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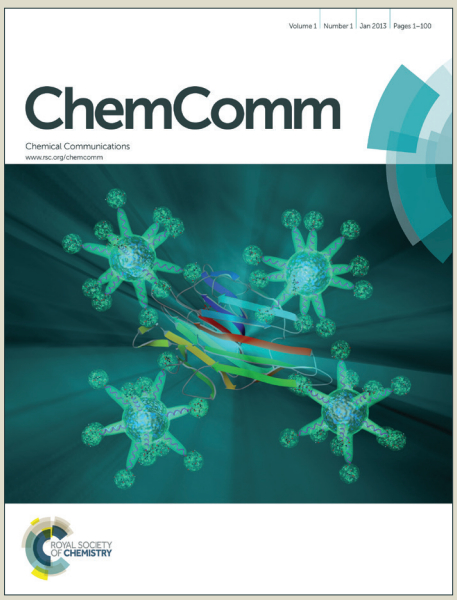

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Aryl-thioether substituted nitrobenzothiadiazole probe for selective detection of cysteine and homocysteine

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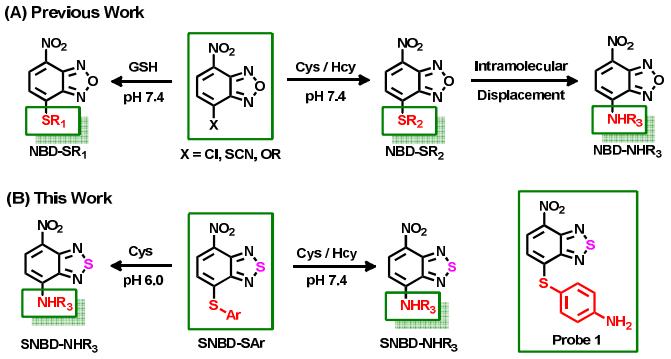
An aryl-thioether substituted nitrobenzothiadiazole was synthesized and employed to detect cysteine and homocysteine selectively in living cells. Interestingly, both cysteine (Cys) and homocysteine (Hcy) promote an enhancement of the fluorescence intensity of the probe at pH 7.4 while only Cys gives rise to this enhancement under weakly acidic conditions (pH 6.0).

Biothiols are a class of substances that are crucial for maintaining life owing in part to an equilibrium that exists in biological systems between their reduced free thiol and oxidized disulfide forms.[1] As a result, biothiols act as antioxidants to maintain normal levels of peroxy radicals, aid in the inhibition of cellular apoptosis, and participate in the construction of complicated multi-dimensional protein structures.[2] Among the many biological thiols that exists in living systems, glutathione (GSH), cysteine (Cys) and homocysteine (Hcy) have received greatest attention owing to the fact that abnormal concentrations of these three species are considered to be signals for many diseases such as cancer, AIDS, osteoporosis, as well as Alzheimer's, liver, heart, inflammatory bowel and cardiovascular diseases.[3] Owing to this feature, the development of methods for selective and sensitive detection of biothiols is of great significance in the area of clinical diagnosis.

Fluorescent probes[4] employing different fluorophores are useful tools to monitor levels of intracellular thiols.[5-8] The most typical strategy used to design fluorescent probes of this type relies on the high nucleophilicity of thiols.[8] An example of this strategy is found in the use of commercially available and fluorescent 4-chloro-7-nitrobenzo[*c*][1,2,5]oxadiazole (**NBD-Cl**, Scheme 1) which, owing to its electron-deficient nature, participates in nucleophilic substitution reactions with thiols that promote changes in emission intensities. For example, hydrogen sulfide (H_2S) reacts with **NBD-Cl** to yield the adduct **NBD-SH**.[9] Likewise, the biothiol GSH also serves as a nucleophile in a substitution reaction with **NBD-Cl** to generate the corresponding thioether **NBD-SG** (Scheme 1(A)). In contrast, reactions of Cys or Hcy with **NBD-Cl** initially generate adducts **NBD-SR$_2$**, which then undergo intramolecular aromatic nucleophilic substitution reactions to form secondary products, **NBD-NHR$_3$**.[10] Furthermore, some derivatives of **NBD-Cl** also serve as substrates for these same types of reactions.[11] Considering the absence of thiophenols in the cells, the detection of biothiols with arylthioether substituted nitrobenzothiadiazoles (**SNBD-SAr**) is not interfered by thiophenols in living cells.

Based on the results of the investigations described above, we designed the new nitrobenzothiadiazole fluorescent probe **1** (Scheme 1(B)), which contains a *p*-aminophenylthioether linkage as a reactive center. The results of a study of probe **1** demonstrate that it only reacts with Cys under pH 6.0 conditions to form an adduct **SNBD-NHR$_3$** in association with a red shift in its maximum absorption from 430 to 475 nm and a remarkable fluorescence enhancement at 535 nm, In contrast, Cys and Hcy both react with this probe at pH 7.4 to bring about similar changes in its absorption and emission properties. Finally, the fluorescence of **1** does not change in the presence of GSH.

Probe **1** was synthesized by reaction of *p*-aminothiophenol with 4-chloro-7-nitrobenzo[*c*][1,2,5]thiadiazole in THF at reflux (93%). The NMR and mass spectra of this substance clearly show that it has the structure represented by **1** (ESI in Figure S1-S3). Analysis of the frontier molecular orbital profiles, derived by using TD-DFT theoretical calculation at the B3LYP/6-31G* level (ESI in Figure S4), suggests that this substance is capable of participating in a PET process, implying that it should be only weakly fluorescent.



Scheme 1. NBD-based fluorescent probes for thiols.

The UV/vis absorption spectrum of **1** contains a strong absorption maximum at 430 nm in HEPES (0.01 M, pH 7.4) containing 1% DMSO. In contrast to other amino acids, addition of only Cys and Hcy to DMSO solution induce a red shift in the absorption

maximum of **1** to 475 nm (Figure 1a). In addition, GSH causes no obvious change in the Uv/vis spectrum of the probe. These result show that **1** has good water solubility and that it reacts in a highly selective manner with Cys and Hcy.

As expected (see above), **1** is non-fluorescent in HEPES (0.01 M, pH 7.4) containing 1% DMSO. Among other amino acids, only Cys and Hcy in HEPES at pH 7.4 induce a remarkable enhancement of the fluorescence intensity of **1** at 535 nm corresponding to the development of yellow/green emission (Figure 1b). Importantly, no change in the emission occurs when 10 equiv of GSH is added to a HEPES solution of probe **1** (ESI in Figure S5-S11). Finally, proof that the absorption and emission changes caused by the addition of Cys and Hcy are a consequence of the operation of aromatic nucleophilic substitution reactions is gained by using ESI mass spectrometry (ESI in Figure S15 and S16).

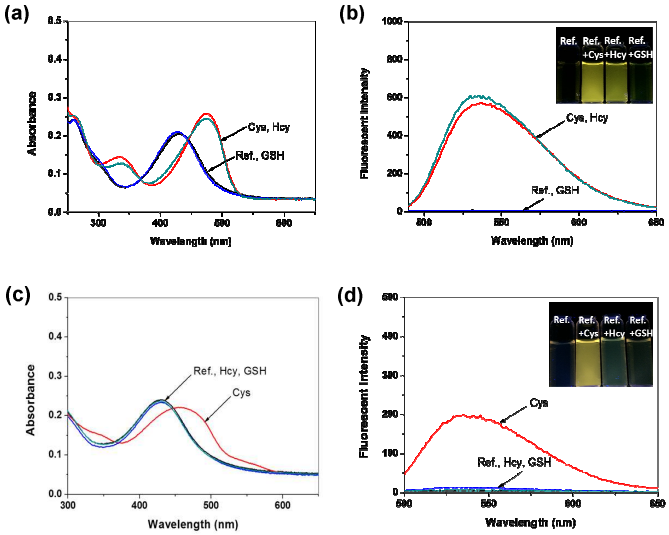


Figure 1. UV/Vis absorbance (a) and fluorescence changes (b) of probe **1** (10 μM) upon the addition of Cys, Hcy and GSH (10 eq) in HEPES (0.01 M, pH 7.4) containing 1% DMSO (Excitation wavelength: 475 nm) Inset: images of fluorescence from probe **1** in the presence of Cys, Hcy and GSH; UV/Vis absorbance (c) and fluorescence changes (d) of probe **1** (10 μM) upon the addition of Cys, Hcy and GSH (10 eq) in citric acid-Na_2HPO_4 (0.01 M, pH 6.0) containing 1% DMSO. (Excitation wavelength: 475 nm) Inset: fluorescence images of probe **1** in the presence of Cys, Hcy and GSH.

Observations made in earlier studies have shown that, owing to pK_a value differences, Cys (pK_a 8.53) is more reactive in nucleophilic substitution reactions than is Hcy (pK_a 10.00).[12] In addition, the existence of a *p*-amino group in **1** indicates that its chemical properties would change in low pH solutions. We felt that a combination of these factors could serve as the basis for a selective response of **1** to Cys. Indeed, the absorption and fluorescence spectra of **1** in a citric acid-Na_2HPO_4 (0.01 M, pH 6.0) solution containing 1% DMSO, is altered by the addition of only Cys and not Hcy (Figures 1 c and 1d, andESI in Figure S12-S14).

Next, variations in the fluorescence of probe **1** caused by additions of Cys or Hcy (0-50 eq) in HEPES (0.01 M, pH 7.4) containing 1% DMSO were explored. As can be seen by viewing Figure 2, additions of Cys or Hcy to the solution of **1** result in concentration dependent increases in the emission intensity of the probe at 535 nm. It is important to note that **1** has a low detection limit of *ca.* 0.1 μM for Cys or Hcy, suggesting that it can be used to monitor levels of these intracellular thiols (ESI in Figure S6 and S7).

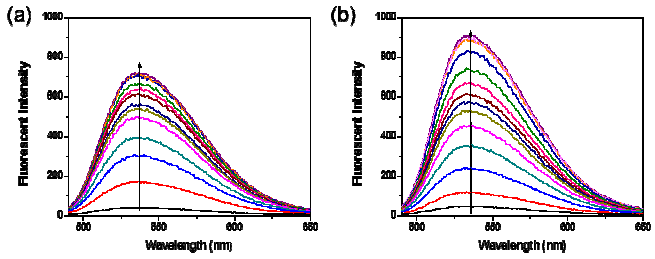


Figure 2. (a) (a) Fluorescence titrations of probe **1** (10 μM) with Cys (0-50 eq.) in HEPES (0.01 M, pH 7.4) containing 1% DMSO. (b) Fluorescence titrations of probe **1** (10 μM) with Hcy (0-50 eq.) in HEPES (0.01 M, pH 7.4) containing 1% DMSO. (Excitation wavelength: 475 nm)

In order to demonstrate its potential biological relevance, probe **1** was employed to monitor the levels of thiols in living cells. As can be seen by viewing the confocal fluorescence microscope and bright field images shown in Figure 3a, significant green fluorescence is produced in the cytoplasm of HeLa cells when they are incubated with probe **1** (3 μM) for 30 min. The results suggest that this probe is capable of permeating into cells and reacting with endogenous thiols to produce discernible fluorescence responses. Subsequent control experiments were performed to gain further support for this conclusion. In one control experiment, the HeLa cells were pretreated with the thiol-blocking reagent *N*-ethylmaleimide (NEM) for 30 min and then incubated with probe **1** for 30 min. The confocal microscope image of cells treated in this manner does not display green fluorescence (Figure 3b). Moreover, addition of Cys or Hcy (300 μM) to the NEM-pretreated HeLa cells gives rise to a significant increase in green emission (Figure 3c and 3d), while no obvious changes are observed upon the addition of GSH (Figure 3e). These results indicate that **1** has good water solubility, cell-penetration ability and biocompatibility, and that it can be utilized as a tool to monitor levels of thiols in living cells. Subsequently, a similar bioimaging in the condition of pH 6.0 was performed in living cells. As expected, probe **1** displays a high selectivity for Cys (ESI in Figure S17). Moreover, it is worth mentioning that probe **1** shows a lower cytotoxicity (ESI in Figure S18).

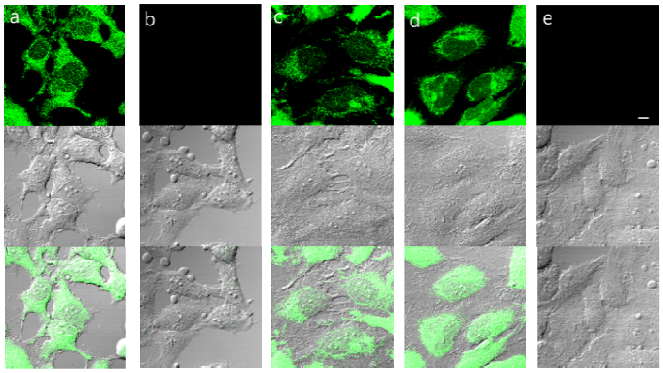


Figure 3. Fluorescence of probe **1** in HeLa Cells. (a) HeLa cells were incubated with 3 μM probe **1** for 30 min, followed by treatment with 1 mM NEM for 30 min (b-e) ; (b) only NEM, (c) incubated with 300 μM cysteine, (d) 300 μM homocysteine and (e) 300 μM GSH-MEE for 30 min additionally. (Scale bar: 10 μm.)

Conclusions

In summary, we have developed the aryl-thioether substituted nitrobenzothiadiazole **1** as a fluorescence turn-on probe to monitor biothiols. The result of the investigation show that Cys and Hcy induce an enhancement of fluorescence intensity of **1** in pH 7.4 solutions and that only Cys promotes this emission enhancement in

weakly acidic solution (pH 6.0). Moreover, because of its good water solubility, cell-penetration ability and biocompatibility, **1** serves as a fluorescent sensor to visualize Cys and Hcy in live cells. Finally, we believe that the new NBD-based aryl thioether probe will prove useful in explorations designed to gain a better understanding of biological processes involving Cys and Hcy.

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Aryl-thioether substituted nitrobenzothiadiazole probe for selective detection of cysteine and homocysteine

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