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In situ Photoacoustic Imaging of Cysteine to Reveal the Mechanism of Limited GSH Synthesis in Pulmonary Fibrosis

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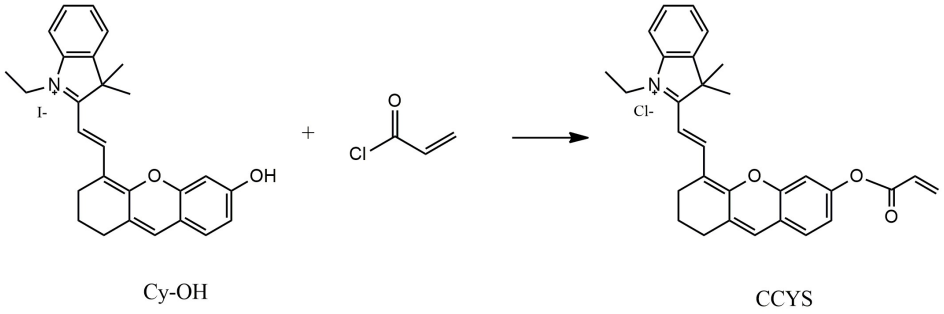
Experimental Procedures

Materials and Instruments.

All reagents used were commercial purchased. Bleomycin were purchased from HISUN (Zhejiang, China). Lipopolysaccharide (LPS) and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sangon Biotech. Lyso Tracker and Mito Tracker were purchased from Beyotime. *N*-ethylmaleimide (NEM) were purchased from Macklin. Cysteine Kit (A126) and γ-glutamylcysteine ligase test kit (A091) were purchased from Nanjing Jiancheng Bioengineering Institute.
^{1}H NMR and ^{13}C NMR spectra were detected on Bruker NMR spectrometers at 400 and 100 MHz. The high-resolution mass spectra (HRMS) were obtained using a BrukerMaxis ultrahigh-resolution TOF MS system. An FLS-920 Edinburgh fluorescence spectrometer was used to measure the fluorescence spectra. Intracellular fluorescence imaging was performed using a Zeiss LSM 880 NLO confocal laser scanning microscope. *In vivo* fluorescence imaging of mice was carried out on an *in vivo* live Imaging system, the Perkinelmer IVIS Spectrum. *In vivo* photoacoustic imaging of mice was carried out on a photoacoustic tomography system, the Endra Nexus 128.

Synthesis of CCYS.



Cy-OH CCYS

Scheme S1. Synthesis of CCYS.

The CCYS probe is composed of Cy-OH and an acrylate-functional group and is synthesized with a yield of 48%. Cy-OH was synthesized according to a reported synthetic route.[1] To a solution of Cy-OH (0.62 mmol) and triethylamine (1.24 mmol) in THF (5 mL) at -30 °C, acryloyl chloride (0.62 mmol) was added dropwise over 20 min. Then, the mixture was stirred at room temperature (r.t.) for 6 h. The solution was removed under reduced pressure, and the residue was purified by column chromatography with CH_2Cl_2/CH_3OH (v/v, 10:1) as the eluent. The probe CCYS was obtained as a blue solid (135 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.65 (d, J=15.1, 1H), 7.52 (t, J=6.0, 1H), 7.50 (s, 1H), 7.48 (d, J=8.0, 2H), 7.44 (s, 1H), 7.16 (d, J=14.0, 2H), 7.07 (d, J=8.4, 1H), 6.71 (q, J=16.2, 2H), 6.37 (d, J=17.3, 1H), 6.14 (d, J=10.5, 1H), 4.69 (q, J=7.1, 2H), 2.87 (t, J=5.8, 2H), 2.76 (t, J=6.0, 2H), 1.96 (m, 2H), 1.81 (s, 6H), 1.56 (t, J=7.2, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 177.8, 164.0, 160.1, 153.0, 152.5, 146.4, 142.2, 140.9, 134.0, 131.2, 130.3, 129.5, 128.3, 128.0, 127.3, 122.6, 119.8, 119.0, 115.6, 113.2, 109.4, 106.1, 51.0, 42.4, 28.1, 24.9, 22.7, 20.1, 14.1, 13.3. HRMS: m/z $[M]^+$ calculated for $C_{30}H_{30}NO_3^+$:452.2226; measured:452.2193.

Measurement of relative fluorescence quantum yield[2]

Fluorescence quantum yield was determined by using ICG (Φ_f =0.13 in DMSO) for CCYS as a fluorescence standard. The quantum yield was calculated using the following equation:

$\Phi_x = \Phi_s (A_s F_x / A_x F_s) (n_x/n_s)^2$

Where Φ_x is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve and n is the refractive index of the solvents used. Subscripts S and X refer to the standard and to the unknown, respectively.

Calculation of the limit of detection.

The detection limit of CCYS as a fluorescent and photoacoustic probe was calculated with the equation $LOD=3S_0/K$.[3] S_0 is the standard deviation of the fluorescent intensity and the photoacoustic signal of CCYS. K is the slope of the linear regression equation, which was determined by the reaction between CCYS and cysteine (Cys).

Specificity of CCYS for Cys.

The fluorescence response of CCYS (20 μM, pH 7.4, 1% DMSO, v/v) to different analytes was measured after incubation at 37 °C for 7 min in PBS. The analytes include 1 mM glutathione (GSH), 100 μM homocysteine (Hcy); 100 μM Cys; 100 μM H_2O_2, .OH, ClO^-, t-BuOOH and $O_2^{\bullet-}$; 10 μM $ONOO^-$; 100 μM metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, K^+, Mg^{2+}, Na^+, Zn^{2+}); and 100 μM amino acids (Arginine, Aspartic, Glutamic, Glycine, Glutamine, Histidine, Isoleucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, Valine and Cystine). The fluorescent intensity was measured at 680 nm with 710 nm excitation. Each experiment was repeated at least three times.
The photoacoustic response of CCYS to 100 μM GSH, Hcy and Cys was also determined. After incubation of CCYS with GSH, Hcy and Cys at 37 °C in PBS, the photoacoustic signal was detected every 5 minutes at a wavelength of 700 nm by using an Endra Nexus128 PA tomography system. Each experiment was repeated at least three times.

Cell culture.

Human lung adenocarcinoma cells (A549), human hepatic cells (HL-7702) and human hepatoma cells (SMMC7721) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2/95% air incubator MCO-15AC (SANYO, Tokyo, Japan).

Cell viability.

The cytotoxicity of CCYS up to 20 μM to HL-7702 cells was determined by a methyl thiazolyl tetrazolium (MTT) assay. First, we seeded the HL-7702 cells (10^6 cell mL^{-1}) into replicate 96-well microtiter plates at a total volume of 100 μL $well^{-1}$. After 12 h of cultured, various concentrations of compound probe (0.1, 1, 5, 10 and 20 μM) made in DMEM were added to the cells and incubated for 24 h. The standard MTT operation was performed.[4] Each experiment was repeated at least three times.

Confocal fluorescent imaging of cells.

Colocalization experiments were performed using HL-7702 cells. CCYS (1 μM) and commercially purchased LysoTracker (2 μM) or MitoTracker (2 μM) were co-incubated with cells for 20 min before imaging.[5] Pearson's colocalization coefficient was automatically calculated by a Zeiss LSM 880 NLO confocal laser scanning microscope.

HL-7702 cells were treated with five kinds of conditions.[6] (a) Blank: HL-7702 cells, (b) NEM-cells: cells pretreated with NEM for 30 min, (c) NEM-Cys cells: NEM-cells with 100 μM Cys added, (d) NEM-GSH cells: NEM-cells with 100 μM GSH added, and (e) NEM-Hcy cells: NEM-cells with 100 μM Hcy added. Then, CCYS (1 μM) was added to the dishes 10 min before fluorescent imaging.

A549 cells were treated with three kinds of conditions. (a) Blank: A549 cells, (b) NEM-cells: cells pretreated with NEM for 30 min, and (c) NEM-Cys cells: NEM-cells with 100 μM Cys added. Then, CCYS (1 μM) was added into dishes for 10 min before fluorescent imaging.

Mice.

All female KM mice (18-20g) were purchased from the Animal Living Center of Shandong University. The animal experiments were performed in compliance with the relevant laws and guidelines issued by the Ethical Committee of Shandong University and were in agreement with the guidelines of the Institutional Animal Care and Use Committee.

Pulmonary fibrosis and pneumonia model mice.

The mice were randomly divided into a pulmonary fibrosis group and a control group. The mice in the pulmonary fibrosis group (PF-mice) were intravenously injected with 100 μL of bleomycin (37.5 mg mL^{-1} in normal saline), and the control mice were injected with saline (Blank mice). After 28 days, three PF mice and Blank mice were randomly selected to euthanize by excess chloral hydrate. The lung tissue of PF mice and Blank mice were removed for section staining to ensure that the PF mice suffered from pulmonary fibrosis.[7]

The mice were randomly divided into pneumonic and control groups. The mice in the pneumonic group (LPS-mice) were injected with LPS (167 μg $μL^{-1}$ in saline) through the intranasal route, and the control mice were injected with saline (saline mice). After 24 hours, mice were randomly selected to euthanize by excess chloral hydrate. The lung tissue of LPS-mice and saline mice was removed for section staining to ensure that the LPS-mice suffered from pneumonia.

***In vivo* PAI of Cys.**

The mice were intravenously injected with CCYS (50 μM, 100 μL) and anaesthetized with 10% chloral hydrate. They were placed in the Endra Nexus 128 PA tomography system, and the endogenous mouse signals were detected at a wavelength of 700 nm at various time points after injection of CCYS. Each experiment was repeated at least three times.

Results and Discussion

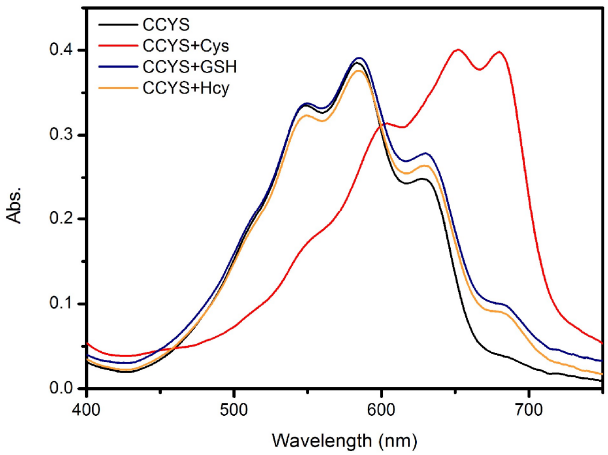


Figure S1. Absorption spectra of CCYS (20 μM) treated with Cys, GSH or Hcy (100 μM).

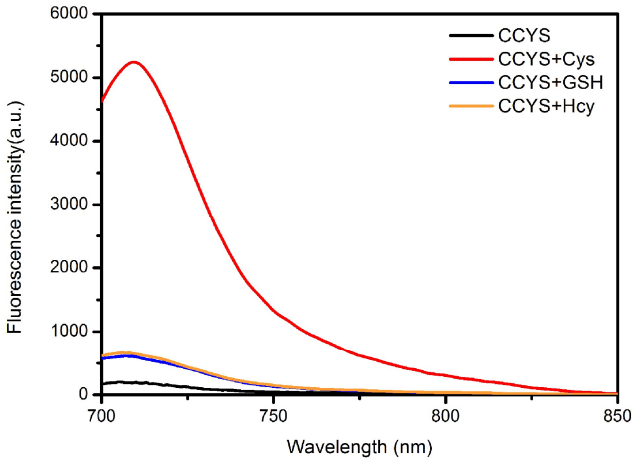


Figure S2. Fluorescence spectra of CCYS (20 μM) treated with Cys, GSH or Hcy (100 μM).

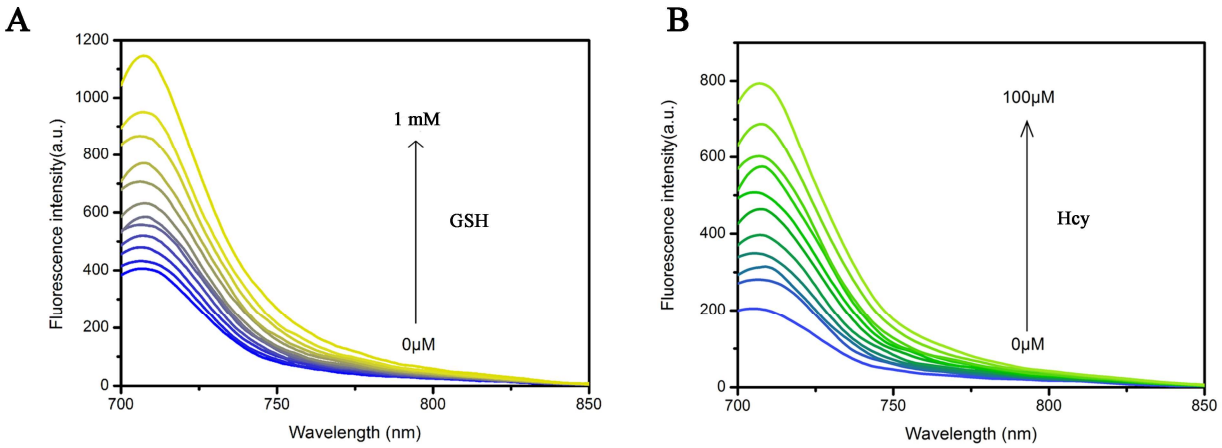


Figure S3. (A) Fluorescence spectra of CCYS (20 μM) treated with different concentration of GSH (0-1 mM). (B) Fluorescence spectra of CCYS (20 μM) treated with different concentrations of Hcy (0-100 μM).

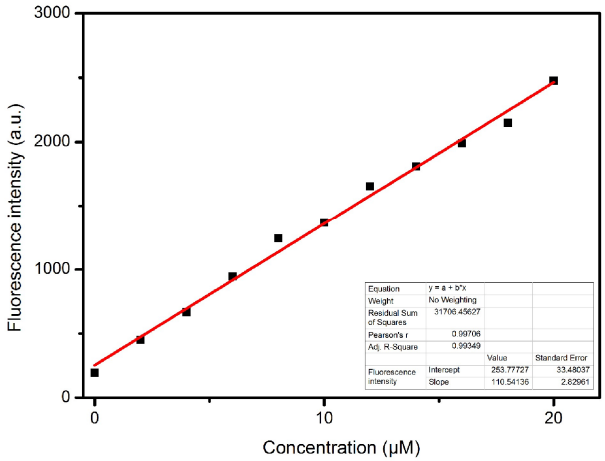


Figure S4. The linear relationship between the fluorescence intensity of CCYS (20 μM) and Cys concentration. CCYS showed a gradual fluorescent enhancement in response to increasing Cys concentrations with a correlation coefficient of 0.99.

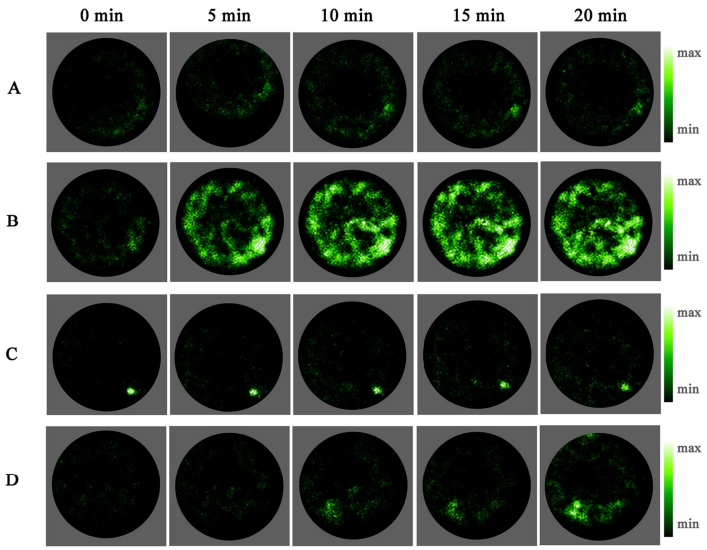


Figure S5. PA images of CCYS (20 μM) treated with (A) None, (B) Cys, (C) GSH and (D) Hcy (100 μM) for 0 min, 5 min, 10 min, 15 min and 20 min in PBS (1 ×, pH=7.4, 1% DMSO, v/v) at 37 °C.

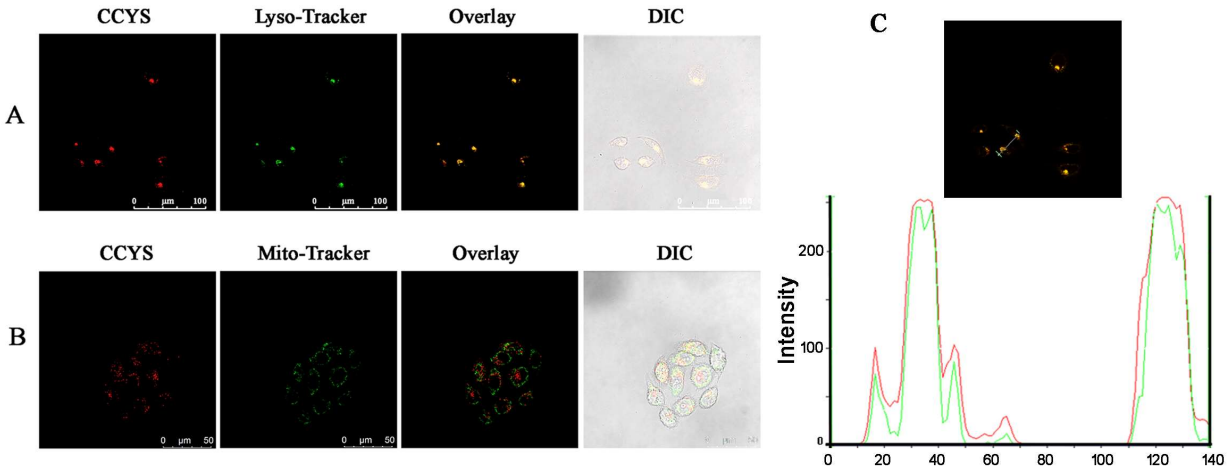


Figure S6. Confocal fluorescence images of HL-7702 cells showing co-staining of CCYS (1 μM, Ex = 633 nm, Em = 700–750 nm) with organelle dyes, including (A) Lyso-Tracker Green (100 nM, Ex = 488 nm, Em = 500–550 nm) and (B) Mito-Tracker Green (100 nM, Ex = 488 nm, Em = 500–550 nm). We co-stained cells with MitoTracker and CCYS (Figure S6B), and the results demonstrated poor overlap (Pearson's colocalization coefficient of 0.21). These data indicate that CCYS possesses excellent lysosome-localization capability. (C) Intensity profile of regions of interest (ROI) across HL-7702 cells.

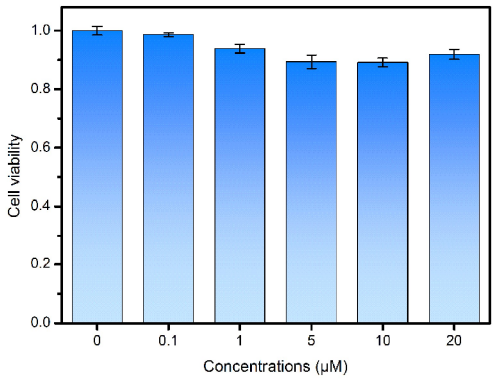


Figure S7. The MTT assay of HL-7702 cells with different concentrations of CCYS. Error bars represent standard deviation (n = 3).

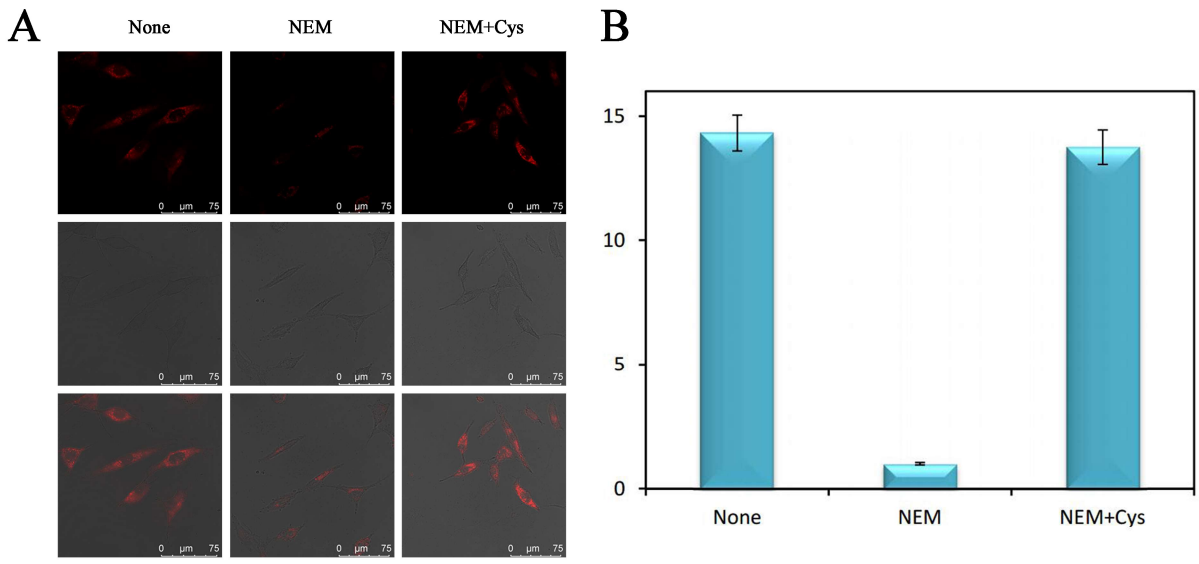


Figure S8. (A) Confocal fluorescence Images of A549 cells treated with different methods then added CCYS (1 μM) for 10 min. (B) The fluorescence intensity enhanced ratios in A. Error bars represent standard deviation (n = 5). Human lung adenocarcinoma cells (A549) were used to verify the feasibility of CCYS in fluorescent imaging. As shown in Figure S8, CCYS exhibited extremely weak fluorescence in NEM-treated A549 cells and emitted significant fluorescence (14-fold enhancement) after Cys was added again.

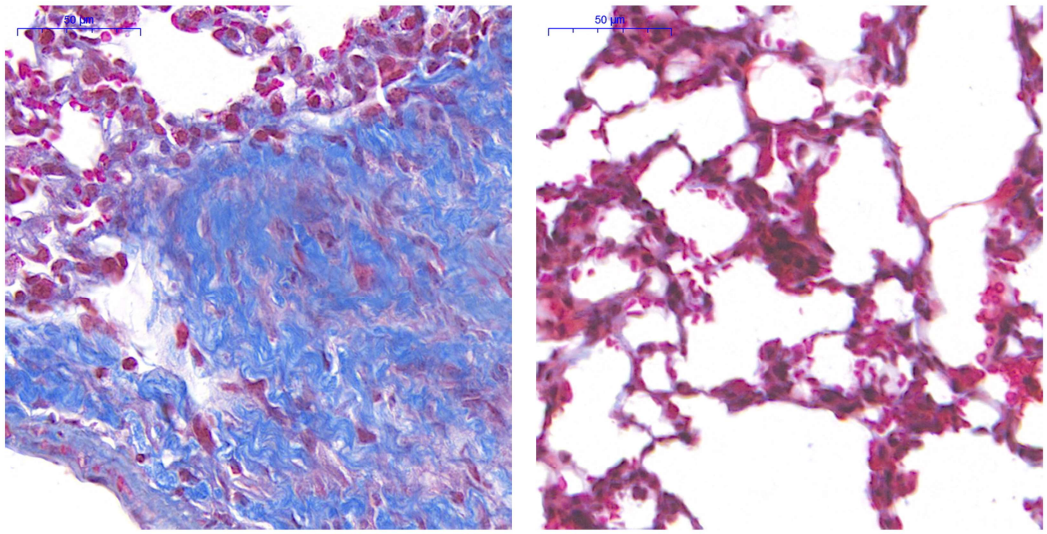


Figure S9. Masson's Trichrome-stained histological sections of lungs from PF mice and Blank mice.

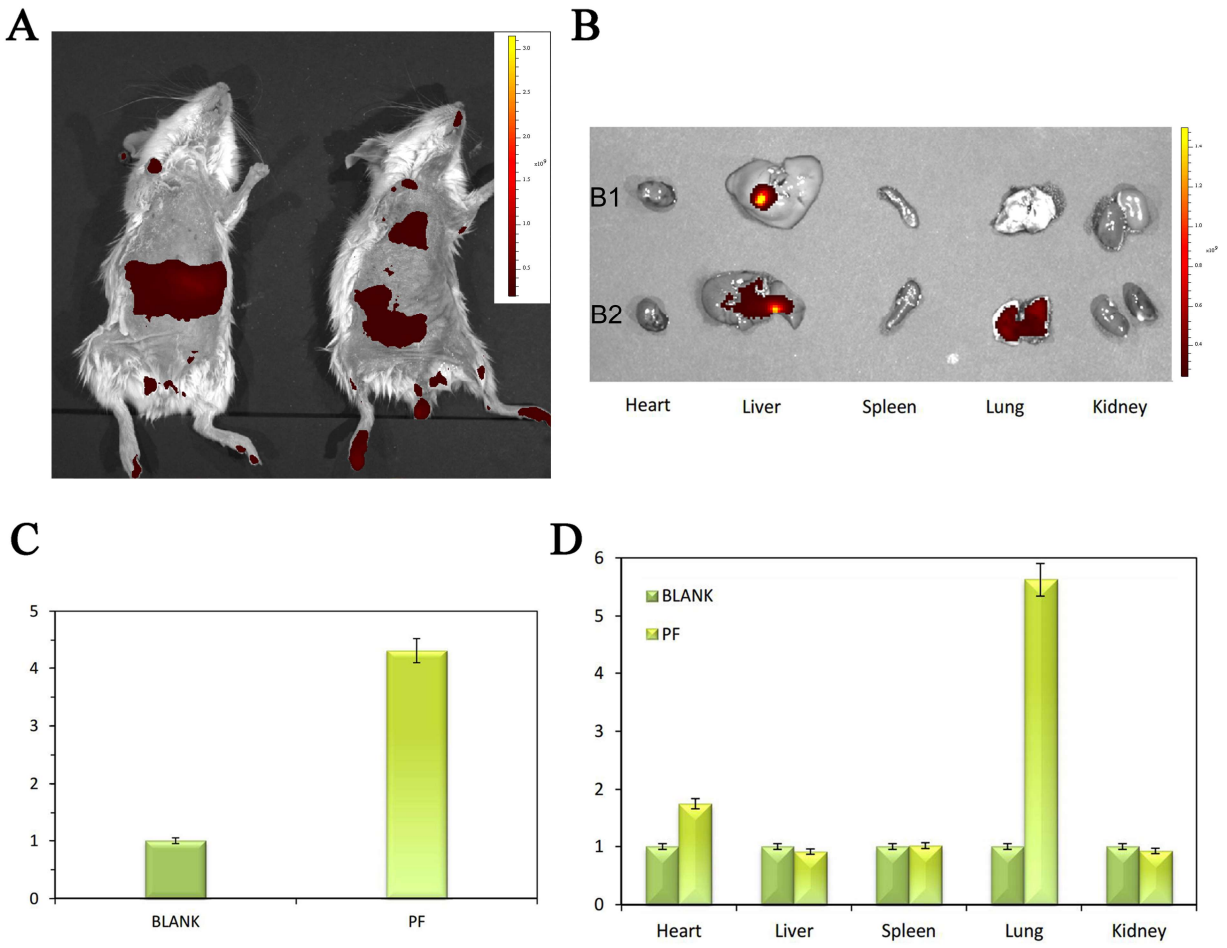


Figure S10. (A) Fluorescent detection of Cys in PF mouse (right) and Blank mouse (left) after vein injection of CCYS (50 μM, 100 μL) for 30 min. (B) Fluorescence images of the different organs after the injection of CCYS for 30 min. B1: Blank mouse. B2: PF mouse. (C) The fluorescence intensity enhanced ratios in A. (D) The fluorescence intensity enhanced ratios in B. The fluorescence intensity was obtained by *in vivo* live Imaging system. At first, we calculated the average fluorescence intensity of three different regions in each of the organs in B. Then the ratio of average FI of each organ in PF group to blank group was calculated. Error bars represent standard deviation (n = 3).

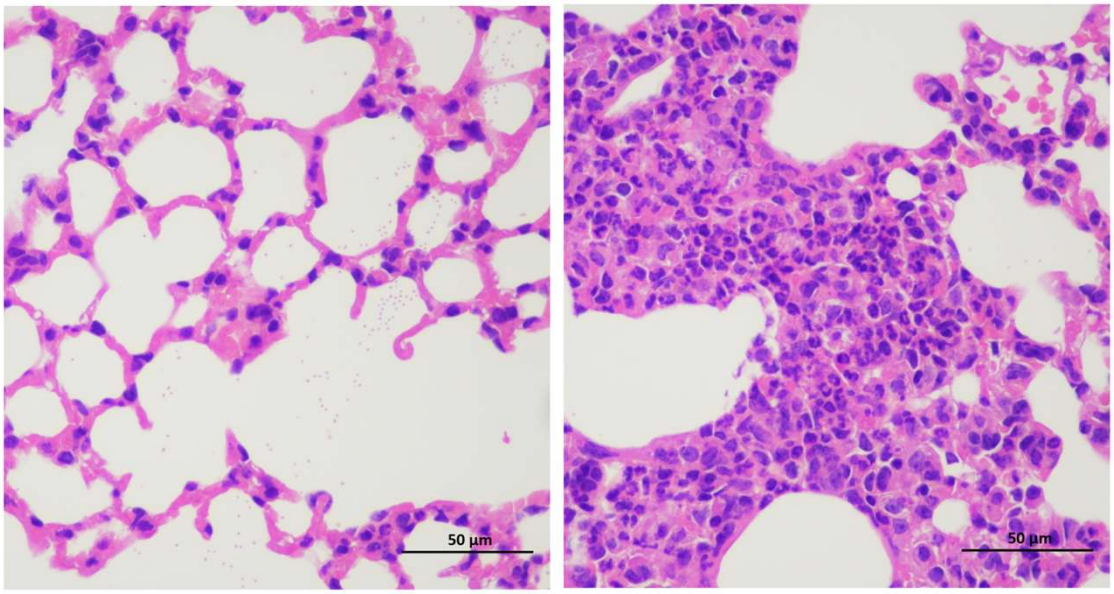


Figure S11. Hematoxylin- and eosin-stained histological sections of lungs from Pneumonia mice and Blank mice. Six to eight-week-old female KM mice were injected with LPS via the intranasal route for 24 hours to construct pneumonic mice. The lung tissue of pneumonic mice and healthy mice was removed for section staining to ensure that the mice suffered from pneumonia.

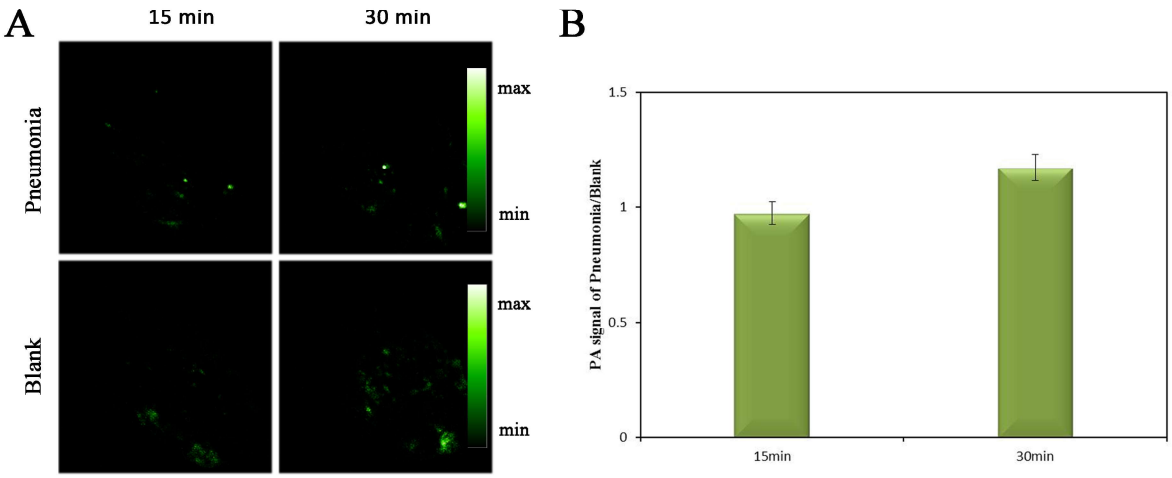


Figure S12. (A) PA images of pneumonia and Blank mouse after vein injection of CCYS (50 μM, 100 μL) for 15 and 30 min. (B) The PA intensity enhanced ratios in A. Error bars represent standard deviation (n = 3).

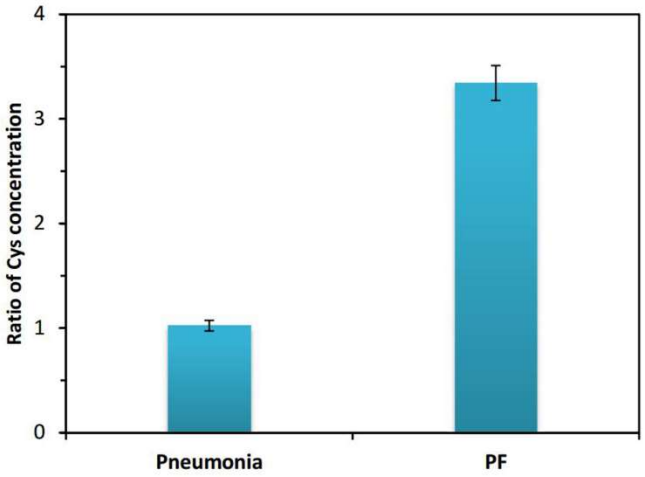


Figure S13. The ratio of Cys concentrations between pneumonia mice/Blank mice and PF-mice/Blank mice. Error bars represent standard deviation (n = 3).

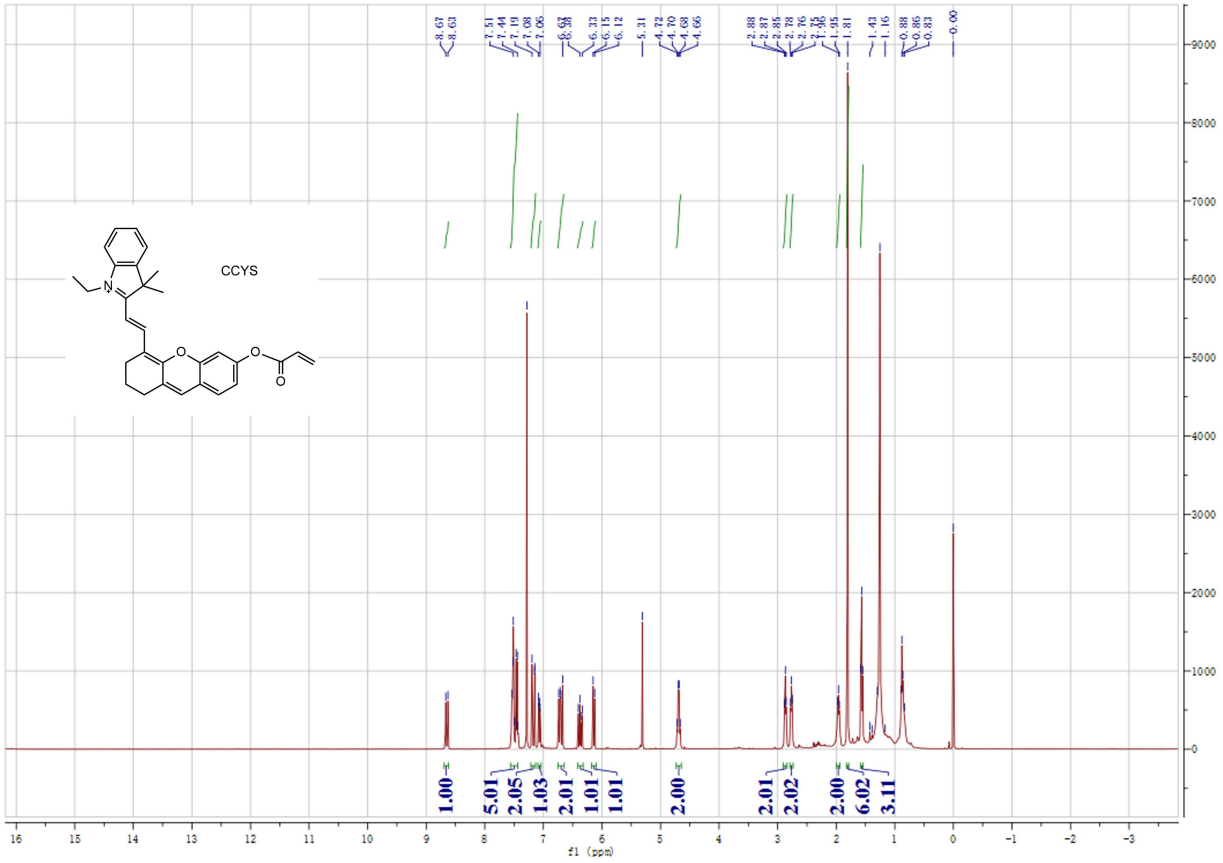


Figure S14. ^{1}H NMR spectrum of CCYS in $CDCl_3$.

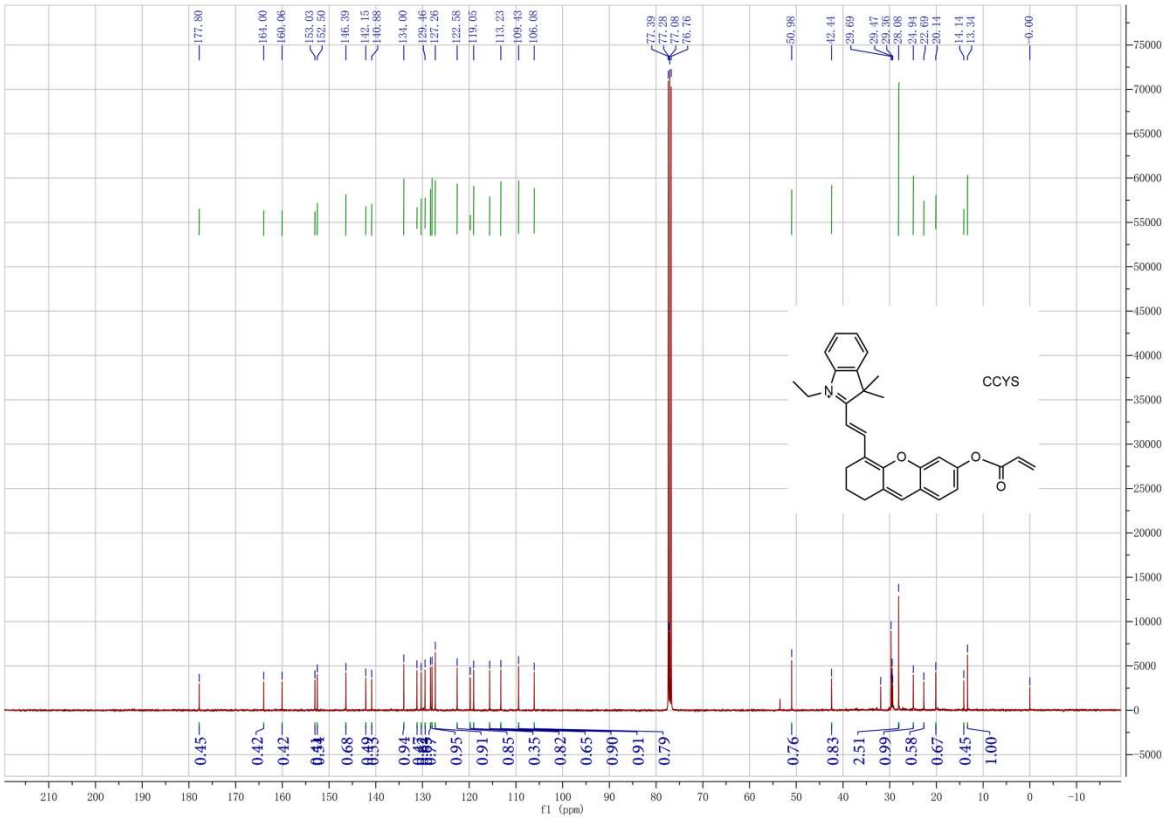


Figure S15. ^{13}C NMR spectrum of CCYS in $CDCl_3$.

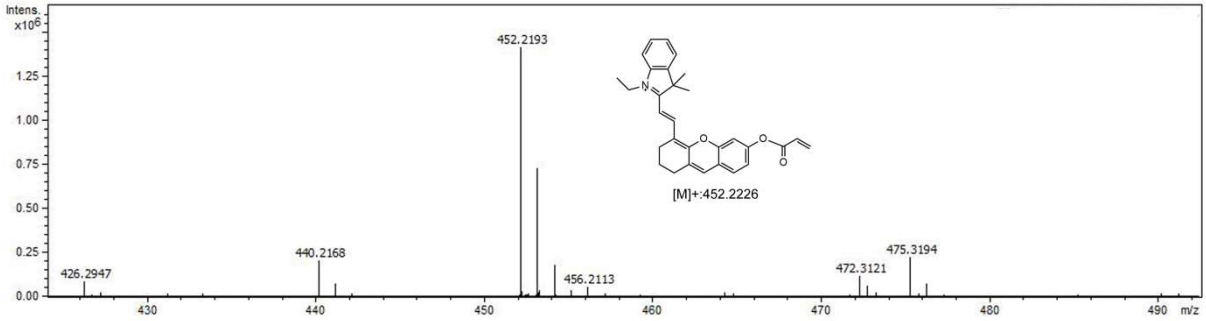


Figure S16. Mass spectrum of CCYS.

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