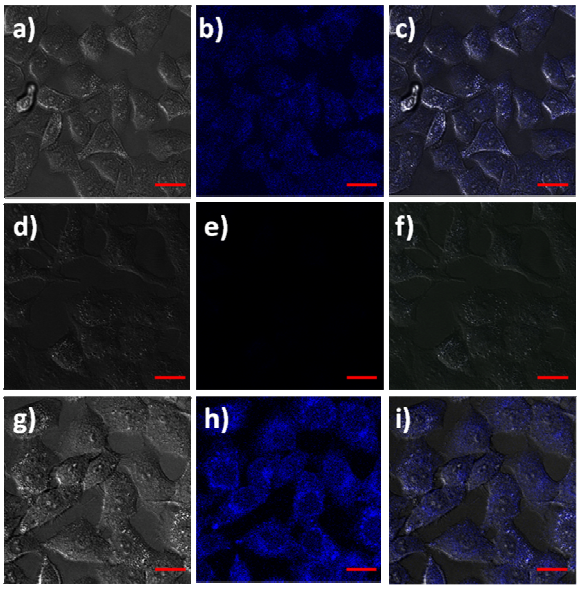

Fig. 4. Fluorescence images of HeLa cells. (a-c) HeLa cells incubated with probe **AHBO** (20 μM) for 30 min; (d-f) images of cells pretreated N-ethylmaleimide (NEM) for 30 min and then incubated with **AHBO** for 30 min; (g-i) images of cells pretreated N-ethylmaleimide (NEM) for 30 min and then incubated with **AHBO** and GSH (100 μM) for 30 min; (a, d and g) Bright-field images of the HeLa cells in samples; (b, e and h) images taken in blue field; (c, f and i) are the overlap of brightfield and fluorescence. Images were acquired by using excitation and emission windows of λ_{ex} = 405 nm and λ_{em} = 420-475 nm, respectively. Scare bar: 20 μm.

To further investigate the biological application of **AHBO**, the fluorescence microscopy experiment in living cells was carried out. When HeLa cells were incubated with 20 μM **AHBO** in culture

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medium at 37 °C for 30 min, significant blue fluorescence is produced in HeLa cells. In control experiment, the HeLa cells were pretreated with the thiol blocking reagent N-ethylmaleimide (NEM) for 30 min and then incubated with **AHBO** for 30 min. The confocal microscopy image of cells does not display fluorescence (Fig. 4). Moreover, addition of Cys or GSH (100 μM) to the NEM-pretreated HeLa cells gives rise to a significant fluorescence increase. These results suggest that **AHBO** is capable of permeating into cells and reacting with endogenous GSH or Cys to produce discernible fluorescence responses.

In summary, we have developed a simple azido-substituted ESIPT fluorescent sensor to selectively turn-on detect GSH and Cys. The results of the investigation show that the turn-on fluorescence at 488 nm is assigned to the tautomer emission, providing a large Stokes shift. Compared to the reported azido-substituted fluorescent sensors for detection of sulfide, **AHBO** showed no obvious fluorescence change upon addition of sulfide. It is interpreted that the amino-substituted product **1** remains showing very weak fluorescence. The selective fluorescence response of **AHBO** to GSH and Cys over Hcy is attributed to the nucleophilic substitution-rearrangement reactions that take place in the case of Hcy but not in the case of GSH and Cys. Finally, **AHBO** can be served as a fluorescent sensor to visualize Cys and Hcy in living cells. Although the excited wavelength of the sensor is in ultraviolet region, the thiol-azide reaction provides a new strategy to design fluorescent sensors for detection of thiols. Further work along exploration of this kind of sensors with longer wavelength fluorescence is in progress in our laboratory.

This work was supported by the National Natural Science Foundation of China (Grant No. 21206137 and 21476185), the Fundamental Research Funds for the Central Universities (2014YB027 and 2452013py014), Shaanxi Province Science and Technology (No. 2014K11-01-02-06) and the Scientific Research Foundation of Northwest A&F University (Z111021103 and Z111021107).

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