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Supporting Information

Visualization diagnosis in acute cerebral ischemia via sulfane sulfur activated photoacoustic imaging

Cheng Song, Lejuan Liao, Yidong Bin, Zongyi He, Jing Hua, Shulin Zhao,* Hong Liang*

State Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, Collaborative Innovation Center for Guangxi Ethnic Medicine, School of Chemistry and Pharmaceutical Sciences, Guangxi Normal University, Guilin 541004, China

EXPERIMENTAL SECTION

Materials and reagents. Potassium carbonate, dithiosalicylic acid, and iodoethane were purchased from Sigma-Aldrich. Phosphorus oxychloride, methyl sulfoxide, *n*-butanol, and cyclohexane were supplied by Xilong Chemical Co., Ltd. Cyclohexanone, acetonitrile, dithiosalicylic acid and 1-(3-Dimethylaminopropyl)- 3-ethylcarbodiimide hydrochloride were provided by Aladdin Reagent Co., Ltd. 1,1,2-Trimethylbenz[e]indole was purchased from Arlingwei Ultra Fine Materials Ltd. Resorcinol and dichloromethane were supplied by Sahn Chemical Technology Co., Ltd.. N,N-Dimethylformamide (DMF) was purchased from Anhui Zesheng Technology Co., Ltd. The water used in all experiments was processed using a Millipore filter and had a resistance of at least 18.2 MΩ. All chemicals were used as received, without further purification.

Equipment. UV-Vis absorption spectroscopy was performed on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, United States). A nuclear magnetic resonance spectrometer (Bruker DRX-500) and an electrospray ionization mass spectrometer (ESI-MS) were supplied by Bruker Daltonics Inc. The Kodak in vivo FX Pro imaging system (Bruker, Germany) was used for *in vivo* imaging. A Cary Eclipse fluorescence spectrometer was purchased from Agilent Technologies Inc. A multispectral photoacoustic tomography imaging system (inVision 256-TF) was acquired from iThera Medical (Germany).

Synthesis and characterization of the HCy-SH probe. *Synthesis of Compound 1*: A combination of 20 mL dichloromethane and 20 mL DMF was added to a round bottom flask, which was placed in an ice bath and stirred for 15 min. Subsequently, 18.5 mL of phosphorus trichloride was dissolved in 17.5 mL dichloromethane. The solution was placed in the funnel and then added to the round bottom flask. After the dropwise addition was completed, the ice bath was removed, and 5 g cyclohexanone was added to the mixed solution. The reaction solution was gradually heated to 40 C and refluxed for 4 h. A 500 mL ice–water mixture was added to the solution after the latter was cooled to room temperature. Subsequently, the solution was aged overnight,

and the product was filtered without purification to obtain an orange solid compound referred to as Compound 1(4.30 g, the yield was 50%).

Synthesis of Compound 2: 1,1,2-Trimethyl-1H-benz[e]indole (6.26 g, 30 mmol) and iodoethane (4.80 g, 30 mmol) were placed into a 50 mL round bottom flask, and dissolved fully by using 20 mL anhydrous acetonitrile. The mixed solution was stirred and reacted at 80 °C for 8 h until gray–white solid precipitation was produced. After cooling to room temperature, the product was washed with petroleum ether and then filtered to obtain a dry gray–white solid compound, referred to as Compound 2 (6.36 g, the yield was 88%).

Synthesis of Compound 3: Compound 1 (0.86 g, 5.0 mmol), Compound 2 (2.38 g, 10.0 mmol), n-butanol (105 mL), and cyclohexane (45 mL) were added into a round bottom flask, and the mixture was reacted, with stirring at 117 °C for 4 h. The solvent was removed by steaming under reduced pressure, and the product was separated by wet column chromatography, with dichloromethane/methanol (100:1 to 25:1) as the eluting reagent. After distillation under reduced pressure, a dark green solid was obtained (2.08 g, the yield was 68%).

Synthesis of Compound 4: Under N_2 gas protection, resorcinol (332 mg, 3.0 mmol) was dissolved in anhydrous acetonitrile (30 mL). Anhydrous potassium carbonate (413 mg, 3.0 mmol) was added and stirred at 35 °C for 30 min. An anhydrous solution of acetonitrile (5 mL) containing Compound 3 (0.917 g, 1.5 mmol) was added and heated up, with stirring for 4 h at 50 °C after the reaction, the solvent was cooled to room temperature. The solvent was subsequently removed under reduced pressure. Column chromatography using dichloromethane/methanol (100:1 to 20:1, v/v) was used as the eluting reagent to obtain a blue solid compound referred to as Compound 4 (1.08 g, the yield was 80%).

Synthesis of Compound 5 (HCy-SH): Compound 4 (100 mg, 0.2 mmol) was placed in a 25 mL round bottom flask and then dissolved in 10 mL anhydrous dichloromethane. After the compound was completely dissolved, dithiosalicylic acid (90 mg, 0.3 mmol) was added to the reaction system. 4-Dimethylaminopyridine and

dicyclohexylcarbodiimide in appropriate amounts were added, with stirring, and then reacted at 25 °C for 12 h. After the aforementioned reaction, appropriate amounts of dithiothreitol and absolute ethanol were added to the reaction system, and the reaction was continued for 2 h. Subsequently, the solvent was removed by distillation under reduced pressure. Column chromatography was conducted using methanol/dichloromethane (10:1, V: V) as the eluting reagent to obtain a blue–purple solid (37 mg, the yield was 32%). LC-MS (ESI, positive mode): m/z calcd 585. 65, found 585. 23 for $[M]^+$ (Figure S1). ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.25 (d, J = 2.9 Hz, 1H), 8.08 (d, J = 15.2Hz, 1H), 7.97 (dd, J = 9.6 Hz, 6.8 Hz, 1H), 7.94 (d, J = 8.8 Hz,1H), 7.65 (d, J = 9.2 Hz, 1H), 7.62 (d, J = 8.4 Hz, 1H), 7.55 (d, J = 9.2 Hz, 1H), 7.52 (t, J = 7.2 Hz, 1H),7.51-7.70 (m, 2H), 7.21 (dd, J = 8.4 Hz, 6.4 Hz, 1H), 4.00 (d, J = 15.2 Hz, 1H), 4.07 (s, 6H), 2.81-2.83 (m, 2H), 1.47-1.57 (m, 4H), 2.50-2.51 (m, 2H), 2.00 (s, 6H),1.86-1.88 (m, 2H), 1.46 (t, J = 6.8 Hz, 3H) (Figure S2). ^{13}C NMR (151 MHz, DMSO-d_6): δ 180.010, 158.591, 155.705, 154.620, 153.851, 145.067, 142.460, 139.969, 138.969, 137.299, 133.078, 131.387, 130.557, 130.432, 130.332, 130.250, 129.915, 128.622, 127.435, 126.909, 122.988, 122.504, 120.311, 117.338, 114.804, 113.080, 108.917, 106.383, 55.376, 52.940, 29.185, 27.345, 24.007, 20.320, 13.648 (Figure S3).

Absorption/fluorescence spectroscopy and *in vitro* PA imaging. HCy-SH was dissolved in DMSO to obtain an HCy-SH reserve solution with a concentration of 1 mM. This solution was stored in the dark at 4 °C. An appropriate amount of the HCy-SH reserve solution was diluted with DMSO to prepare 10 μM of the test solution. Na_2S_4 was dissolved in HEPES buffer (DMSO: HEPES=1:1, V/V, pH=7.4).

The Na_2S_4 solution was added to the HCy-SH solution. The absorption and fluorescence spectra of the solution were then measured after the solution was incubated for 30 min in a 37 °C water bath. For PA imaging, the test sample was transferred to a transparent suction pipe 3 mm in diameter, which was embedded in an agar phantom, attached to a bracket, and immersed in water at 25 °C.

In vitro toxicity assessment of HCy-SH. The cytotoxicity of HCy-SH was evaluated using MTT assays in SH-SY5Y cells. The cells were seeded onto 96-well cell culture plates and incubated for 24 h. Cells representing the control group were left untreated, and HCy-SH was added to the experimental wells at concentrations of 0, 5.0, 10.0, 20.0, 30.0, and 40.0 μM. MTT (20 μL, 5 mg/mL) was subsequently added to each well. After the cells were incubated for 4 h, the medium was removed, and 150 μL DMSO was added to each well. The absorbance of each well was then measured at 570 nm by using a Multiskan Mk3 microplate reader.

In vivo toxicity assessment of HCy-SH. For acute toxicity assessment, both groups of mice were injected with saline (control group) or the HCy-SH probe solution (2 mg/kg). After 24 h, the mice were euthanized, and major organs (heart, liver, spleen, lung, and kidney) were extracted. Tissue sections measuring 3 μm in thickness were fixed in a paraformaldehyde solution and then embedded in paraffin. The sections were successively H&E-stained and examined under the microscope, and images were captured.

For systemic toxicity assessment, both groups of mice were injected with saline (control) and HCy-SH (1 mM, 2 mL/kg) via the tail vein. The body weight was measured daily for 7 d and observed for clinically relevant abnormal responses. Experiments on the mice were stopped once they were unable to eat, suffered injuries, and died.

Experimental animals. The female Kunming mice (15–20 g) aged 6–8 weeks were purchased from Hunan Slake Jingda Experimental Animal Co., Ltd. All animals were given *ad libitum* access to food and water and were housed in an environment at room temperature under sterile conditions. All animal experiments were approved by the Animal Ethics Committee of Guangxi Normal University (No. GXNU-2023-11-003).

Establishment of a mouse model of acute cerebral ischemia: The mice were first anesthetized, with their heads secured and their necks exposed. The carotid artery was accessed by dissecting the skin and superficial tissue. It was then tied with a surgical

cord to block it. After a specific ischemic period, the binding of the carotid artery could be released. The subsequent physiological, histological, and behavior were assessed to ensure the success of the cerebral ischemia model in mice.

PA imaging of the mouse brain for the detection of acute cerebral ischemia. Kunming mice were divided into two groups: the ischemic group and the control group. The ischemic group of mice established the mouse cerebral ischemia model. The control mice underwent no treatment. The mice in the ischemic group were anesthetized, and HCy-SH was injected into their tail veins. The mice were daubed with ultrasound gel and coated with a transparent film, placed in 36.5 °C water, perpendicular to the scanning plane. PA imaging of the brain area was conducted at 0, 5, 10, 15, 20, 25, and 30 min after carotid artery ligation.

Data analysis. All PA data were reconstructed using a standard back-projection algorithm. The data were subsequently analyzed using ordinary one-way ANOVA, and the results are presented as mean ± standard deviation.

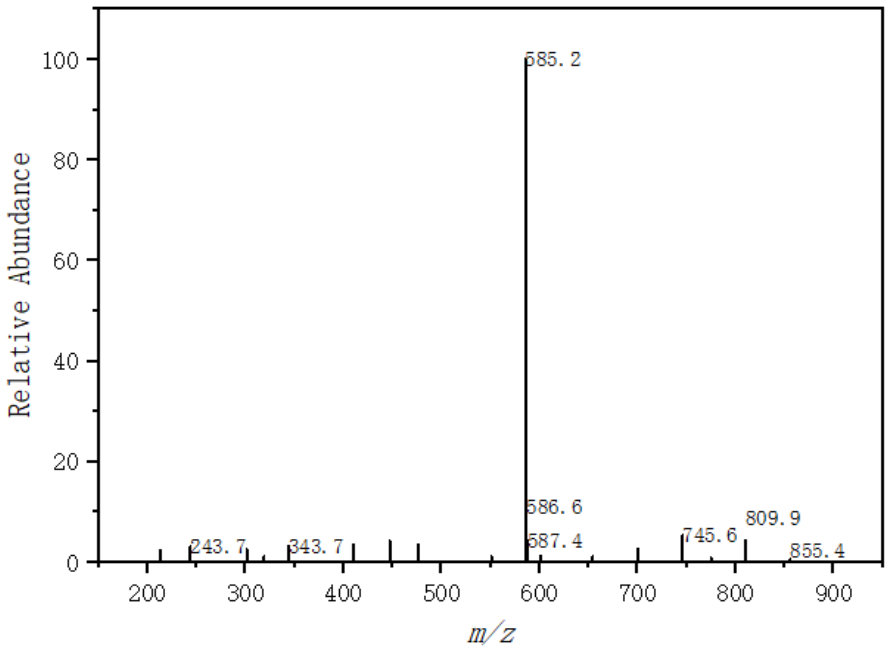

Fig. S1 HRMS spectrum of compound 5 (HCy-SH).

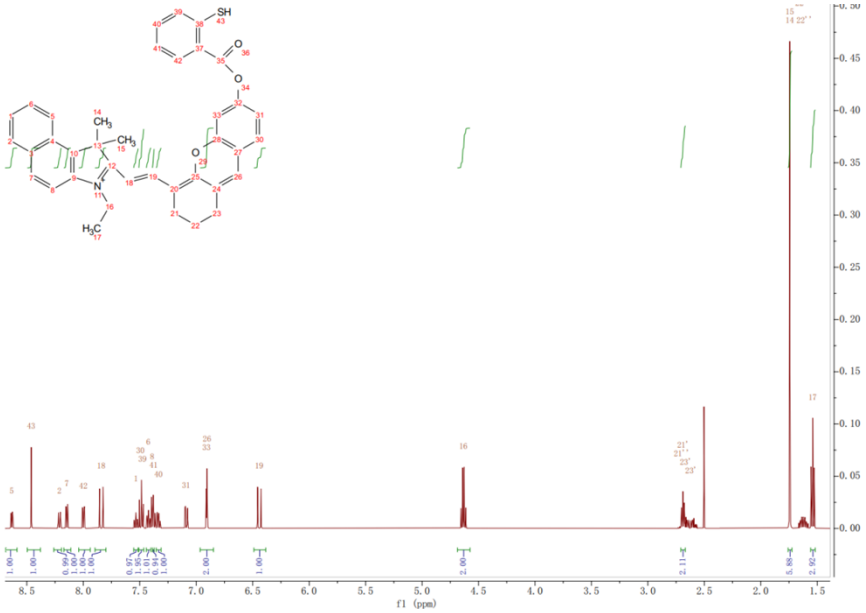

Fig. S2 ^{1}H NMR spectrum of HCy-SH in DMSO-d6, 298K, 600 MHz.

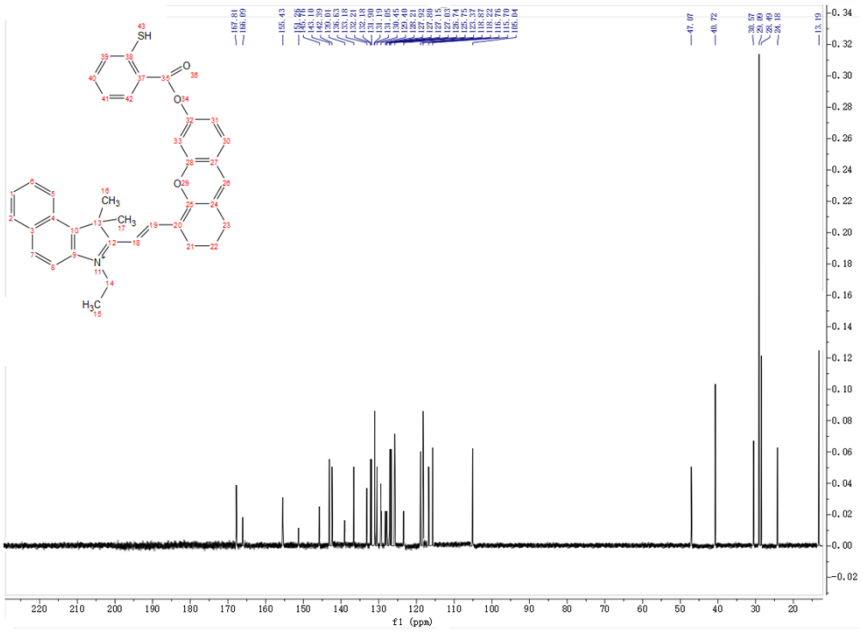

Fig. S3 ^{13}C NMR spectrum of HCy-SH in DMSO-d6, 298K, 151 MHz.

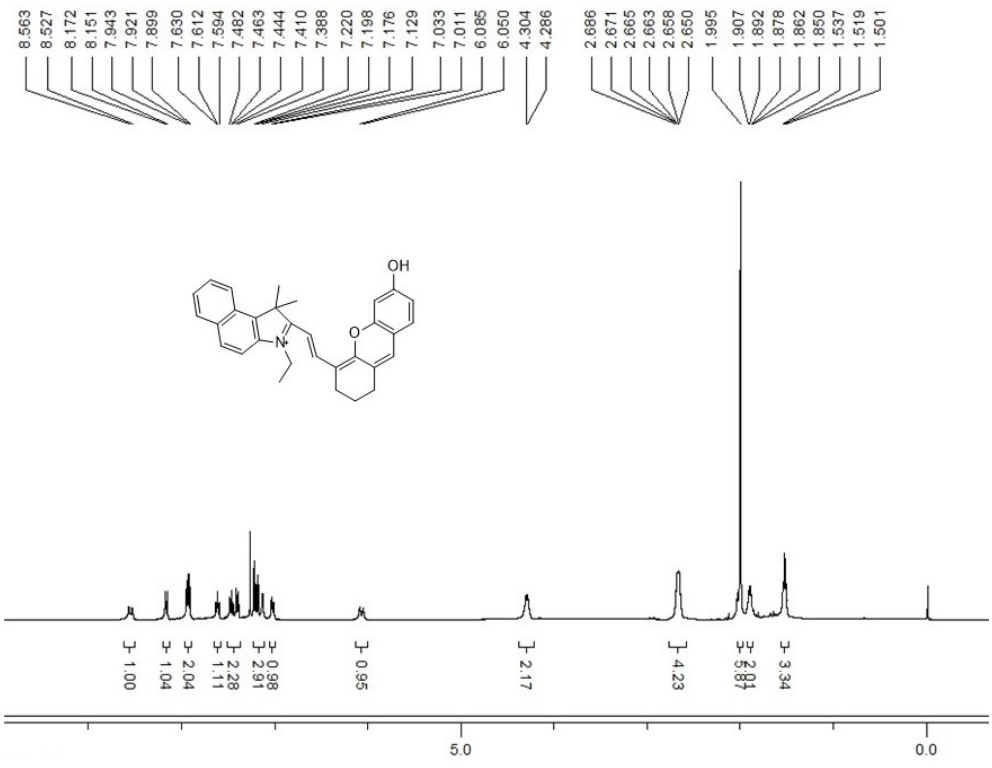

Fig. S4 ^{1}H NMR spectrum of HCy-OH (compound 4) in $CDCl_3$.

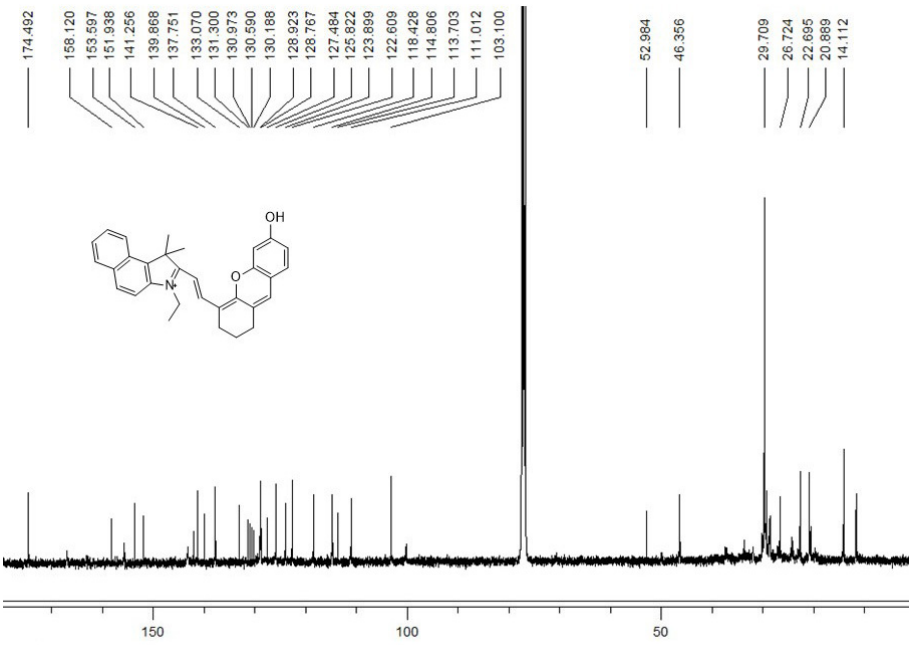

Fig. S5 ^{13}C NMR spectrum of HCy-OH in $CDCl_3$.

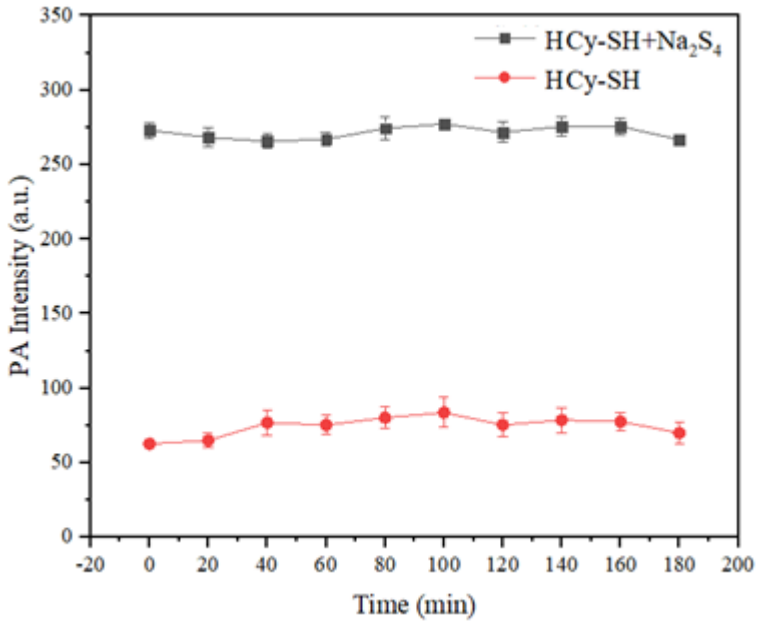

Fig. S6 Effect of the reaction time on the PA intensity of product for HCy-SH reaction with Na_2S_4 in plasma.

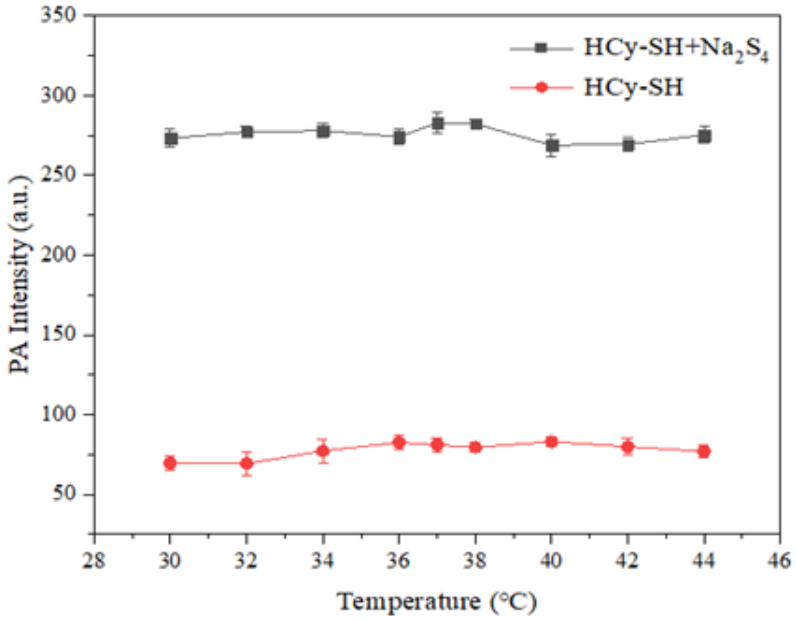

Fig. S7 PA intensity of HCy-SH and its reaction product with Na_2S_4 at different temperatures.

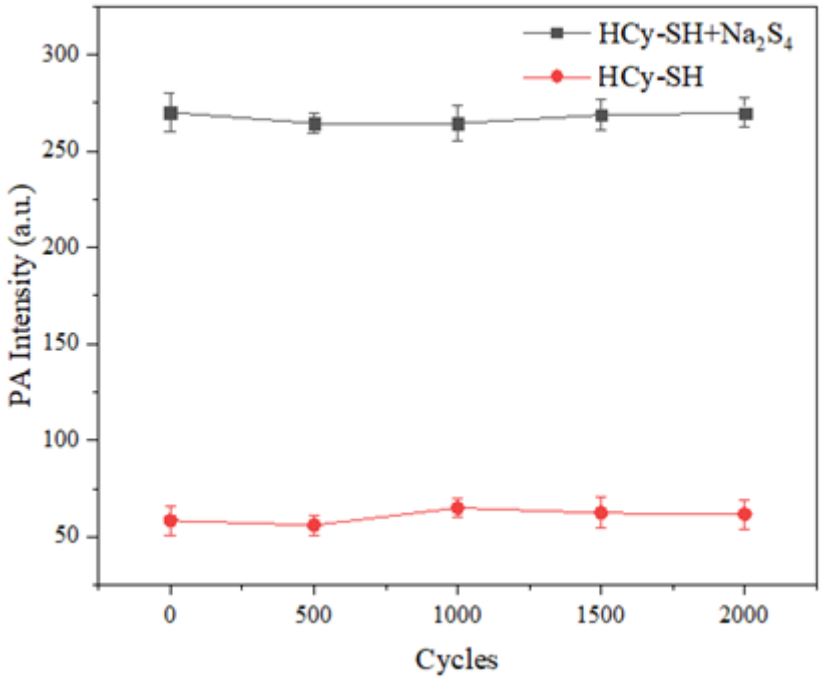

Fig. S8 Photostability of HCy-SH and its reaction product with Na_2S_4 (2000 consecutive irradiation by 720 nm laser).