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Electronic Supplementary Information

A nitrobenzoxadiazole-based near-infrared fluorescent probe for specific imaging of H_2S in inflammatory and tumor mice

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1. Experimental Section.

Reagents and instruments. Cyclohexanone, phosphorus tribromide (PBr_3), 2-hydroxy-4-methoxybenzaldehyde, cesium carbonate (Cs_2CO_3), boron tribromide (BBr_3), 4-(diethylamino) salicylaldehyde, ethyl acetoacetate, piperidine, 4-chloro-7-nitrobenzofurazan were purchased from Aladdin (Shanghai, China). Unless otherwise stated, all chemicals were purchased from commercial suppliers and used without further purification.

Nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance II NMR spectrometer (Germany). Mass spectra (MS) were acquired on a Bruker Autoflex MALDI-TOF mass spectrometer (Germany). High-resolution mass spectra (HRMS) were obtained on an Agilent 1260II/6230B LC-MS spectrometer (USA). HPLC was carried out on a Agilent 1260 LC system with a C18 column (USA). The absorption spectra were determined by using a PerkinElmer Lambda 25 UV-vis spectrophotometer (USA). The fluorescence spectra were determined by using a PerkinElmer LS-55 fluorescence spectrometer (USA). The fluorescence images of cells and zebrafish were performed by using a Nikon confocal fluorescence microscope (Japan). The fluorescence images of mice were carried out on an IVIS Lumina XR small animal optical in vivo imaging system (USA).

Determination of the detection limits. The detection limit was calculated according to the fluorescence titration. The emission intensity of CX-N in the absence of H_2S in PBS buffer solutions (10 mM, pH 7.4) was calculated ten times, and the standard deviation of blank measurements was determined. Three independent duplication measurements of emission intensity were performed in the presence of H_2S, and each average value of the intensities was plotted as a concentration of H_2S for determining the slope. The detection limit

is then calculated with eq 1:

$$\text{Detection limit} = 3\sigma/k \quad (1)$$

Where σ is the standard deviation of the emission intensity of CX-N and k is the slope between the emission intensity and concentration.

Preparation of test solution. The stock solution of CX-N was obtained in DMSO. The stock solution of H_2S was obtained by dissolving NaHS with deionized water. The test solution was prepared with PBS buffer solution (10 mM, pH 7.4) in a 10 mL volumetric flask. The concentration CX-N were 1.0×10^{-5} M and H_2S were 3.0×10^{-5} - 5.0×10^{-7} M.

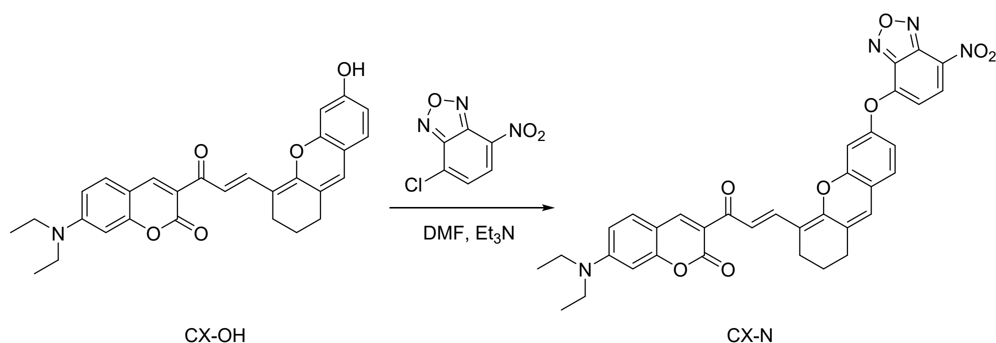
Imaging H_2S in living cells. HeLa cells were cultured in the DMEM medium with the addition of fetal bovine serum (10 %) and antibiotics (1%) at 37 °C in atmosphere of 5% CO_2. For imaging exogenous H_2S, the cells were separated into four groups. The first group was only incubated with CX-N (10 μM) for 30 min at 37 °C. The second to fifth groups were pretreated with H_2S (10, 20 and 30 μM, respectively) for 30 min at 37 °C, and then incubated with CX-N (10 μM) for 30 min. To image endogenous H_2S, one group of cells were treated with Cys (200 μM) for 1 h at 37 °C, followed by incubation with CX-N (10 μM) for 30 min. another group of cells with 1 mM DL-propargylglycine (PAG) for 1 h and then 200 μM Cys for another 1 h at 37 °C before incubation with CX-N (10 μM) for 30 min. To image H_2S level in inflammatory cells, the cells were pretreated with LPS (1.0 μg/mL) for 0 h, 3 h, 6 h, 12 h and then incubated with CX-N (10 μM) for 30 min at 37 °C. All the cells were washed three times with PBS buffer solution (10 mM, pH 7.4) before imaging. Cell selection and calculation of the mean fluorescence intensity were done using ImageJ.

Imaging H_2S in zebrafish. Zebrafish were divided into four groups. The first group was

treated with CX-N (10 μM) for 30 min at 37 °C. The second group was pretreated with H_2S (50 μM) for 30 min at 37 °C before incubation with CX-N (10 μM) for 30 min. The third group was pretreated with Cys (200 μM) for 1 h at 37 °C before incubation with CX-N (10 μM) for 30 min. The fourth group was pretreated with 1 mM DL-propargylglycine (PAG) for 1 h and then 200 μM Cys for another 1 h at 37 °C before incubation with CX-N (10 μM) for 30 min. All zebrafish were washed three times with embryo media before imaging. The mean fluorescence intensity was calculated using ImageJ.

Imaging H_2S in mice. For imaging exogenous H_2S in the normal mice, the left and right sides of the back of mice were subcutaneously injected with CX-N (100 μM) at the same time. Then, H_2S (100 μM) was injected into the same area on the right side. For imaging H_2S in inflammatory mice, the mice were subcutaneously injected with LPS (2.0 mg/mL) for 12 h in the abdominal cavity to induce an inflammation model. Then, the inflammation areas were injected with CX-N (100 μM). For imaging H_2S in the tumor-bearing mice, the mice were injected with cancer cells in the armpit to establish tumor model. Then the mice was intratumorally injected with CX-N (100 μM). The mice were anesthetized with isoflurane and remained anesthetized throughout the image period. All experiments were carried out in accordance with "Regulations of Hunan province on the administration of experimental animals".

2. Synthesis of probe CX-N.



Scheme S1. Synthesis of probe CX-N.

CX-OH was synthesized according to our reported method (Chem. Commun. 2021. 57, 13768-13771). CX-N was synthesized through a route described in Scheme 2. CX-OH (94 mg, 0.20 mmol), 4-chloro-7-nitrobenzofurazan (40 mg, 0.20 mmol) and triethylamine (Et_3N, 0.2 mL) were dissolved in DMF (5 mL). After the solution was stirred at room temperature for 6 h, the solvent was concentrated. The product was further purified by silica gel column chromatography (CH_2Cl_2) to obtain a red solid. Yield: 75 mg (60%). ^1H NMR (400 MHz, $CDCl_3$, Fig. S1) δ 8.50 (d, J = 11.1 Hz, 2H), 8.21 (d, J = 15.1 Hz, 1H), 7.57 (d, J = 15.4 Hz, 1H), 7.39 (d, J = 9.1 Hz, 1H), 7.15 (d, J = 8.1 Hz, 1H), 7.02 (s, 1H), 6.88-6.85 (m, 1H), 6.76 (d, J = 8.3 Hz, 1H), 6.61-6.58 (m, 1H), 6.44 (d, J = 13.3 Hz, 2H), 3.47-3.42 (m, 4H), 2.56 (t, J = 5.0 Hz, 4H), 1.84-1.78 (m, 2H), 1.24 (t, J = 3.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, Fig. S2) δ 186.2, 161.0, 158.5, 154.1, 153.3, 152.8, 151.4, 148.3, 145.2, 144.2, 137.7, 133.3, 131.6, 127.4, 122.4, 121.7, 121.1, 117.5, 115.1, 112.9, 109.8, 109.0, 108.7, 107.8, 96.6, 45.1, 30.1, 25.3, 20.7, 12.5. HRMS (ESI) m/z calcd for $C_{35}H_{28}N_4O_8$ $[M + Na]^+$: 655.1799, found 655.1810 (Fig. S3).

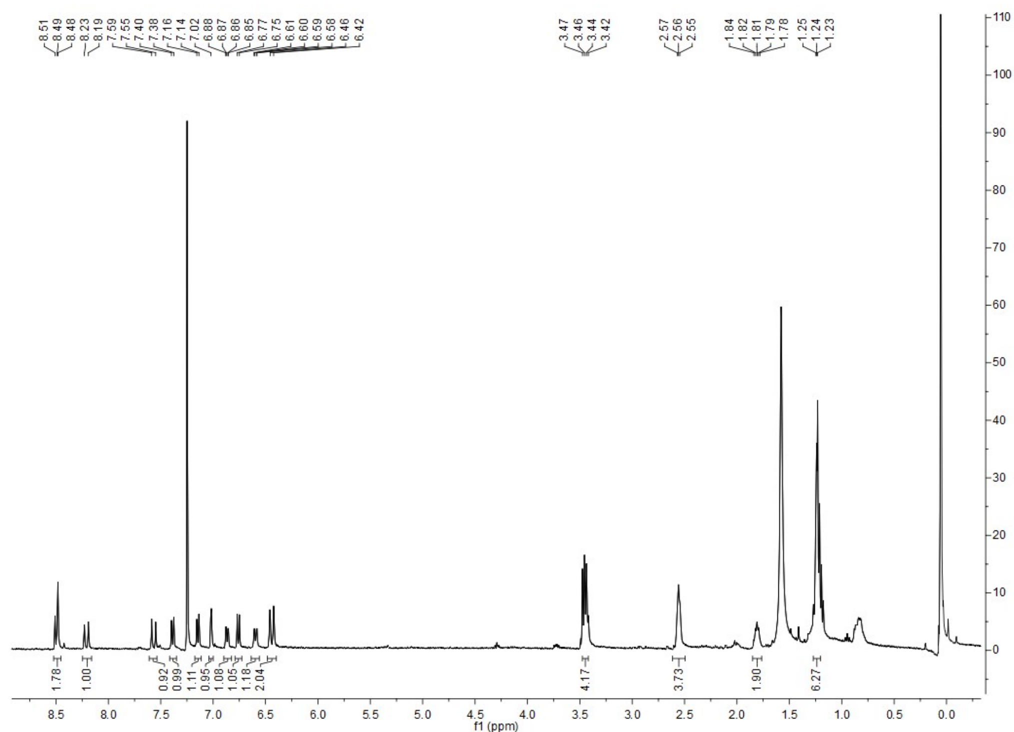


Fig. S1. ^{1}H NMR spectra of CX-N in $CDCl_3$.

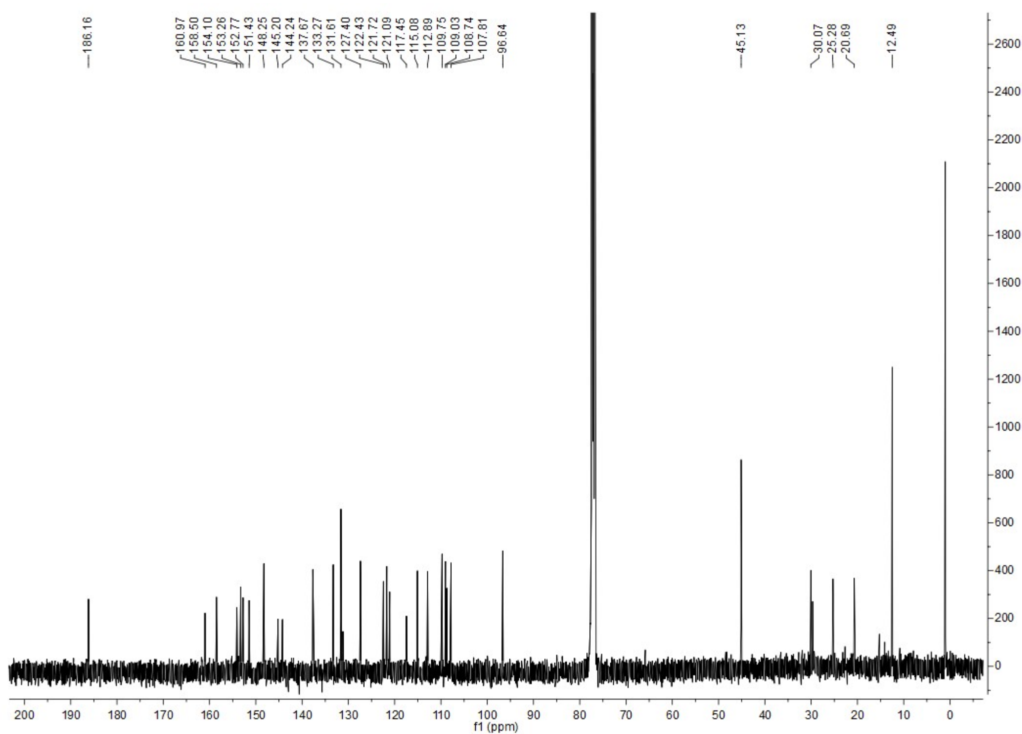


Fig. S2. ^{13}C NMR spectra of CX-N in $CDCl_3$.

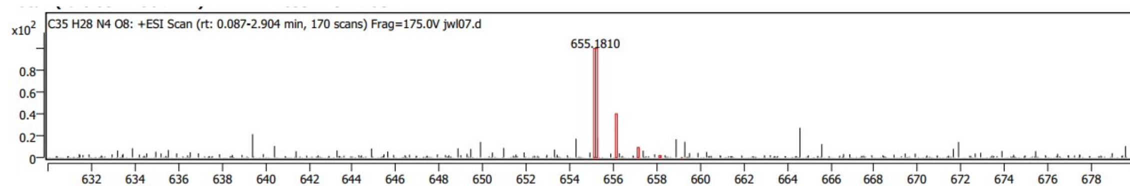


Fig. S3. HRMS spectra of CX-N.

3. Spectral data.

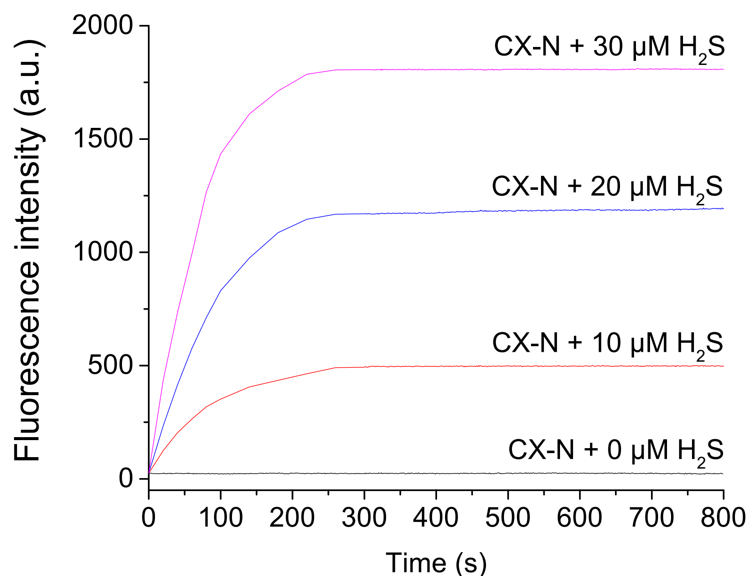


Fig. S4. Time-dependent fluorescence intensity of CX-N (10 μM) upon the addition of H_2S (0, 10, 20, 30 μM) in PBS buffer solution.

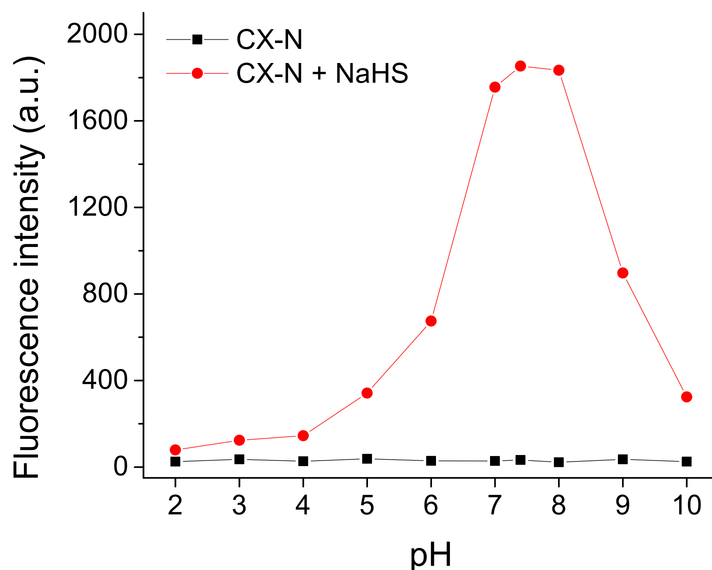


Fig. S5. Effect of pH on the fluorescence intensity of CX-N (10 μM) before and after the reaction with H_2S (30 μM).

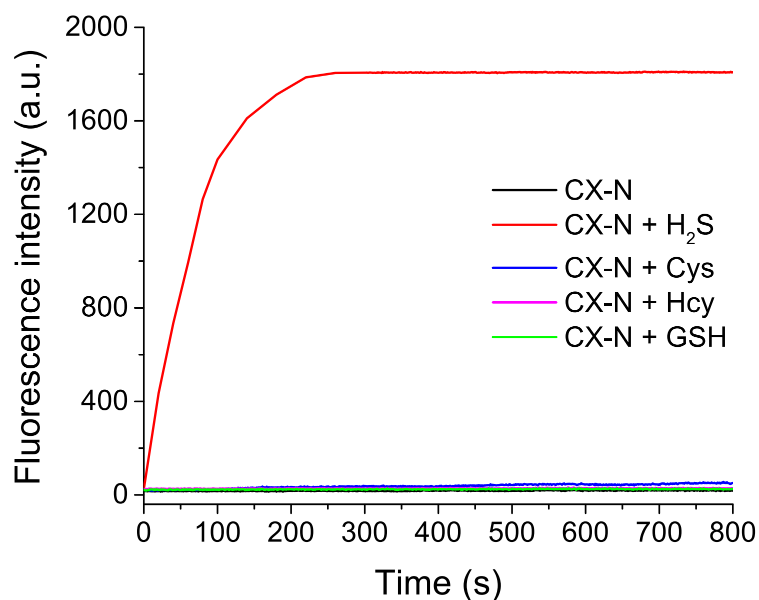


Fig. S6. Time-dependent fluorescence intensity of probe CX-N (10 μM) upon addition of H_2S (30 μM) and biothiols (1 mM) in PBS buffer solution.

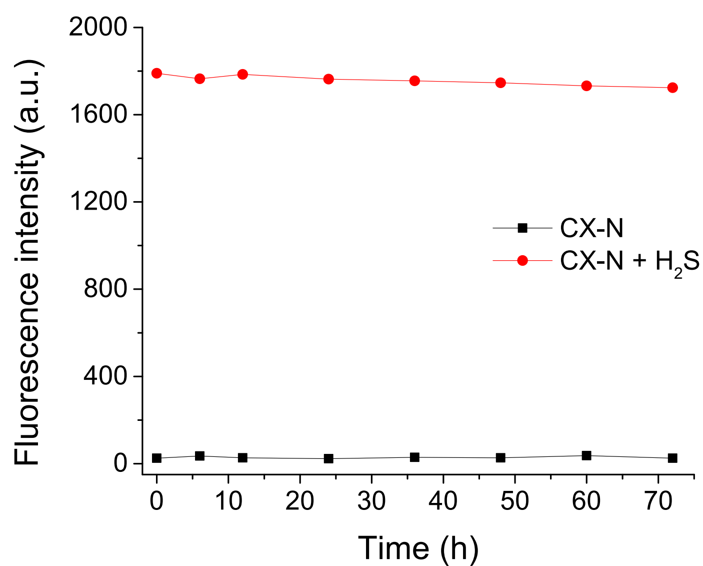


Fig. S7. Time-dependent fluorescence intensity of CX-N (10 μM) before and after the reaction with H_2S (30 μM) during 72 h.

4. Response mechanism.

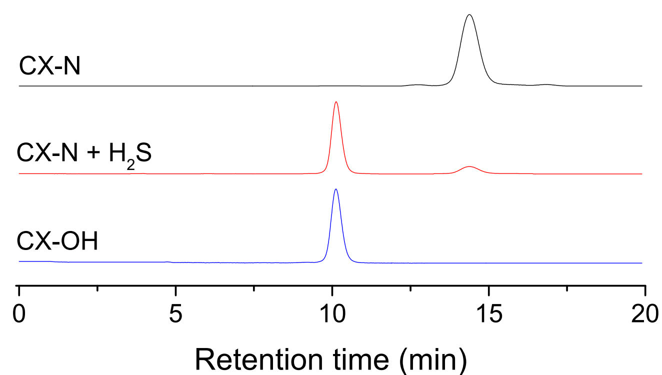


Fig. S8. HPLC chromatograms of CX-N, CX-N reacted with H_2S, and CX-OH. HPLC mobile phase: methanol/H_2O = 90/10 (V/V).

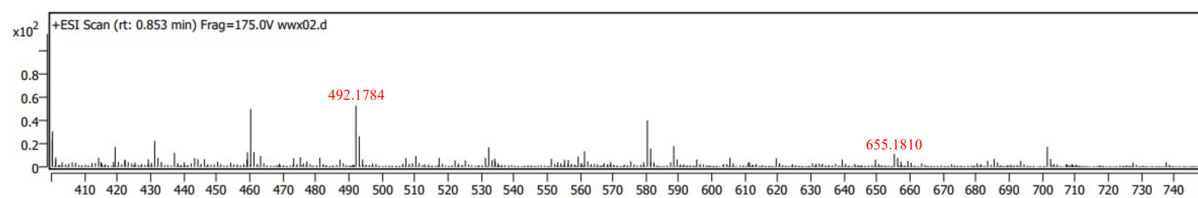


Fig. S9. HRMS spectra of CX-N with H_2S.

5. Biological assays.

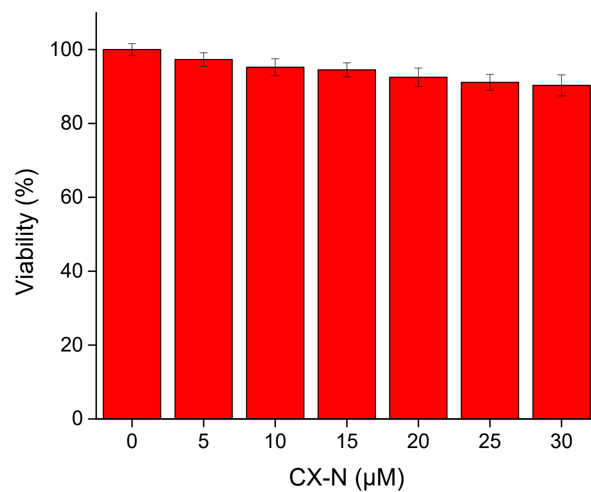


Fig. S10. CCK-8 assay for estimating cells viability (%) of HeLa cells treated with various concentrations of CX-N (0-30 μM) after 24 h incubation.

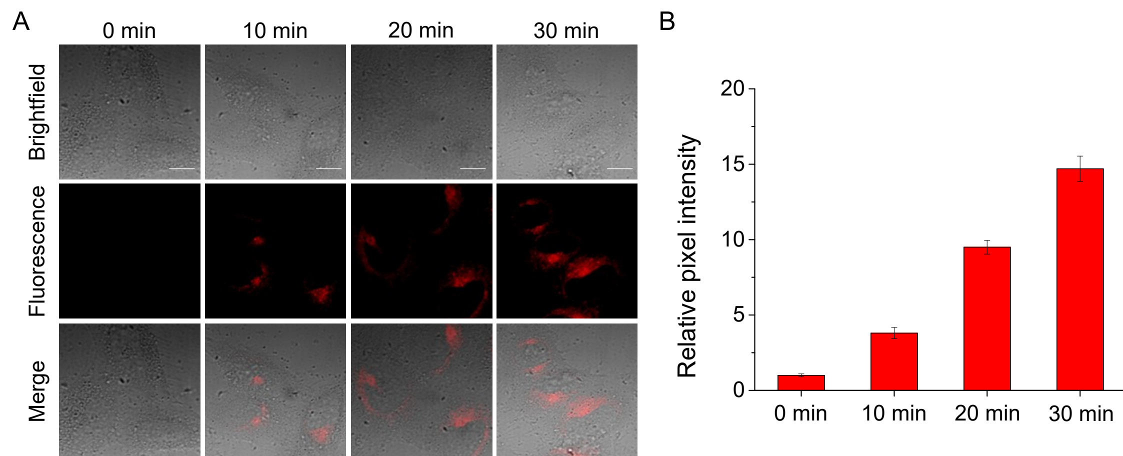


Fig. S11. Fluorescence imaging of endogenous H_2S in HeLa cells. The cells were incubated with Cys (200 μM) and then incubated with CX-N (10 μM) for 0-30 min. λ_{ex} = 560 nm, λ_{em} = 650-750 nm; Scale bar:10 μm.

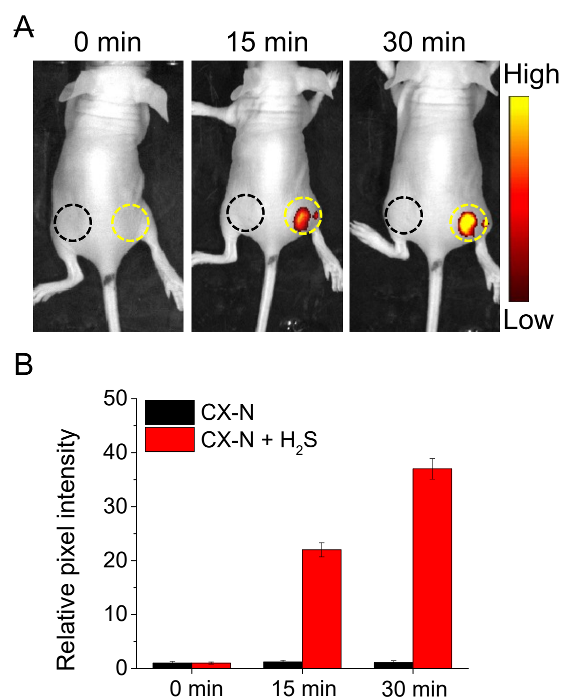


Fig. S12. (A) Fluorescence imaging of exogenous H_2S in normal mice. The left and right sides of the back of mice were subcutaneously injected with CX-N (100 μM) at the same time. Then H_2S (100 μM) was injected into the same area on the right side. (B) Relative pixel intensity. λ_{ex} = 560 nm, λ_{em} = 650-750 nm.