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

Dan Zhang, Zihao Yang, Hongjuan Li, Zhichao Pei, Shiguo Sun and Yongqian Xu*

a College of Science, Northwest A&F University, Yangling, Shaanxi, P.R. China, 712100, xuyq@nwsuaf.edu.cn

Materials. All chemicals and reagents were used directly as obtained commercially unless otherwise stated. All solvents were of reagent grade. Water used was ultra filter deionized.

Measurements

Absorption and emission spectra were collected by using a Shimadzu 1750 UV-visible spectrometer and a RF-5301 fluorescence spectrometer (Japan), respectively. NMR spectra were collected on a Bruker 500 avance III spectrometer. Chemical shifts (δ) were reported as ppm with TMS as the internal standard. Mass spectrometric (MS) data were obtained with HP1100LC/MSD MS and an LC/Q-TOF-MS instruments.

Sample Preparation and Titration. Stock solutions of amino acids and sodium sulfide were prepared in deionized water. The concentration are fixed to 1.0×10^{-2} M. Stock solution of fluorescent sensors (5.0×10^{-4} M) were prepared in CH_{3}CN and then further diluted to 5.0×10^{-6} M for titration experiments. Every time an appropriate volume of each analyte was added to the test solution. UV and fluorescence spectra were monitored within 15 seconds.

Calculation of Quantum Yield. The quantum yield of the sample was measured using quinine sulfate as the standard (Φ = 0.53, 0.1 M H_{2}SO_{4})^{51} and calculated using eq 1:
\[ \Phi_{\text{unk}} = \Phi_{\text{std}} \times \left( \frac{I_{\text{unk}}}{I_{\text{std}}} \right) \times \left( \frac{A_{\text{std}}}{A_{\text{unk}}} \right) \times \left( \frac{n_{\text{unk}}}{n_{\text{std}}} \right)^2 \]  

(1)

Where \( \Phi_{\text{unk}} \) is the fluorescence quantum yield of the sample, \( \Phi_{\text{std}} \) is the fluorescence quantum yield of the standard, \( I_{\text{unk}} \) and \( I_{\text{std}} \) are the integrated emission intensities of the sample and the standard, respectively, \( A_{\text{unk}} \) and \( A_{\text{std}} \) are the absorbance of the sample and the standard at the excitation wavelength, respectively, and \( n_{\text{unk}} \) and \( n_{\text{std}} \) are the refractive indexes of the corresponding solution.

**Preparation of Human Blood Samples.** The procedure for preparation of human blood samples is followed the reported literature.\textsuperscript{S2} Human blood samples were collected from healthy volunteers treated in the local Medical Hospital. All samples were obtained by venipuncture and collected in heparinized vacutainer tubes. Then, a 200 \( \mu \text{L} \) aliquot of the blood was deproteinized by mixing immediately with 400 \( \mu \text{L} \) of cold 10\% Cl\textsubscript{3}CCOOH. After vortex mixing, the mixture was centrifuged at 8000 rpm for 10 min. A total of 400 \( \mu \text{L} \) of the supernatant was collected. The obtained supernatant was ready for assays.

**Cell Culture and Fluorescence Image**

HeLa cells were seeded on 35 mm glass-bottomed dishes (NEST) and incubated in RPMI-1640 in an incubator (37 °C, 5\% CO\textsubscript{2} and 20\% O\textsubscript{2}) for 24 h. The cells were rinsed slightly 3 times with fresh RPMI-1640 and incubated in RPMI-1640 medium spiked with or without sensor (20 \( \mu \text{M} \)) for 30 min, respectively. After washing with fresh RPMI-1640, the cells treated with sensor were further incubated in fresh RPMI-1640 containing 100 \( \mu \text{M} \) GSH or Cys for 0.5 h. Cells were then analyzed by Laser Scanning Confocal Microscope (A1R).

The equimolar amount of 2-aminophenol (436.5 mg, 4 mmol) with 5-aminosalicylic acid (612.6 mg, 4 mmol) was stirred in 15 mL polyphosphoric acid (PPA) at 200 °C for 4 h. Then the cooled mixture was poured into 400 mL cold water and the yellow precipitate separated out. After filtered, the yellow precipitate was washed by 10% Na$_2$CO$_3$. The solid was dissolved in CH$_2$Cl$_2$ and dried by Na$_2$SO$_4$, and the solvent was evaporated under reduced pressure. The obtained crude product was separated by silica gel column chromatography to get compound 1 (yellow solid, 682 mg, 75%).

The pure compound 1 (200 mg, 0.88 mmol) was dissolved in 5 mL DMF, the solution was stirred in ice-salt-bath under the protection of nitrogen. Then 1.5 mL HCl was added into the above solution drop-by-drop. NaNO$_2$ (182.9 mg, 2.65 mmol) dissolved in 5 mL distilled water was added drop-by-drop. The resulting solution was stirred for 40 min, then NaN$_3$ (172.2 mg, 2.65 mmol) dissolved in 5 mL distilled water was added. The mixture solution was stirred at room temperature overnight. The precipitate was filtered, washed by distilled water. The solid was dissolved in DCM and dried by Na$_2$SO$_4$. The solvent was evaporated under reduced pressure. The obtained crude product was separated by silica gel column chromatography to get probe AHBO (light yellow solid, 160 mg, 72%).

The probe AHBO (150 mg, 0.59 mmol) was added into 5 mL acetic anhydride. After the solution was refluxed overnight, the reaction mixture was poured into 50 mL cold water. The pH was changed from 2 to 7 and the white precipitate separated out. After filtered, the precipitate was dissolved by CH$_2$Cl$_2$ and dried by Na$_2$SO$_4$. The
solvent was evaporated under reduced pressure. The obtained crude product was separated by silica gel column chromatography to get probe ABO (white solid, 83 mg, 48%).

**Fig. S1.** The absorption spectra change of AHBO (10 μM) upon addition of GSH in CH₃CN/HEPES Buffer (1:1, v/v, pH 7.4).

**Fig. S2.** Jobs plot for a 1:1 stoichiometry between AHBO and GSH in CH₃CN/HEPES Buffer (1:1, v/v, pH 7.4), λₑₓ=325 nm.
**Fig. S3.** Jobs plot for a 1:1 stoichiometry between AHBO and Cys in CH$_3$CN/HEPES Buffer (1:1, v/v, pH 7.4), $\lambda_{ex}$=325 nm.

**Scheme S1** Electronic transitions in the photoluminescent process of 2-(2’-hydroxyphenyl)benzoxazole. The excited molecules in the enol form (S1 state) are transformed to its tautomeric structure (keto form) through “excited state intramolecular proton transfer” (ESIPT).
**Fig. S4.** The fluorescence spectra change of ABO (10 μM) upon addition of 2 equivalents of GSH in CH$_3$CN/HEPES Buffer (1:1, v/v, pH 7.4), $\lambda_{\text{ex}}$=325 nm.

**Fig. S5.** The effect of pH on the fluorescence intensity changes of AHBO (10 μM) at 488 nm in the absence and presence GSH (2 equiv.) in CH$_3$CN/HEPES Buffer (1:1, v/v, pH 7.4), $\lambda_{\text{ex}}$=325 nm. All the data were obtained after 5 min with addition of GSH.
**Fig. S6.** Absorption spectra change of AHBO (10 μM) upon addition of various amino acids (20 μM). Each spectrum was acquired after 5 min incubation with an analyte in CH$_3$CN/HEPES Buffer (1:1, v/v, pH 7.4), $\lambda_{ex}$=325 nm.

**Fig. S7.** The relative fluorescence intensity of AHBO (10 μM) at 488 nm upon addition of various analysts (20 μM) in CH$_3$CN/HEPES Buffer (1:1, v/v, pH 7.4), $\lambda_{ex}$=325 nm.
Fig. S8. (a) and (b) Time dependent absorption intensity change at 342 nm in the presence of 2 and 10 equivalents of GSH, Cys, Hcy and sulfide. (c) Time dependent fluorescence intensity (488 nm) of AHBO (10 µM) in the presence of 10 equivalents of GSH, Cys, Hcy and sulfide. All the solution is in CH$_3$CN/HEPES Buffer (1:1, v/v, pH 7.4).

Time dependent absorption and fluorescence spectra suggested that AHBO can react with GSH or Cys to produce the corresponding products within 10 min no matter 2 or 10 equivalents of them were added. Interestingly, we found that no absorption spectra change of AHBO in the presence of sulfide was observed within 10 min. Although AHBO can react with sulfide to produce the amine-substituted compound I, the reaction rate is slow.
**Fig. S9.** Fluorescence spectra change for AHBO (10 µM) with different concentration of GSH in diluted deproteinized FBS, $\lambda_{ex}=325$ nm. Inset: The fluorescence intensity change of AHBO (10 µM) at 488 nm upon addition of GSH in diluted deproteinized FBS, indicative of good linear relationship. The image shows the photo of samples before and after addition of GSH in diluted deproteinized FBS, which is excited by hand-held UV lamp (365 nm).

**Fig. S10.** The MS spectrum of AHBO treated with GSH.
Fig. S11. The MS spectrum of AHBO treated with Cys.

Fig. S12. The MS spectrum of AHBO treated with Hcy.
Fig. S13. $^1$H NMR titration of AHBO in DMSO-$d_6$ and D$_2$O (9:1, v/v) upon addition of GSH.

Fig. S14. HPLC spectra of solution of AHBO and its reaction solutions after addition of GSH and Cys. All the solutions were pre-extracted with methanol before HPLC detection. The peaks with retention time of 4.9, 14.3 and 25.7 min are assigned to the corresponding fluorescent product, 1 and AHBO, respectively.
Fig. S15. LC-MS spectra of reaction solution of AHBO after addition of Cys. The solution was pre-extracted with methanol before LC-MS assay. a) LC spectrum of the solution; b) the MS spectrum of peak with retention time of 5.76 min, which is assigned to the corresponding fluorescent product; c) the MS spectrum of peak with retention time of 7.86 min, which is assigned to product 1.
**Fig. S16.** LC-MS spectra of reaction solution of AHBO after addition of GSH. The solution was pre-extracted with methanol before LC-MS assay. a) LC spectrum of the solution; b) the MS spectrum of peak with retention time of 5.41 min, which is assigned to the corresponding fluorescent product.

**Fig. S17.** The MS spectrum of AHBO treated with Hcy. The intermediate product 4 is unstable and further reacts with Hcy to form Hcys-sHcy.
Fig. S18. Fluorescence images of HeLa cells. (a) Bright-field images of the HeLa cells; (b) images taken in blue field; (c) is the overlap of brightfield and fluorescence. Images were acquired by using excitation and emission windows of $\lambda_{\text{ex}} = 405 \text{ nm}$ and $\lambda_{\text{em}} = 420-475 \text{ nm}$, respectively. Scare bar: 20 $\mu$m.

Fig. S19. Confocal fluorescence images of Hela cells. Each panel (a-d) shows a confocal micrograph obtained at a different fluorescence channel of bright-field images of HeLa cells (a), AHBO (b) and Rhodamine 123 (c), and overlay of the two fluorescence images (d). Image of b was acquired by using excitation and emission windows of $\lambda_{\text{ex}} = 405 \text{ nm}$ and $\lambda_{\text{em}} = 420-475 \text{ nm}$, respectively. Image of c was acquired by using excitation and emission windows of $\lambda_{\text{ex}} = 488 \text{ nm}$ and $\lambda_{\text{em}} = 500-525 \text{ nm}$, respectively. Scare bar: 20 $\mu$m.

The co-staining of AHBO and Rhodamine 123 was performed. The fluorescence signals of AHBO and Rhodamine 123 are highly overlapped in the mitochondria regions.
Fig. S20. $^1$H NMR spectra of AHBO.

Fig. S21. $^{13}$C NMR spectra of AHBO.
Fig. S22. MS spectra of AHBO.

Fig. S23. $^1$H NMR spectra of ABO.
Fig. S24. $^{13}$C NMR spectra of ABO.