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A turn on ESIPT probe for rapid and ratiometric fluorogenic detection of homocysteine and cysteine in water with live cell-imaging

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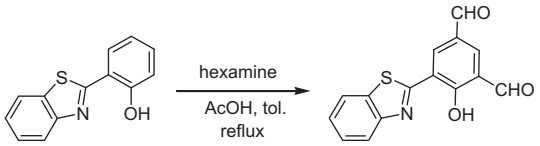
ABSTRACT

5(Benzothiazol-2-yl)-4-hydroxyisophthalaldehyde (BHI), an intense ESIPT containing molecule in mixed media loses its properties due to resonance-assisted H-bond (RAHB) in absolute water. Due to resonance-assisted H-bond the *o*-aldehyde is more reactive than the other one. With addition of cysteine/homocysteine into this solution the *o*-aldehyde group gets transformed into thiazolidine/thiazine ring, respectively, and the phenolic proton becomes free enough for transfer to nitrogen of the benzothiazole ring in excited state, that is, the ESIPT of BHI is turned on. Thus we can detect cysteine/homocysteine in water as well as in live cells.

Inter- and intramolecular proton transfers are fundamental reactions in chemistry and biology that have been broadly investigated.[1] A broad class of molecules that contain an H-chelated ring exhibits both in the gas phase and in nonpolar solvents fast excited-state intramolecular proton transfer (ESIPT).[2] ESIPT compounds have also drawn much attention due to their potential applications in optical devices that may take advantage of the salient properties such as the ultra-fast reaction rate and extremely large fluorescence Stokes shift.[3] Riedle and co-workers[4] have nicely explored this area by taking 2-(2′ hydroxyphenyl)benzothiazole (HBT) as a standard moiety. Taking this advantage of HBT, many researchers have designed anion and cation sensors with ESIPT mechanism.[5]

We also take HBT as an ESIPT containing fluorophore with the addition of aldehyde group for taking advantage of RAHB, that is, resonance-assisted H-bond introduced by Gilli et al.[6] in 1989. This also helps in selective and ratiometric detection of cysteine (Cys) and homocysteine (Hcy) which are essential biological molecules relevant to the growth of cells and tissues in living systems.[7] Cys deficiency is involved in many syndromes, for instances, slow growth in children, liver damage, skin lesions, and weakness.[8] Hcy has also been linked to increased risk of Alzheimer's disease,[9] inflammatory bowel disease, and osteoporosis.[10]



Scheme 1. Synthetic outlook.

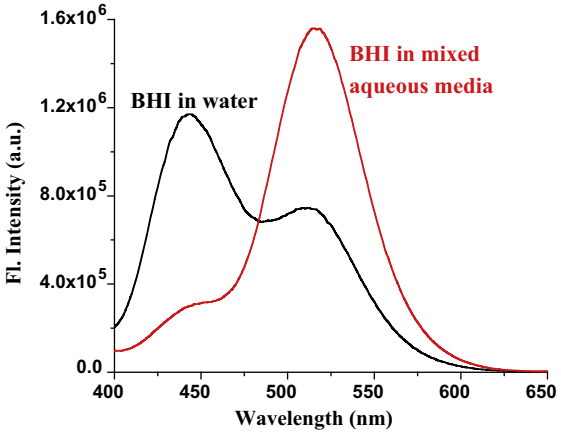


Figure 1. Solvent dependent fluorescence response of BHI ($c = 2.0 \times 10^{-5}$ M).

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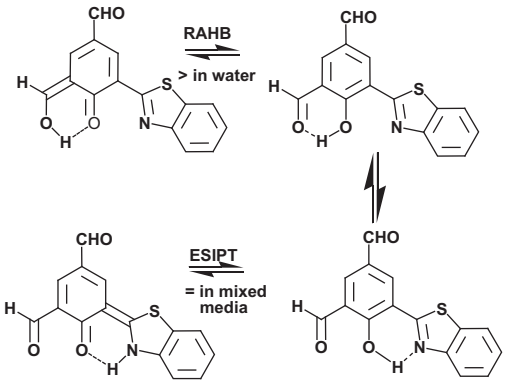
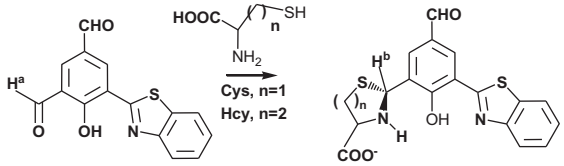


Scheme 2. Probable solvent dependent isomers of BHI.



Scheme 3. Reaction mode of Cys/Hcy with BHI.

Therefore, the detection and discrimination of thiol-containing molecules in biological samples are of great importance. Due to simplicity, sensitivity, and efficiency of fluorescence methods[11] design of fluorescent receptors is desirable for potential applications. In the past few years, various fluorescent chemodosimeter probes for biothiols based on different mechanisms have been developed.[12] From those, a few are also capable of displaying high selectivity over other amino acids by turning on the fluorescence at neutral pH in pure water.[13] But a ratiometric approach toward Cys and Hcy at neutral pH in pure water considering both the strong H-bonding phenomena, that is, RAHB and ESIPT is still a challenge. It is well known that, ratiometric probes allow measurement at two different wavelengths, which could provide a built-in correction for environmental effects and improve the detection sensitivity.[14] Thus, a chemodosimeter exhibiting greatly changed emission intensity with ratiometric manner is ideal for sensing.

Thus considering all the above facts, herein we report a HBT based probe, 5(benzothiazol-2-yl)-4-hydroxyisophthalaldehyde (BHI) for highly selective and sensitive detection of Cys/Hcy in pure water and cells. The probe (BHI) was prepared according to our previously reported procedure (Scheme 1).[15] It has a HBT moiety as a fluorescence signaling unit and salicyldehyde functionality as a reaction unit. Both Cys and Hcy react with the carbonyl group of BHI to afford a five or six-membered ring.

In our previous work,[15] we see that BHI is a strong ESIPT containing agent in mixed aqueous media (CH_3CN/H_2O = 1:1). Upon irradiation (λ_{ex} = 344 nm), BHI generates the excited-state

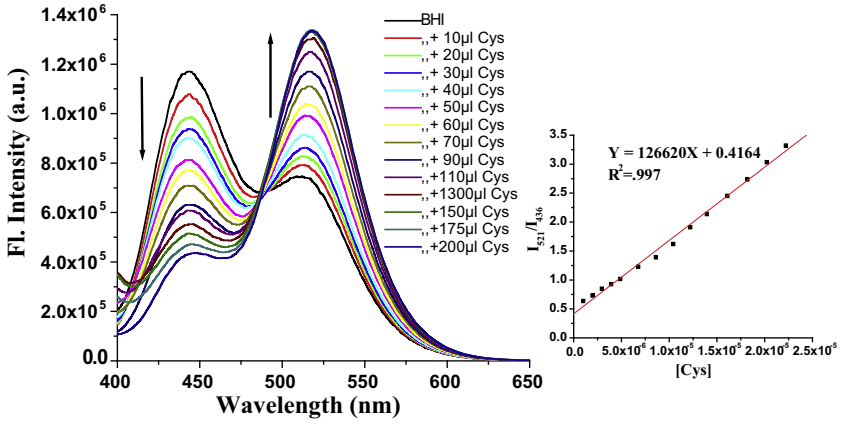


Figure 2. Fluorescence spectra of BHI ($c = 2.0 \times 10^{-5}$ M) in the presence of 1 equiv Cys ($c = 2.0 \times 10^{-4}$ M, total 200 µl in various amounts) in buffered-H_2O (at pH 7.4) and the corresponding ratiometric response (I_{521}/I_{436}) of BHI with the addition of Cys.

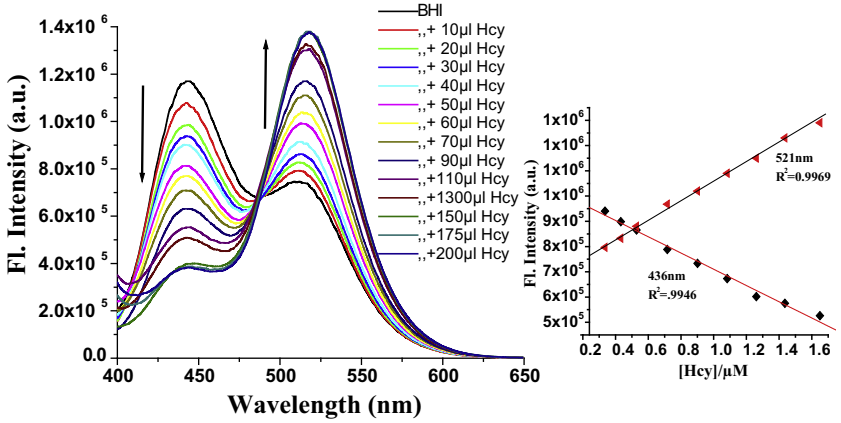


Figure 3. Fluorescence spectra of BHI ($c = 2.0 \times 10^{-5}$ M) in the presence of 1 equiv Hcy ($c = 2.0 \times 10^{-4}$ M, total 200 µl in various amounts) in buffered-H_2O (at pH 7.4) and the corresponding intensity plot as a function of [Hcy] at two different wavelengths.

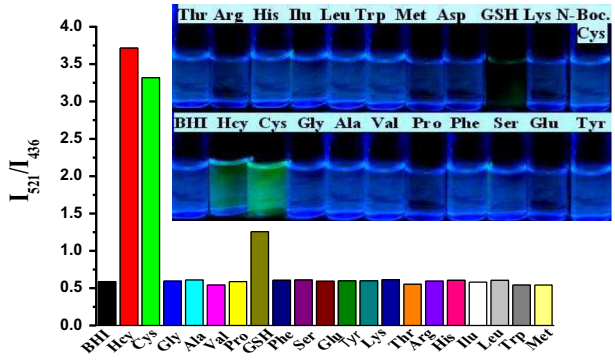


Figure 4. Ratiometric response of BHI toward 1 equiv of various amino acids with their 'naked eye' response under UV-light.

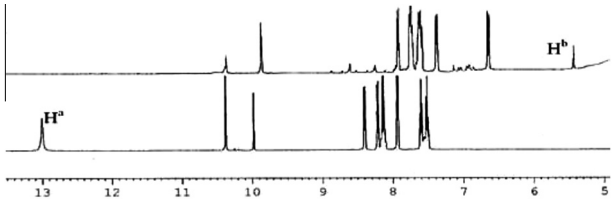


Figure 5. Partial ^{1}H NMR spectrum of BHI and its adduct with Cys in DMSO-d_6.

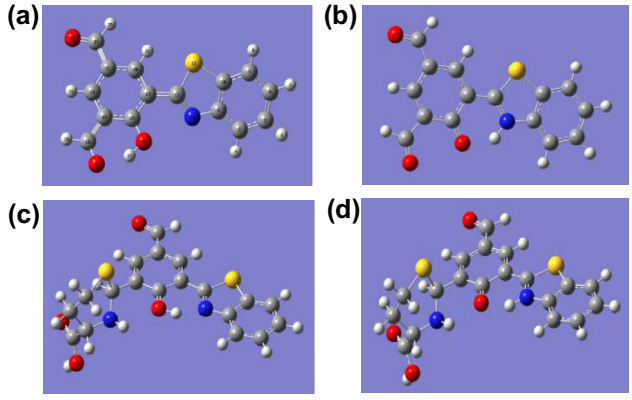


Figure 6. Energy minimized structure of (a) BHI in enol form, (b) BHI in keto form, (c) BHI-CN in enol form, and (d) BHI-CN in keto form from DFT calculation.

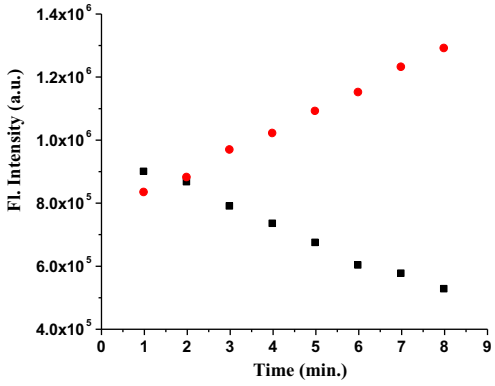


Figure 7. Time-dependent change of fluorescence intensity of BHI (20 μM) at 436 nm (in red) and 521 nm (in black) in the presence of Cysteine (1.0 equiv at a time) at pH 7.4 in H_2O.

intramolecular proton transferred (ESIPT) tautomers (the keto forms) which show fluorescence more strongly at a longer wavelength (at 521 nm) compared to the phenol forms (at 436 nm). The enol isomer, which is lower in energy than the keto isomer in the electronic ground state, undergoes the proton transfer reaction upon excitation to the excited state in mixed aqueous media. But in absolute water the most intense fluorescence peak of BHI at 436 nm confirms the hampered ESIPT of HBT (Fig. 1). Probably in the presence of polar protic solvent, the proton of hydroxyl group is strongly bonded with the nearest carbonyl group (RAHB). Thus the particular proton of BHI whose transfer (ESIPT) is the main cause of longer wavelength fluorescence is engaged in other purposes (Scheme 2).

In this circumstance, when we add Hcy/Cys, the hampered ESIPT of BHI is regained rapidly through the RAHB. Due to strong H-bonding the carbonyl group becomes very electron deficient which is readily converted into thiazoline or thiazolidine in the presence of Hcy or Cys, respectively, (Scheme 3), as it is well known that the reaction of aldehydes with the N-terminal group of Cys forms thiazolidines.[16] Thus the proton of the phenol moiety is free enough to move to nitrogen of bezothiazole moiety which is an essential condition for ESIPT of BHI (Scheme 3).

To measure the fluorescence response of BHI for amino acids, fluorescence titration was carried out with a series of amino acids

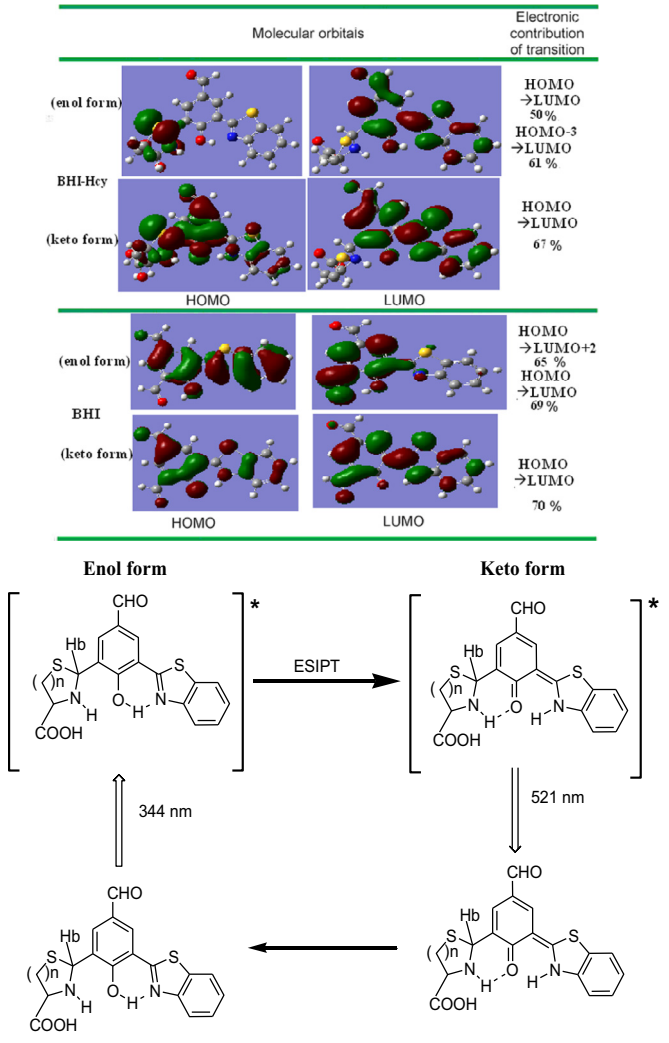


Figure 8. Molecular orbitals and electronic contributions of the relevant excitations for BHI and BHI-Hcy in water with the corresponding 4-level diagram.

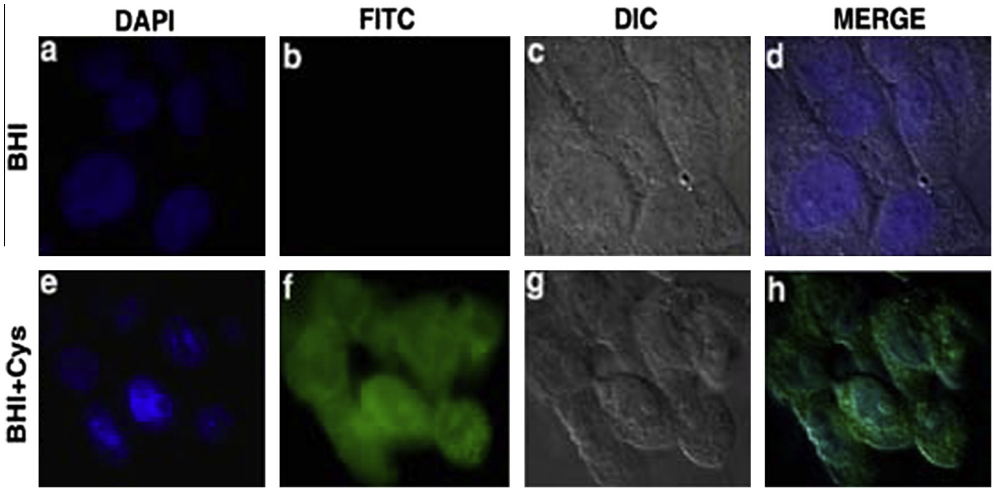


Figure 9. Fluorescence images of HeLa cells incubated with 50 μM of the receptor (BHI) in the absence (a and b) and in the presence (e and f) of 50 μM of cysteine. Corresponding differential interference contrast (DIC) images (c and g) and merge images (d and h) of the cells are shown.

for example Cys, Hcy, N-Boc-Cys, Met, Ser, Thr, Lys, Trp, Asp, Pro, Glu, Leu, Ile, Gly, Ala, His, Phe, Val, Arg, Tyr, and GSH under biological pH at room temperature.

Only with the addition of Hcy/Cys, the emission at 521 nm is increased with a 'naked eye' color change from blue to green (under UV-light) with a well-defined iso-emissive point at 488 nm in water (HEPES buffer, pH 7.4) and gives a better ratiometric response over others (Fig. 4). Glutathione, a thiol containing amino acid also reacts with BHI, but the lower response than Cys/Hcy makes BHI a specific probe for these amino acids. Probably formation of the more stable product (with cyclic structure) is the main reason for this type of preferable reaction over others. Fluorescence changes were monitored by using a 20 μM solution of BHI in water sharply, followed by the ratiometric decrease in the emission at 436 nm, which indicated that the chemical reaction between the following thiol containing amino acids and the aldehyde group interrupted the RAHB and the original ESIPT of BHI is regenerated strongly (Figs. 2 and 3).

The linear ratiometric response (I_{521}/I_{436}) of BHI with increasing concentration of Cys also makes it a unique probe for it (Fig. 2). The fluorescence intensity of BHI is also changed linearly with increased concentration of Hcy between 0.2 μM and 1.6 μM at two different wavelengths (Fig. 3). From the plot, it is clear that 0.7 μM of Hcy is enough for ratiometric response of BHI at physiological condition.

To confirm that the thiazolidine ring was formed by the reaction of Cys with the aldehyde group of BHI, we investigated the ^{1}H NMR spectrum of BHI upon addition of Cys and compared it with that of the probe itself. Complete conversion of BHI to its Cys adduct was accomplished by the addition of Cysto BHI in DMSO-d_6 (Fig. 5). As the aldehyde proton (H^a) at around δ 13 ppm disappeared, the thiazolidine methine proton (H^b) at around δ 5.41 ppm newly appeared and the aromatic protons are up field shifted. Under the same conditions, Hcy also reacted similarly with BHI and formed the six-membered ring of thiazinane. Interestingly, the aldehyde proton *para* to the hydroxyl group remains almost unchanged in the NMR spectrum. The mass spectrum (ESI MS) of the Cys-adduct shows peaks at *m*/*z* 387.04 possibly due to BHI+Cys+H^+ ions, which also proves a single mononuclear addition of cysteine to BHI, *m*/*z* 284.03 (BHI+H^+) (Supplementary data).

DFT and TD-DFT calculations in water as a solvent at the B3LYP/6-311+G(d,p) (PCM) level also support the rapid nucleophilic attack of Hcy to BHI. The electronic transition with main contribution of BHI and BHI-Hcy is shown in Figure 8. Both the forms of BHI-Hcy are much more stable than the corresponding form of BHI (about 1.79 $\times$ 10^6 kJ/mol) which also suggests the rapid response of Hcy to BHI (Figs. 6 and 7).

In order to determine the membrane permeability of BHI and its ability to specifically bind to the Cys in living cells, HeLa cells were first incubated with the cysteine molecules followed by the addition of the receptor. A control incubation of the cells with BHI only was also carried out. As shown in Figure 9, the cells showed intense green fluorescence in FITC channel when they were treated with Cys molecule followed by BHI. Nuclei of the cells stained in DAPI showed deep blue color. No fluorescence was observed when the cells were not treated with the cysteine molecule. The result clearly established that the receptor (BHI) could permeate the plasma membrane of the cells and give specific fluorescence only in the presence of Cys molecule.

In conclusion, we have successfully shown an ESIPT containing receptor (BHI) which lost its ESIPT characteristics (due to RAHB) in water and is recovered (through RAHB) with the addition of cysteine/homocysteine over other amino acids in absolute aqueous solution with live cell-imaging. BHI exhibits a unique rapid ratiometric fluorescence change in the presence of Hcy/Cys demonstrating its excellent selectivity over other amino acids.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.11.055.

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