Fast responsive and cell membrane-targetable near-infrared H_2S fluorescent probe for drug resistance bioassay in chemotherapy

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1 Materials and instruments

All reagents and solvents were of analytical grade and purchased from Aladdin Reagent, Ltd. (Shanghai, China). Thin-layer chromatography (TLC) was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 600 spectrometer, employing TMS as an internal standard. High-resolution mass spectra (HR-MS) were recorded from Thermo Fisher Scientific mass spectrometer of Exactive Plus. UV-vis-NIR spectra were recorded on a UV2550 UV-vis spectrophotometer. Fluorescence spectra were measured by a Hitachi F-4600 fluorescence spectrophotometer. Cells were cultured in a CO_2 incubator (FORMA STERI-CYCLE i160, Thermo Fisher Scienfitic). Fluorescence images of living cells were performed on a Leica confocal laser scanning microscope (TCS-SP8). All procedures involving animals were approved by and performed in compliance with the guidelines of the Animal Care and Use Committee of South-Central Minzu University.

2 Synthesis of CM-H_2S

The synthetic route was shown in Figure S1.

*Compound **3,5,6,7*** These compounds were synthetized according to literatures[1-3].

CM-H_2S Compound 2 (0.104 g, 0.17 mmol) and 60% NaH (6.8 mg, 0.17 mmol) was dissolved in DMF and stirred for 20 min at room temperature. Compound 3 (0.2 g, 0.2 mmol) was then added and the temperature was raised to 60 °C. After reaction for 12 h, the product was poured into water and extracted with ethyl acetate. The organic phase was collected and the solvent was removed through reduced-pressure distillation. 2 mL trifluoroacetic acid (TFA) was then added and stirred for 2 h. After that, TFA was removed by distillation and further purified by silica gel column chromatography. CM-H_2S was obtained as a dark green solid (31mg, 30% yield). ^{1}H NMR (400 MHz, d6-DMSO) δ 7.83 (d, J = 14.1 Hz, 2H), 7.69 (d, J = 9.0 Hz, 2H), 7.52 (d, J = 7.4 Hz, 2H), 7.37 (d, J = 3.9 Hz, 4H), 7.24-7.15 (m, 2H), 7.11 (d, J = 9.0 Hz, 2H), 6.18 (d, J = 14.1 Hz, 2H), 4.19-4.03 (m, 4H), 3.99 (t, J = 6.5 Hz, 4H), 3.45 (s, 2H), 2.82 (s, 8H), 2.72 (s, 8H), 2.26 (t, J = 7.3 Hz, 4H), 2.01-1.92 (m, 2H), 1.67 (m, 4H), 1.54-1.46 (m, 8H), 1.39-1.27 (m, 20H), 1.23 (s, 24H) (Figure S2). ^{13}C NMR (101 MHZ, $CDCl_3$) δ 173.65, 171.09, 168.67, 160.32, 152.78, 152.43, 144.60, 143.46, 142.44, 141.24, 138.49, 135.20, 131.08, 129.64, 128.91, 128.86, 127.84, 125.49, 123.96, 123.81, 123.64, 122.55, 121.42, 119.70, 116.65, 111.18, 104.73, 65.19, 57.87, 52.05, 47.98, 46.24, 46.19, 46.17, 45.89, 44.93, 43.74, 43.59, 34.33, 30.59, 30.18, 30.10, 29.68, 29.66, 29.14, 28.95, 28.88, 28.26, 27.91, 27.51, 27.36, 27.25, 26.97, 25.66, 25.16, 23.54, 19.04, 13.78 (Figure S3). $[M]^+$ calcd for $[C_{68}H_{98}N_7O_6]$ 1220.8825, found 1220.8813 (Figure S4).

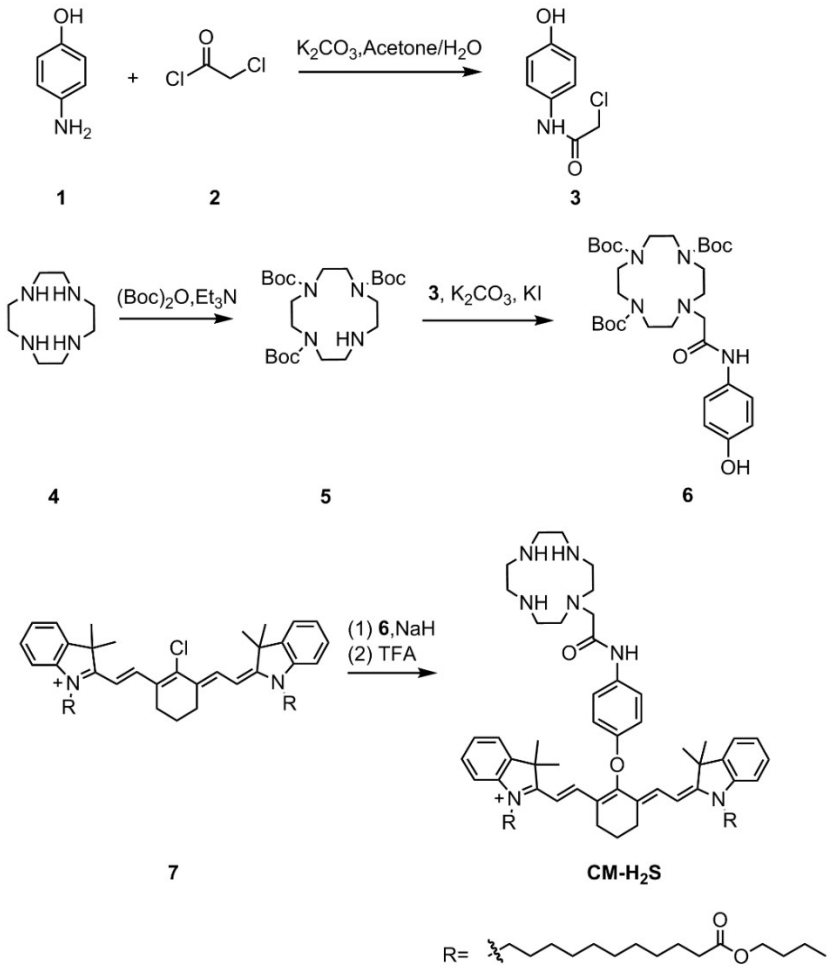

Figure S1 Synthetic route of CM-H_2S.

3 Coordination between CM-H_2S and Cu^{2+}

10 mM CM-H_2S in DMSO was added with 10 mM $CuNO_3 \cdot 3H_2O$ (a.q.). The solution was shaken for 30 min at room temperature to get a stock solution of CMCu-H_2S. $[M]^+$ calcd for $[C_{68}H_{98}CuN_7O_6]$ 1283.8121, found 1283.8143 (Figure S5).

4 H_2S detection *in vitro*

CMCu-H_2S in the mixture of HEPES (10 μM, pH 7.4)/DMSO=1:1 (*v*/*v*) was added with various concentrations of Na_2S. After incubation at 37 °C for 20 min, the fluorescence spectrum were obtained excited at 780 nm.

5 Cell culture

HepG2 cells were cultured in modified Eagle's medium (Gibco) supplemented with 1% diabody solution (streptomycin-penicillin) and 10% calf serum at 37°C in an atmosphere of 5% CO_2 and 95% air or 5% CO_2, at 37 °C.

6 Biocompatibility study

The MTT method was used to measure the biotoxicity of the probe. HepG2 cells with a density of 4×10^4-5×10^4/ml was transferred into 96-well plates (the volume of each parts was equal). The cells were incubated with various concentrations of the probe (0-20 μM) for 24 h. Each concentration was tested in 3 replicates. After that, 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-methyltetrazolium bromide (MTT) solution (5mg/ml in PBS, pH=7.4) was added and incubated for another 4 h. Culture supernatant was carefully discard and 100 μL DMSO was add

to each well. After shaken for 10 min, the crystals were fully melt and the absorption at 490 nm was measured and recorded. Cell viability can be calculated according to the formula: cell viability (%) = (mean Abs. of CMCu-H_2S wells)/(mean Abs. of control groups) ×100%.

To investigate the biocompatibility of the probe, female Kunming mice (~25g) were *i.v.* injected with 50 μL probe (100 μM in physiological saline). The mice were then divided into four groups and normally fed for 5, 10, 15 and 30 days, respectively. The main organs were dissected for H&E staining.

7 Colocalization test

HepG2 cells were incubated with commercial cell membrane-targetable dye, DiL (50 nM), and 10 μM CMCu-H_2S in Eagle's medium for 40 min. After the cells were washed by PBS buffer for 3 times, the confocal fluorescence images were recorded. DiL was excited at 488 nm and the emission from 560 nm-575 nm was collected.

8 Method to obtain NFS1-KD HepG2 cells

NFS1-KD cells were obtained according to the literature.[4] Briefly, about 4×10^7 HepG2 cells were plated in fifteen 10-cm dishes, which ensured the sufficient coverage of sgRNAs. 10 μg/mL polybrene was introduced to enhance infecting. The multiplicity of infection (MOI) was ~0.3 to achieve the high infecting probability. After 48 h, the infected cells were selected with 2 μg/ml puromycin for 7 days to get the positively transduced cells and eliminate the uninfected cells. The genome-edited cell pools were then obtained.

9 H_2S imaging in HepG2 cells.

For exogenous H_2S imaging: HepG2 cells were added with NEM (0.5 mM) for 30 min to deplete endogenous H_2S. After that, various concentrations of Na_2S (0, 5, 10, 20 μM) were incubated for another 30 min and then 10 μM CMCu-H_2S was supplied. After 30 min, the cells were washed with PBS buffer and then used for imaging.

For endogenous H_2S imaging: Normal HepG2 cells weren't treated by NEM, DCS or L-Cys was served as sham group. Other HepG2 cells used in the following experiments were all treated with NEM (0.5 mM) to deplete endogenous H_2S. L-Cys (0.1 mM) was incubated with the HepG2 cells for 30 min to promote H_2S production and served as control group. Two methods was employed to study NFS1 level-mediated [H_2S] change. One is pretreating HepG2 cells by DCS (0.1 mM), a commercial NFS1 inhibitor, for 1 h before adding L-Cys. The other is knocking down NFS1 in HepG2 cells and then incubated with L-Cys (0.1 mM) for 30 min. For the negative group, NFS1 in HepG2 cells was not successfully knocked down and the cells was then also incubated with L-Cys. All of above cells were incubated with 10 μM CMCu-H_2S for 30 min and washed with PBS for 3 times before conducting confocal fluorescence imaging. All of the fluorescence images were excited by 552 nm laser, and emission in the range of 780-800 nm was collected.

10 H_2S detection in adriamycin-based chemotherapy

Chemotherapy was performed employing a generally used drug, Adriamycin. After incubating HepG2 cells with various Adriamycin (0, 0.1, 0.5, 0.7, 1 μM) for 30 min. The fluorescent probe (10 μM) was added and incubated for 30 min. After the free probe was washed by PBS buffer, the fluorescence images were recorded. When investigating the role of NSF1 in drug resistance, the HepG2 cell with or without NSF1-knockdown were treated by 1 μM Adriamycin for 1h. Other procedures to obtain the fluorescence images were identify with that described above. The fluorescence was excited by 552 nm laser and the signal in the range of 780-800 nm was collected.

11 Cell apoptosis assessment

Various kinds of HepG2 cells were seeded in 6-well plates with a density of about 1.0×10^6 cells/well and incubated in culture medium with 10 μM probe and 5 μM adriamycin for 30 min (adriamycin was not added in the sham group). The cells were then washed and then stained by AnnexinV-FITC/PI Cell Apoptosis Detection Kit and then measured by flow cytometry.

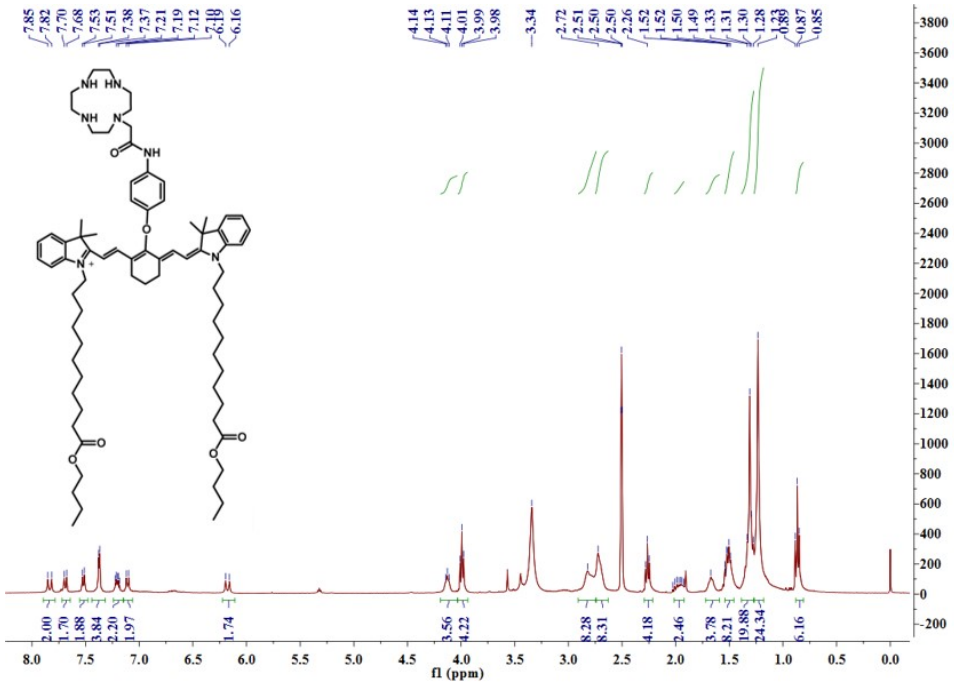

Figure S2 ^{1}H NMR of CM-H_2S.

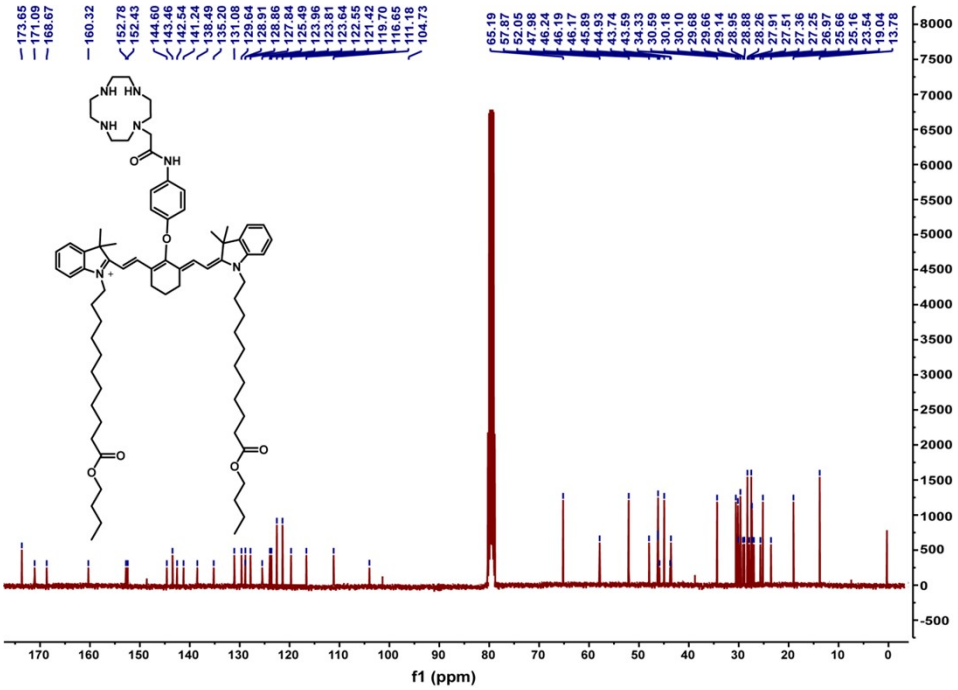

Figure S3 ^{13}C NMR of CM-H_2S.

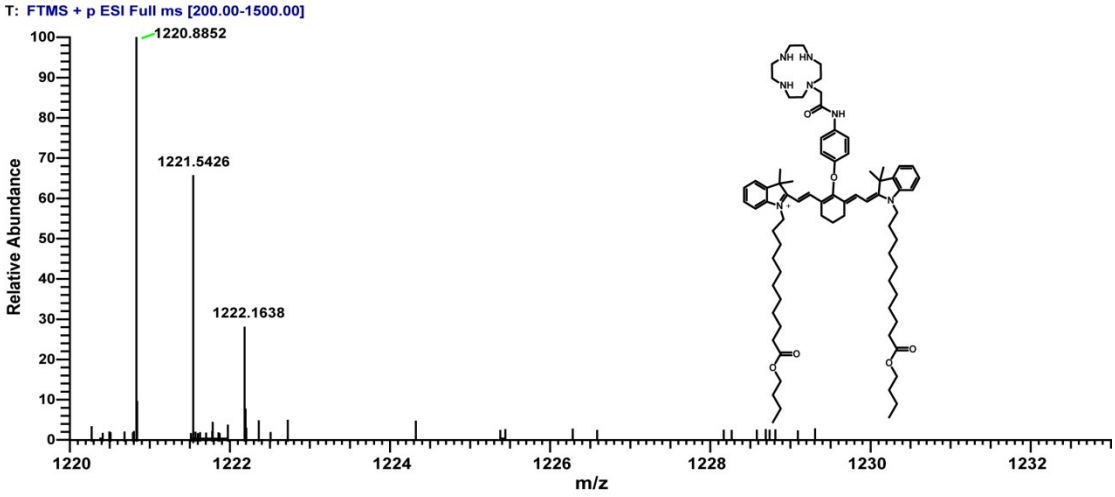

Figure S4 High resolution mass spectrum (HRMS) spectra of CM-H_2S.

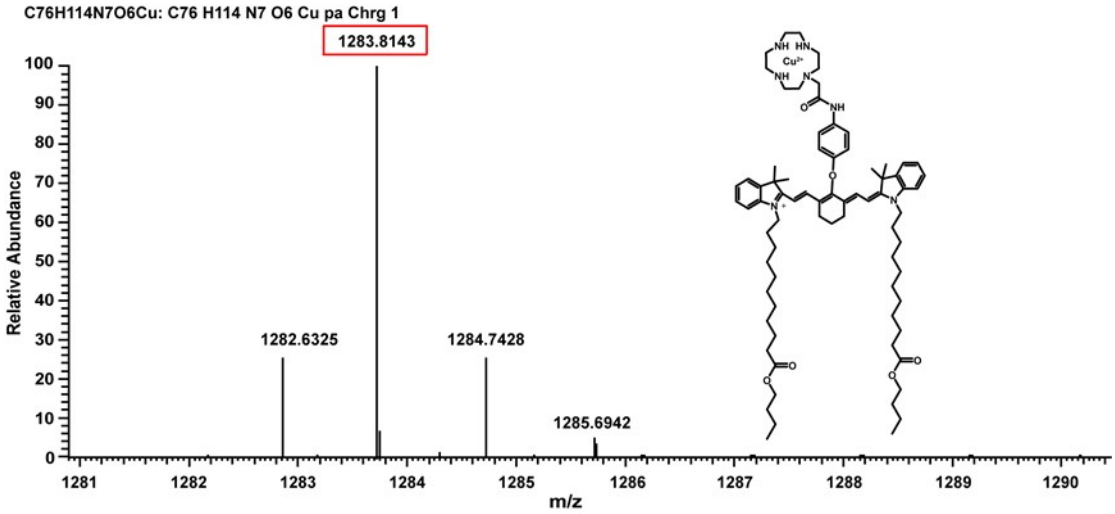

Figure S5 High resolution mass spectrum (HRMS) spectra of CMCu-H_2S.

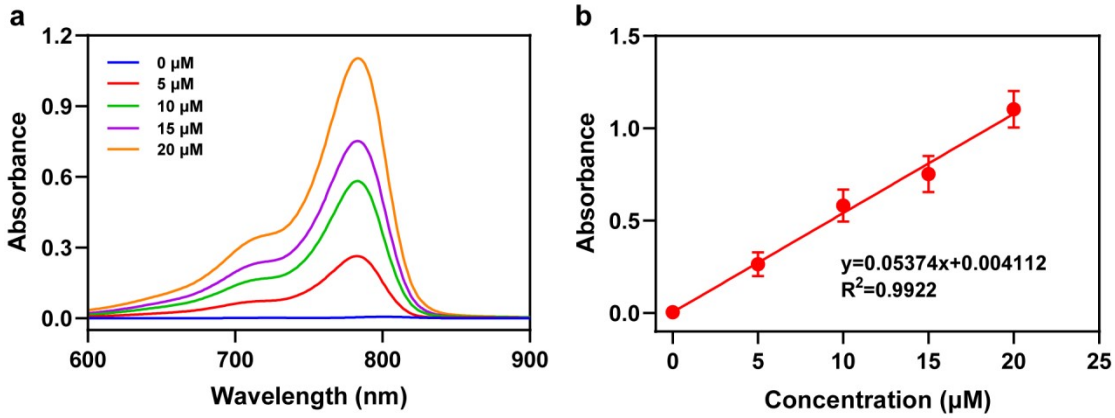

Figure S6 (A) Absorption spectrum and (B) the corresponding absorption at 780 nm of CM-H_2S with various concentration in DMSO.

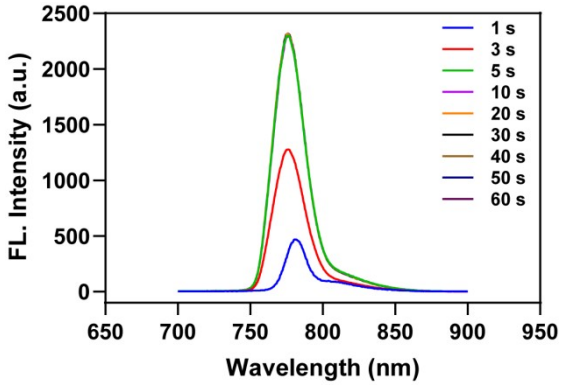

Figure S7 Fluorescence spectrum of CMCu-H_2S (10 μM) after reacting with 10 μM Na_2S for different time.

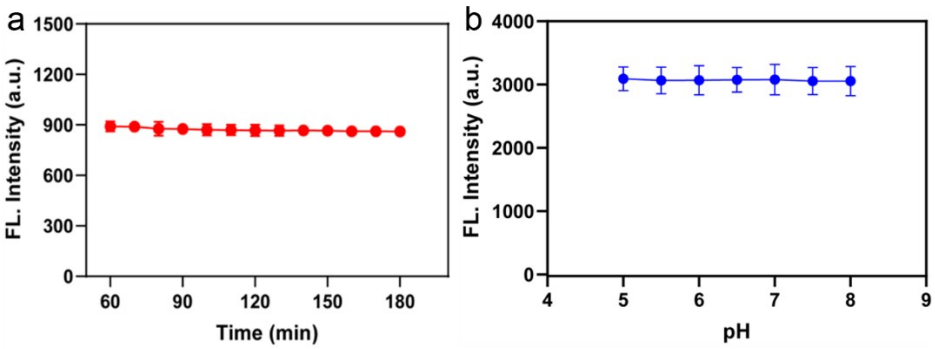

Figure S8 (a) Thermodynamic stability and (b) pH stability tests of CMCu-H_2S in HEPES.

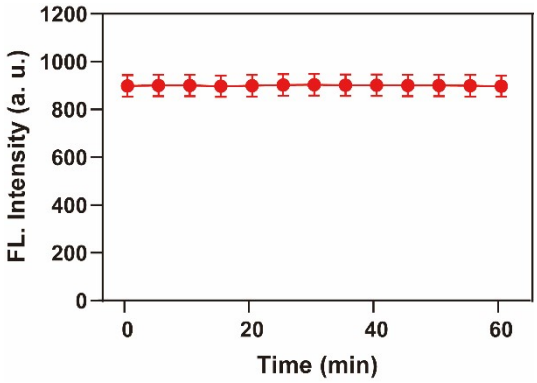

Figure S9 Photostability test of CMCu-H_2S in HEPES under 780 nm irradiation.

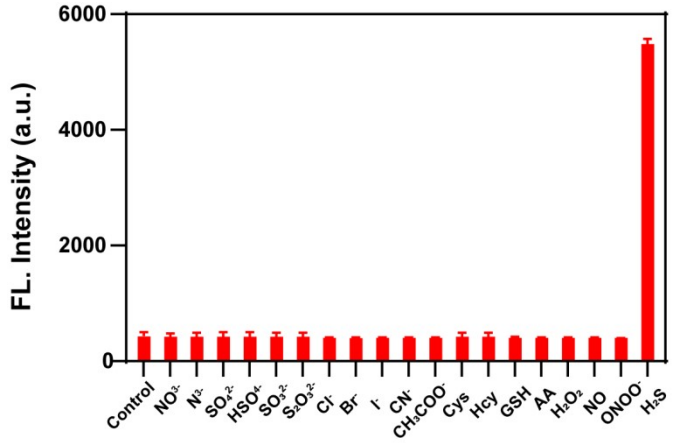

Figure S10 Fluorescence intensity of probes (10 μM) at 790 nm after reaction with 10 μM Na_2S and 100 μM interfering substances.

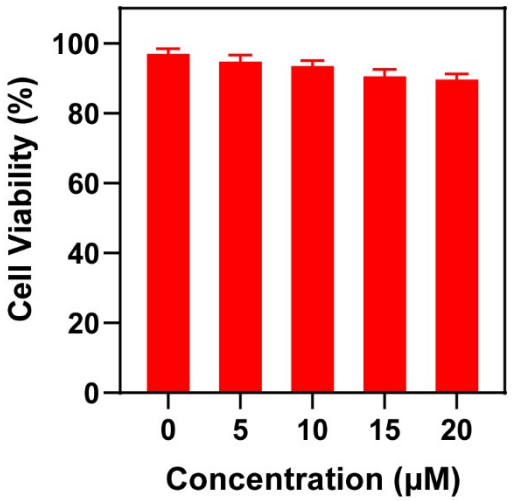

Figure S11 MTT test of CMCu-H_2S in HepG2 cells after incubation for 24 h.

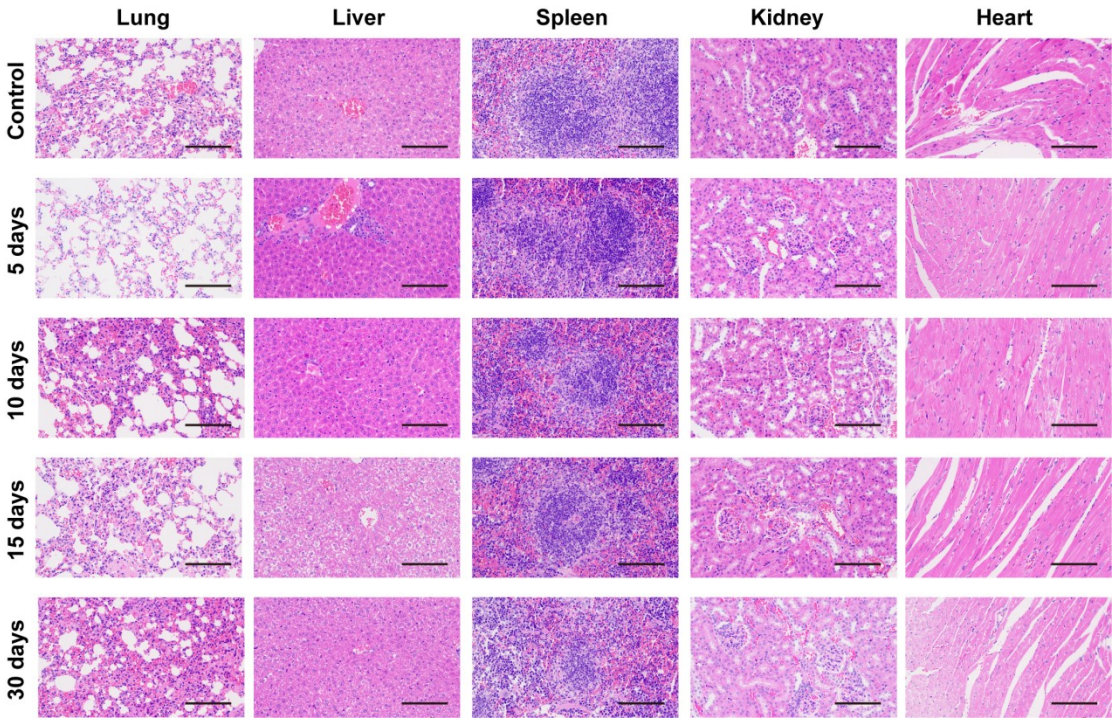

Figure S12 H&E staining of main organs of mice after feed with **CMCu-H_2S** for different time. The scar bar is 50 μm.

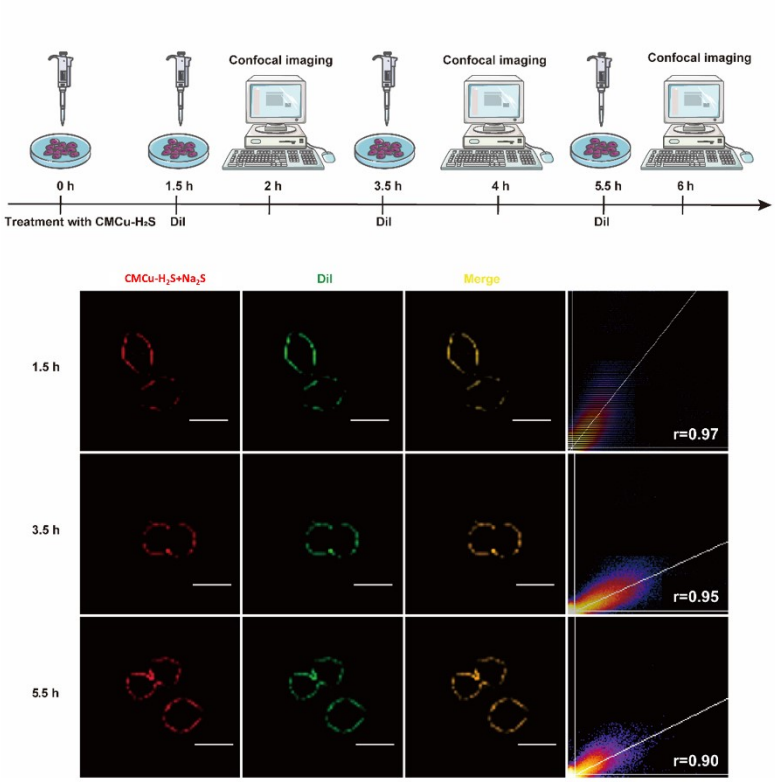

Figure S13 Cell membrane-target ability test for various incubation time. The scar bar is 50 μm.

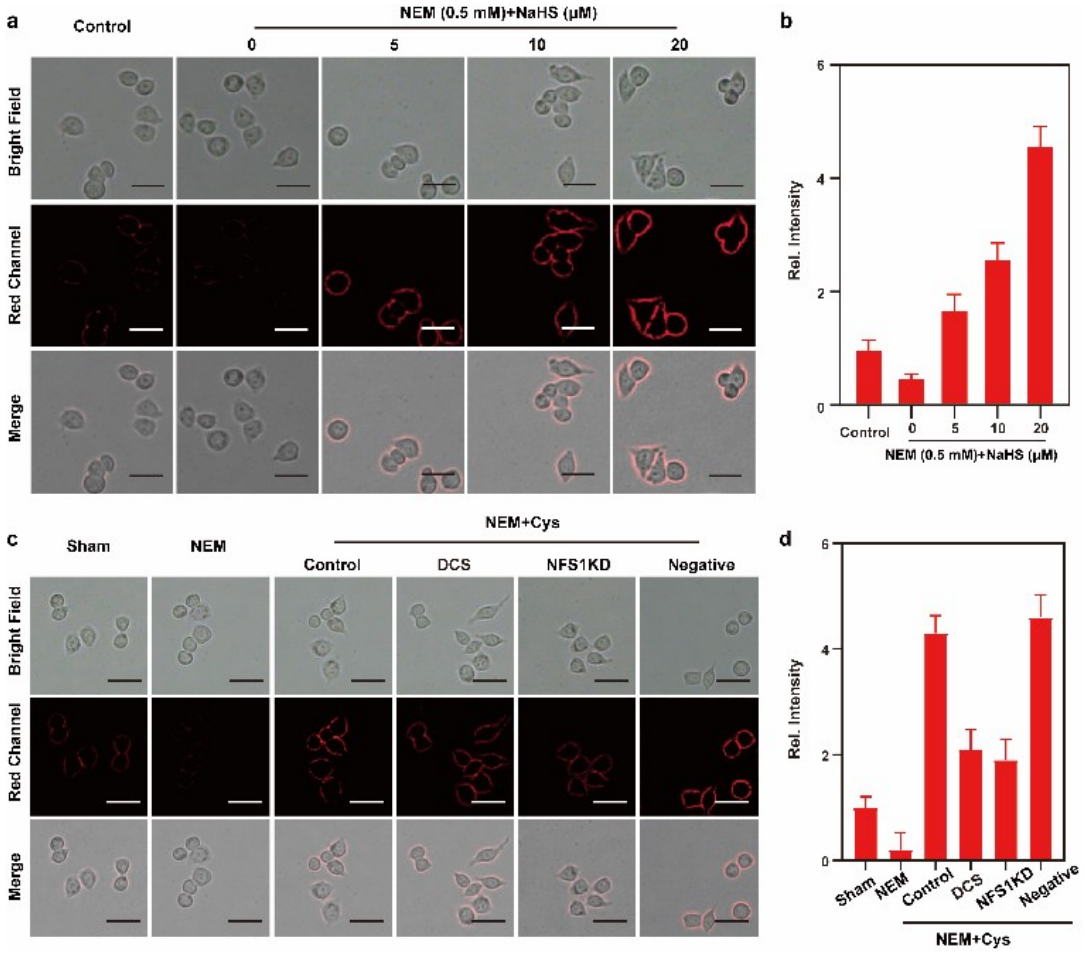

Figure S14 (a) Fluorescence imaging and (b) corresponding intensity of HepG2 cells after sequentially treated with 0.5mM NEM for 30 min, various concentrations of NaHS for 30 min and 10 μM CMCu-H_2S for 30 min. (c) Endogenous H_2S imaging and (d) corresponding intensity in HepG2 cells. Sham group: incubation with CMCu-H_2S for 30 min; NEM group: incubation with 500 μM NEM for 30 min and then CMCu-H_2S for 30 min; Control, NFS1KD and negative groups: incubation with 500 μM NEM for 30 min, Cys for 30 min and then CMCu-H_2S for 30 min; DCS group: incubation with 500 μM NEM for 30 min, DCS for 30 min, Cys for 30 min and then CMCu-H_2S for 30 min. The scar bar is 50 μm.

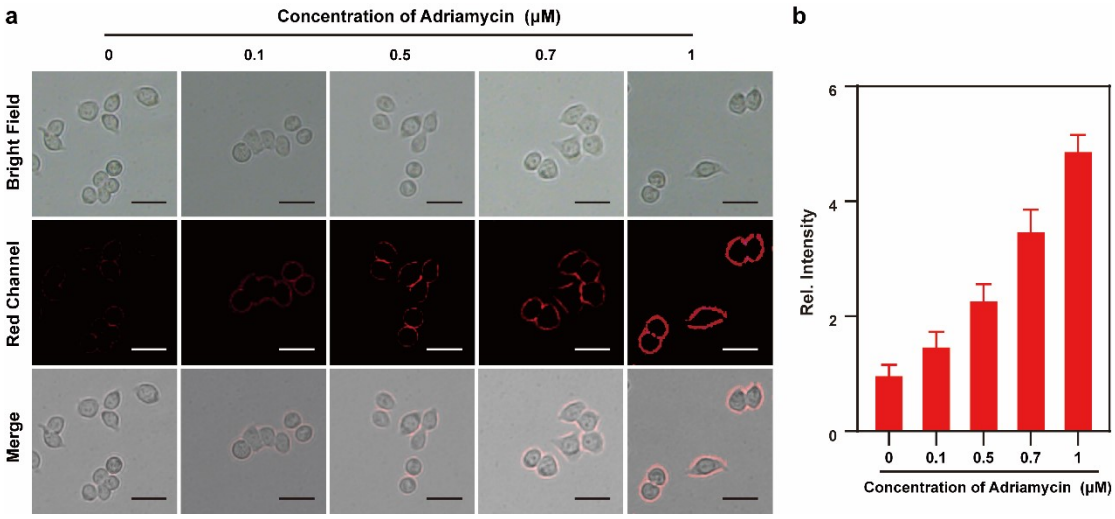

Figure S15 (a) Confocal fluorescence images of HepG2 cells pretreated with various concentrations of adriamycin for 1h and then stained with CMCu-H_2S for 30 min. (b) The corresponding fluorescence intensity (780-800nm) in (a). λ_{ex}=552 nm.

References

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