Electronic Supplementary Information for:

Near-Infrared Ratiometric Self-assembled Theranostic Nanoprobe: Imaging and Tracking Cancer Chemotherapy

Xinwei Tian, Zhao Li*, Ning Ding and Jiahang Zhang

Shaanxi Engineering Laboratory for Food Green Processing and Safety Control, College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an 710062, China. lizhao0309@snnu.edu.cn

Table of contents

1. Apparatus and reagents

^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (^{1}H NMR and ^{13}C NMR) spectra were performed on a Brucker DMX-600 spectrometer with CD_3OD as a solvent. Ultraviolet-visible absorption of the sample solutions was measured by using a Hitachi U-3010 spectrophotometer. Fluorescent spectra were recorded on HITACHI F-7000 Fluorescence Spectrometer (Hitachi Limited Ltd., Japan), the slit-width was set as 1 nm with a scan speed of 2000 nm min^{-1}. MTT analysis was recorded on a microplate reader (BIO-TEK Synergy HT, USA). Cell fluorescence imaging was conducted on a confocal laser scanning microscope (Leica, Germany) with 635 nm excitation and mice imaging was performed on an IVIS Lumina LT Series imaging system.

Triphosgene, 2, 2'-dithiodiethanol, N, N-diisopropylethylamine (DIEA), IR-780 iodide and N, N'-Dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich. 3-Nitrophenol, chlorambucil (Cb), 4-dimethylaminopyridine (DMAP) were purchased from J&K Scientific Co. Ltd. Dichloromethane (CH_2Cl_2) was dried by refluxing with anhydrous sodium sulfate and distilled just before use. Stock solution (1 mM) of AX-S-Cb was prepared by dissolving a refined calculation of probe in deoxygenated dimethyl sulphoxide (DMSO). Reactive oxygen species including peroxynitrite anion ($ONOO^-$), hydrogen peroxide (H_2O_2, 30% aqueous solution), hydroxyl radical (·OH) and sodium hypochlorite (NaClO) were prepared according previous literatures.[1] Ultrapure water (18.5 MΩ cm) was purified with Milli-Q reference system (Millipore). All other reagents and solvents were purchased from the domestic suppliers and used as received.

2. Synthesis of AX-S-Cb and NAX-S-Cb

2.1 Synthesis of fluorophore AXPI.

Compound AXPI was synthesized according to our previous reported method.[2]

2.2 Synthesis of AX-S.

To a mixture of AXPI (90 mg, 0.22 mmol), triphosgene (202 mg, 0.66 mmol) and dry acetonitrile (5 mL) was added DIEA (389 mg, 3.1 mmol) dropwise under an argon atmosphere at

room temperature. The resulting solution was refluxed under argon protection for 3 h. After removal of unreacted phosgene gas by flushing argon gas, a solution of 2, 2'-dithiodiethanol (396 mg, 2.34 mmol) in acetonitrile was added to the mixture and the reaction mixture was stirred overnight at room temperature. After removing the solvent under reduced pressure, the crude product was purified by silica gel chromatography using CH_2Cl_2/MeOH as an eluent to afford (*E*)-2-(2-(6-(((2-((2-hydroxyethyl)disulfanyl)ethoxy)carbonyl) amino)-2,3-dihydro-1*H*-xanthen-4-yl) vinyl)-3,3-dimethyl-1-propyl-3*H*-indol-1-ium (AX-S) as a mazarine solid (31.2 mg, yield 24 %).

^{1}H NMR (600 MHz, CD_3OD) δ 8.63 (d, *J* = 14.9 Hz, 1H), 7.76 (s, 1H), 7.68 (d, *J* = 7.4 Hz, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 7.57-7.51 (m, 1H), 7.46 (t, *J* = 7.4 Hz, 1H), 7.38 (d, *J* = 8.5 Hz, 1H), 7.29 (s, 1H), 7.19 (dd, *J* = 8.4, 1.9 Hz, 1H), 6.47 (d, *J* = 14.9 Hz, 1H), 4.37 (t, *J* = 6.5 Hz, 2H), 4.33 (t, *J* = 7.4 Hz, 2H), 3.81 (t, *J* = 6.4 Hz, 2H), 3.01 (t, *J* = 6.5 Hz, 2H), 2.89 (t, *J* = 6.4 Hz, 2H), 2.78-2.71 (m, 2H), 2.64 (t, *J* = 6.0 Hz, 2H), 1.93 (dt, *J* = 11.8, 6.6 Hz, 4H), 1.82 (s, 6H), 1.08 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz, CD_3OD) δ 179.32 , 162.71 , 155.09, 154.96 , 146.99 , 144.34, 143.46 , 143.02, 134.66 , 130.34 , 129.52 , 129.33 , 128.58 , 123.93 , 118.48 , 117.10 , 115.75 , 114.16 , 105.36 , 105.11 , 64.43 , 61.29, 55.91, 52.11 , 47.80, 43.89, 42.38, 38.33, 18.84 , 17.40, 13.26 , 11.73 . ESI-MS, m/z calcd. for AX-S ($C_{33}H_{39}N_2O_4S_2^+$, $[M]^+$): 591.23; found: 591.2334 (Fig. S1-S4).

2.3 Synthesis of probe AX-S-Cb.

Cb (82 mg, 0.27 mmol) and DCC (67 mg, 0.32 mmol) were dissolved in dried $CHCl_3$ (10 mL), and the mixture was stirred at 0 °C. After 30 min, the mixture was added to a solution of AX-S (48.4 mg), and the resulting solution was stirred for 48 h at room temperature in the dark. Then the reaction mixture was filtered to remove white solid (dicyclohexylurea) and the filtrate was concentrated under vacuum. The crude product was purified by column chromatograph using dichloromethane (CH_2Cl_2) and methanol (CH_2Cl_2: MeOH, 20:1 v/v) as the eluent. The product was collected and the solvent was removed by rotary evaporation to afford (*E*)-2-(2-(6-(((2-((2-((4-(4-(bis(2-chloroethyl)amino)phenyl) butanoyl)oxy)ethyl)disulfaneyl)ethoxy)

carbonyl)amino)-2,3-dihydro-1*H*-xanthen-4-yl)vinyl)-3,3-dimethyl-1-propyl-3*H*-indol-1-ium (**AX-S-Cb**) as a mazarine solid (27.6 mg, yield 38 %).

^{1}H NMR (600 MHz, CD_3OD) δ 8.70 (d, *J* = 14.9 Hz, 1H), 7.86 (s, 1H), 7.64 (d, *J* = 7.4 Hz, 1H), 7.56 (d, *J* = 7.9 Hz, 1H), 7.53 (t, *J* = 7.6 Hz, 1H), 7.46-7.43 (m, 1H), 7.41 (d, *J* = 8.5 Hz, 1H), 7.32 (s, 1H), 7.20 (dd, *J* = 8.4, 1.9 Hz, 1H), 6.98 (d, *J* = 8.6 Hz, 2H), 6.60 (d, *J* = 8.6 Hz, 2H), 6.51 (d, *J* = 14.9 Hz, 1H), 4.42 (t, *J* = 6.4 Hz, 2H), 4.33 (dd, *J* = 10.8, 4.5 Hz, 4H), 3.66 (t, *J* = 6.9 Hz, 4H), 3.60 (t, *J* = 6.6 Hz, 4H), 3.23 (q, *J* = 7.4 Hz, 4H), 3.04 (t, *J* = 6.4 Hz, 2H), 2.75 (d, *J* = 5.8 Hz, 2H), 2.68 (s, 2H), 2.48 (t, *J* = 7.5 Hz, 2H), 2.29 (t, *J* = 7.3 Hz, 2H), 1.93 (dd, *J* = 14.3, 6.9 Hz, 4H), 1.80 (s, 6H), 1.08 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz, CD_3OD) δ 179.38 , 175.04 , 164.88 , 162.87 , 155.20 , 155.01 , 147.11 , 146.04 , 144.38 , 143.46 , 143.05 , 134.71 , 131.11 , 130.65 , 130.31, 129.50, 128.55, 126.32, 123.89, 118.56 , 117.21 , 115.77 , 114.10 , 113.51, 105.09 , 64.28, 63.39 , 55.91, 54.54 , 52.09, 43.87, 41.80 , 38.51, 35.03 , 34.36, 28.47 , 27.96, 22.37, 18.79, 17.36 , 13.21, 11.69 . ESI-MS, m/z calcd. for AX-S-Cb ($C_{47}H_{56}Cl_2N_3O_5S_2^+$, $[M]^+$): 876.30; found: 876.3016 (Fig. S5-S7).

2.4 Preparation of Nanoprobe NAX-S-Cb.

The nanoparticle was prepared by the procedures reported in literature.[3] mPEG-DSPE (6.7 mg) was dissolved in ultrapure water (10 mL) and sonicated for 20 min. After that, the **AX-S-Cb** (0.08 mg) was rapidly poured into the mixture and sonicated for another 20 min. Then the solution was filtered with a polyvinylidene fluoride (PVDF) syringe (0.22 μm) and dialyzed with ultrapure water for 12 h to remove free dyes. Eventually, the concentration of nanoparticle **NAX-S-Cb** was determined according to the UV-vis standard absorption curve.

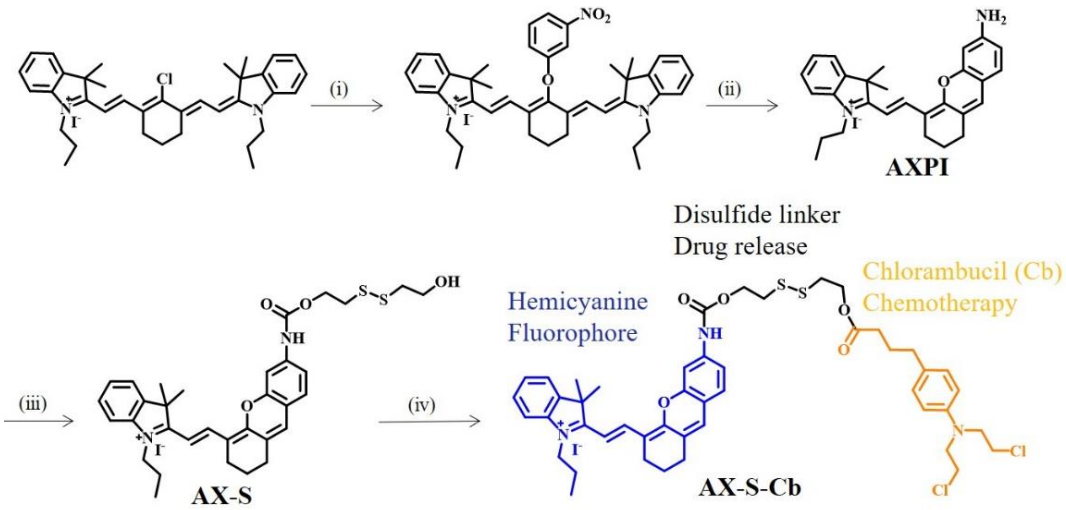

Fig. S1 Synthesis of AX-S and AX-S-Cb. Reagents and conditions: (i) 3-Nitrophenol, K_2CO_3, CH_3CN, rt, 4 h; (ii) $SnCl_2$, HCl, CH_3OH, 70℃, overnight; (iii) triphosgene, DIEA, 2,2'-dithiodiethanol, CH_3CN, rt, 8 h; (iv) Cb, DCC, DMAP, CH_3CN, rt, 48 h.

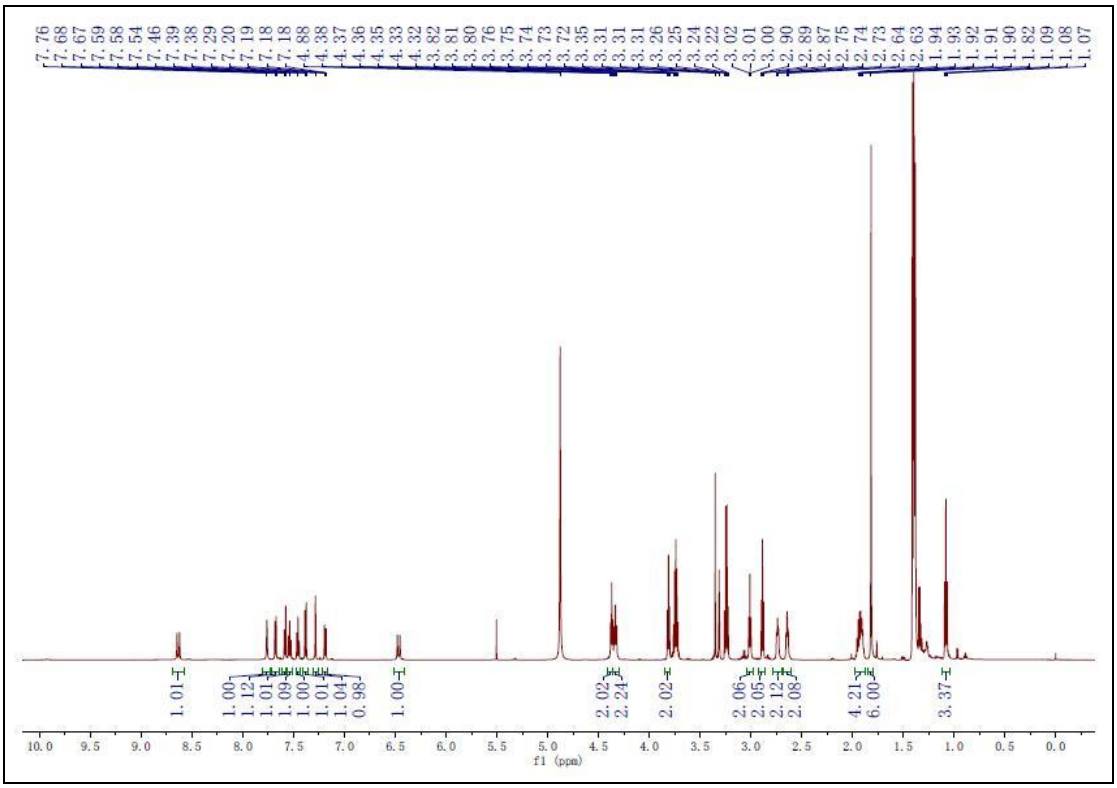

Fig. S2 ^{1}H NMR spectrum of AX-S in CD_3OD

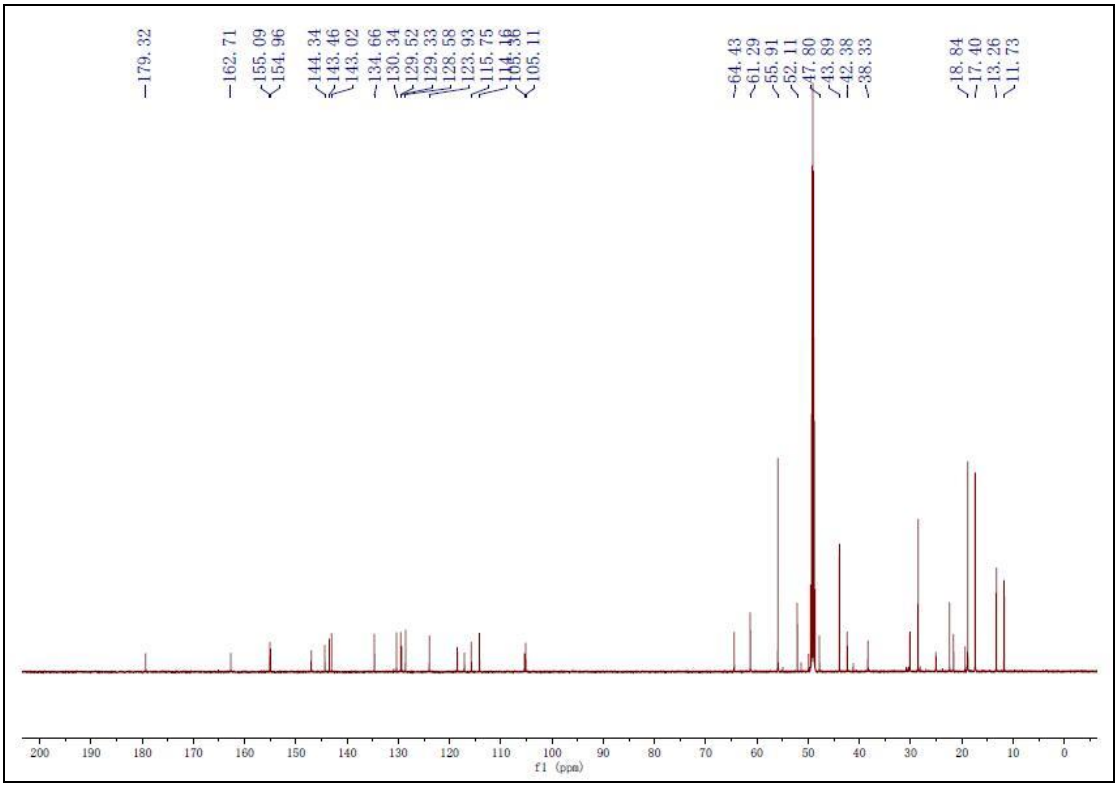

Fig. S3 ^{13}C NMR spectrum of AX-S in CD_3OD.

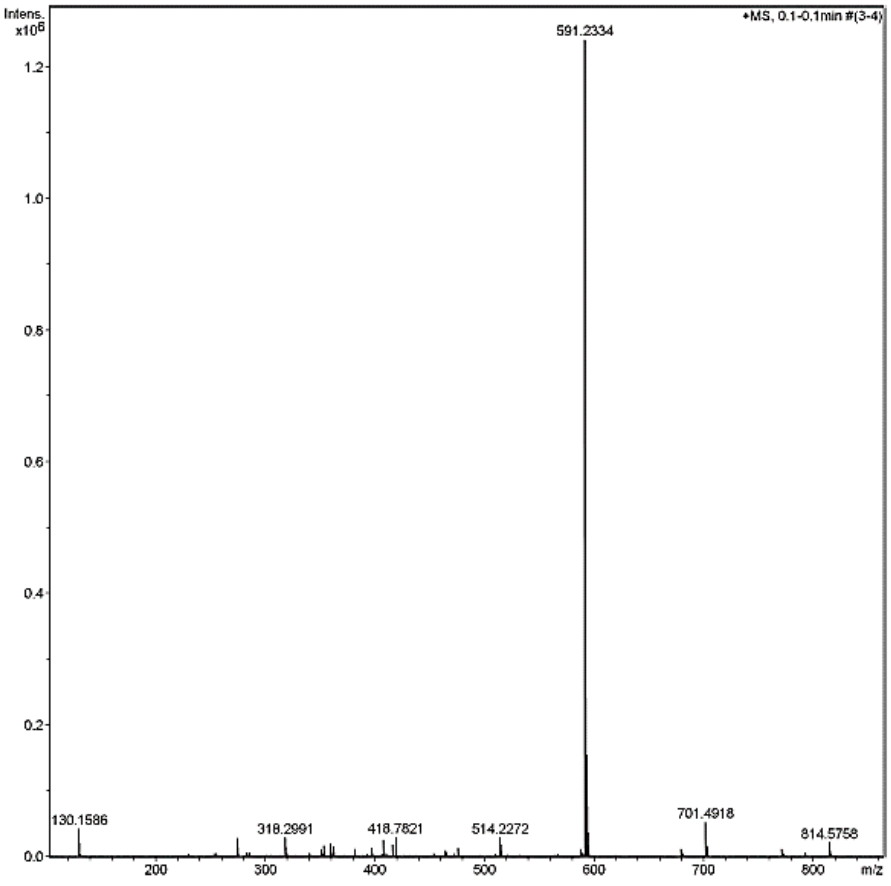

Fig. S4 Electrospray ionization mass spectrum of AX-S.

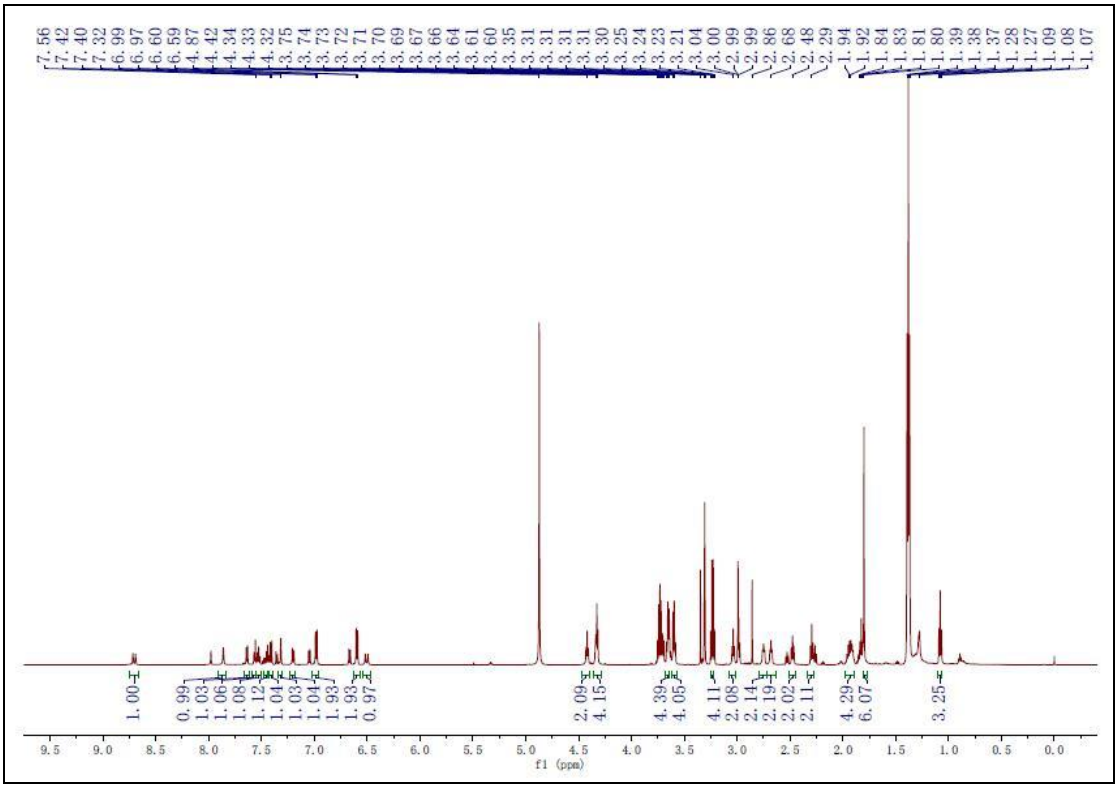

Fig. S5 ^{1}H NMR spectrum of **AX-S-Cb** in CD_3OD.

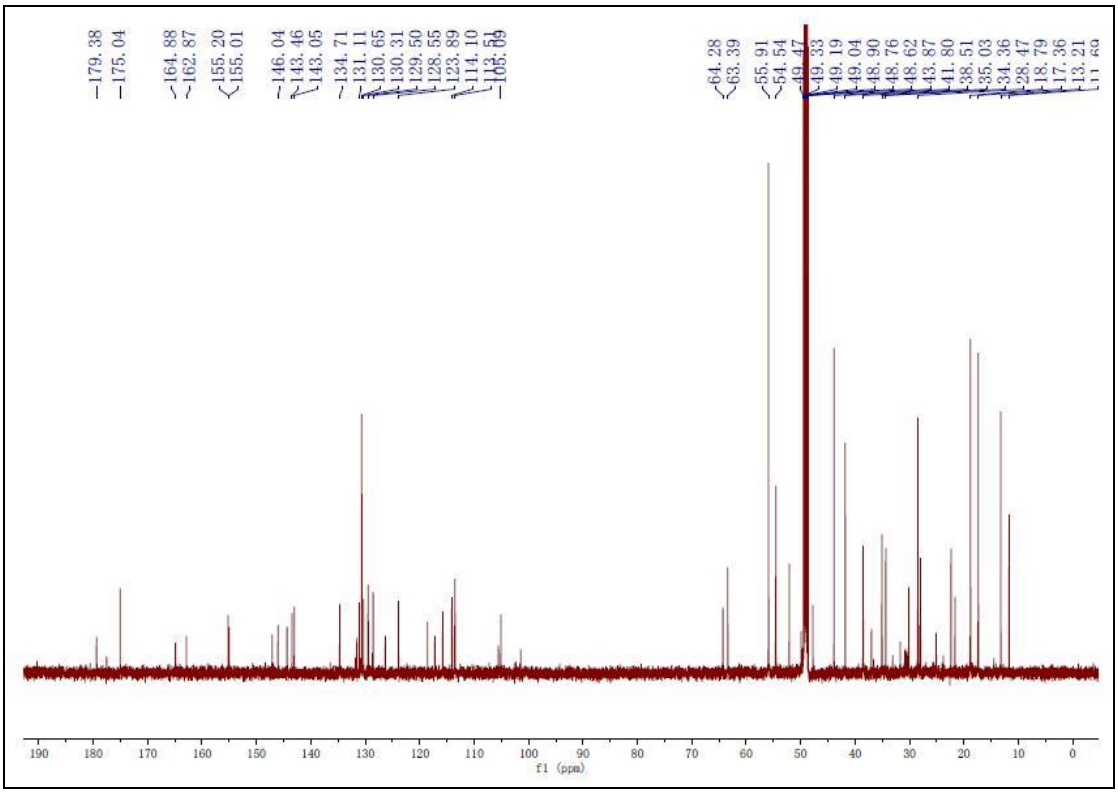

Fig. S6 ^{13}C NMR spectrum of **AX-S-Cb** in CD_3OD.

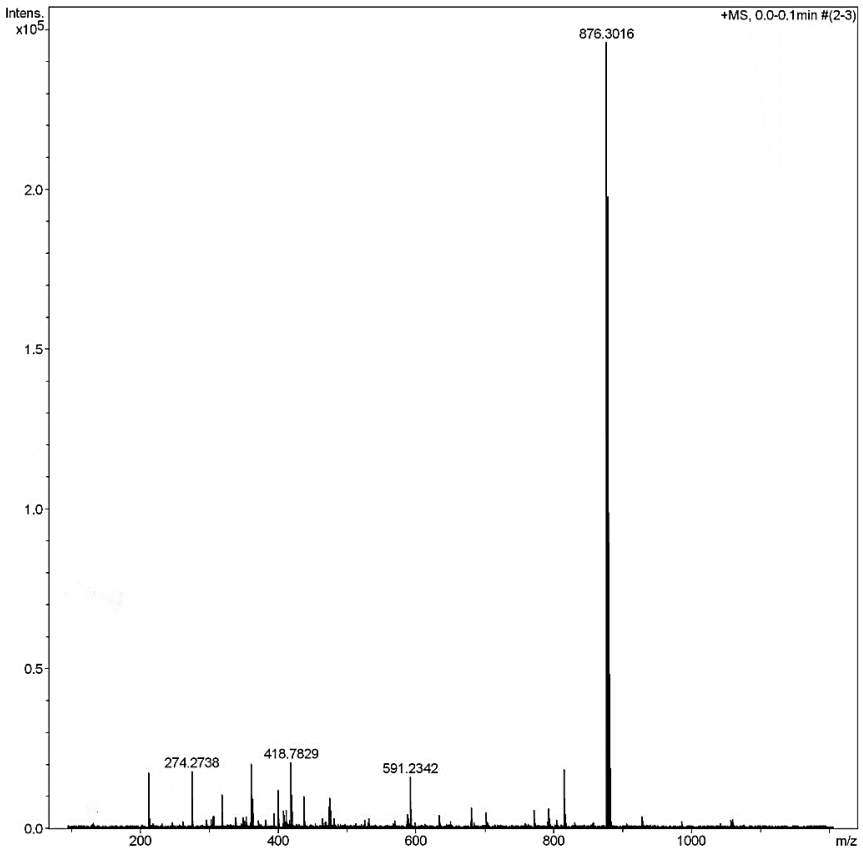

Fig. S7 Electrospray ionization mass spectrum of **AX-S-Cb**.

3. General Procedure for AX-S-Cb or NAX-S-Cb Assay

Fluorescence and absorption spectrums were obtained with a HITACHI F-7000 Fluorescence Spectrometer and Hitachi U-3010 spectrophotometer respectively. After dilution to 10 μM of the **AX-S-Cb** or **NAX-S-Cb** with PBS buffer solution (10 mM, pH=7.4), various amounts of GSH and other analytes were added for measurements. The solution containing no analyte was measured as comparison.

4. Electrospray ionization mass spectrum of the reaction solution of AX-S-Cb

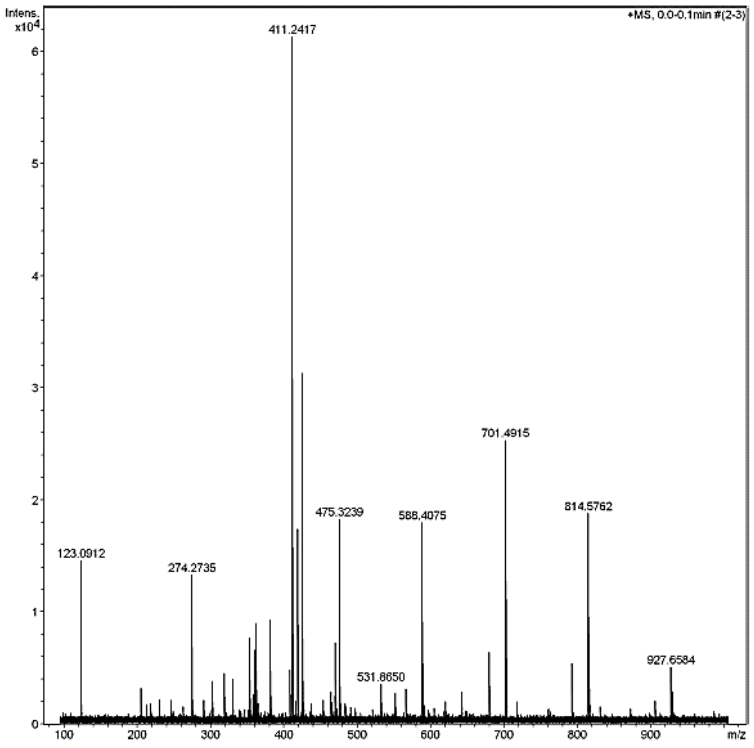

Fig. S8 ESI-MS of the reaction solution of **AX-S-Cb** (10 μM) with GSH (2.4 mM).

5. Fluorescence curves of AX-S-Cb reacting with GSH

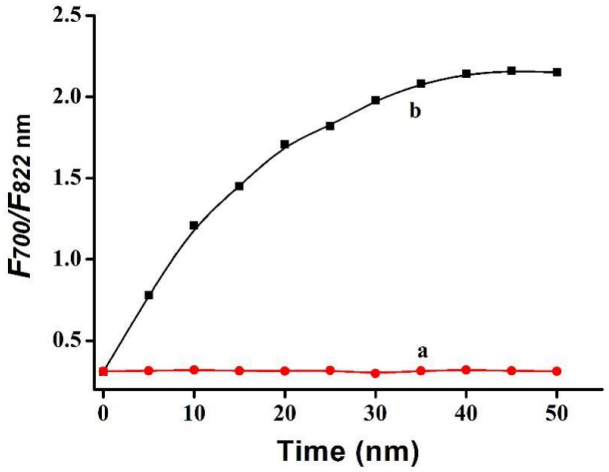

Fig. S9 Plots of fluorescence intensity of **AX-S-Cb** (10 μM) *vs.* the reaction time in the presence of GSH (2.4 mM): **AX-S-Cb** only (a), reaction soulution (b). The measurements were performed at 37 °C in 10 mM PBS (pH 7.4).

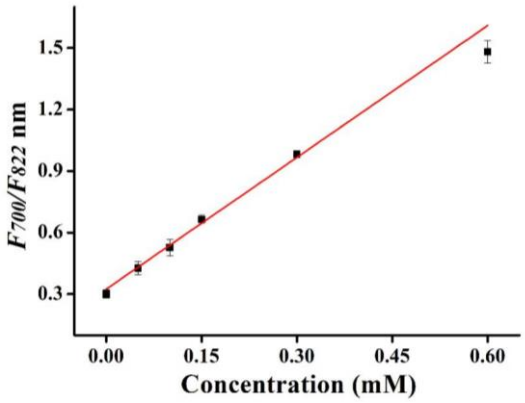

Fig. S10 The linear fitting curve of F_{700}/F_{822} nm towards the concentration of GSH between 0-0.6 mM.

6. TEM imaging and stability of NAX-S-Cb

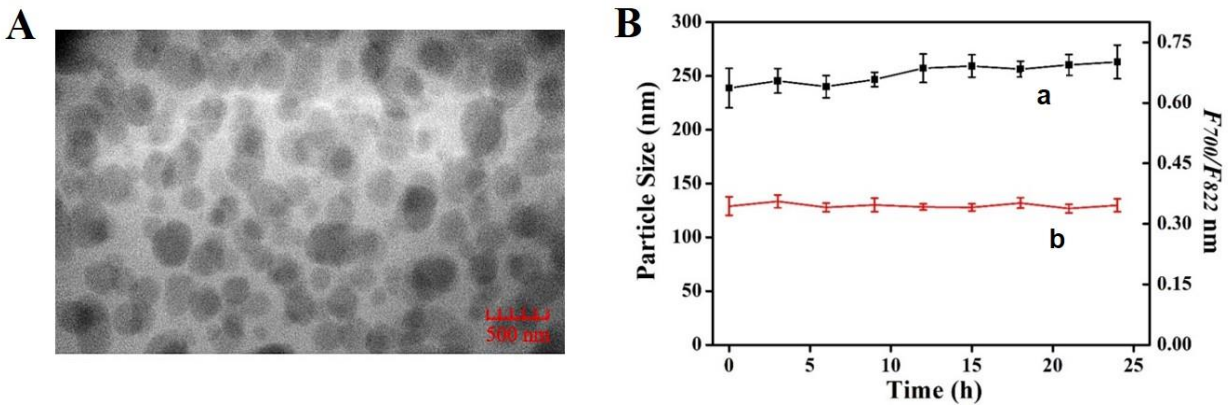

Fig. S11 (A) TEM imaging of **NAX-S-Cb**. (B) a: The average diameter fluctuation of the nanoprobe stored in PBS for different time periods, the error bars represent the standard deviation of three separate measurements; b: Time dependent fluorescence ratio (F_{700}/F_{822} nm) of **NAX-S-Cb** in PBS (10 mM, pH 7.4)

7. Selectivity of NAX-S-Cb

The testing analytes including inorganic salts ($ZnSO_4$, $CaCl_2$, $MgCl_2$, $FeCl_3$), common species (serine, lactose, tyrosine, arginine), reactive oxygen species (H_2O_2, •OH, $ONOO^-$, ClO^-), some reducing species and enzymes as ascorbic acid (Vc), $FeCl_2$, dithiothreitol (DTT), cysteine (Cys) and homocysteine (Hcy).

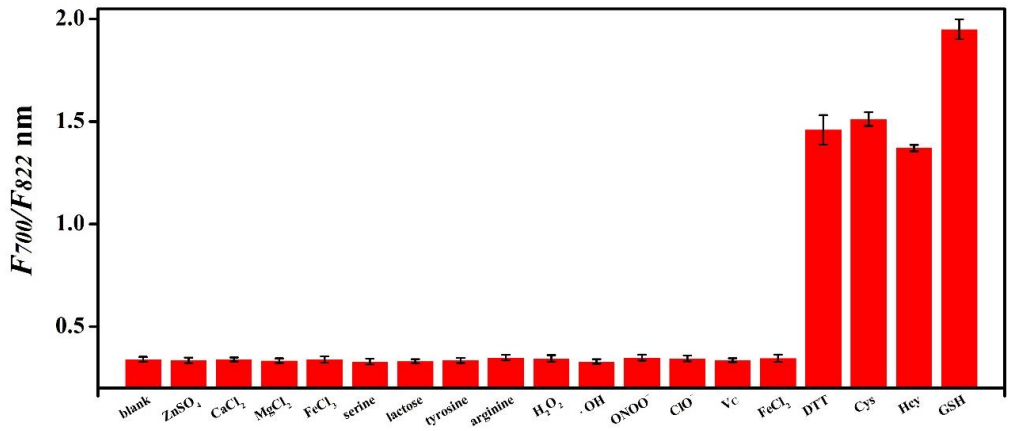

Fig. S12 Fluorescence responses of **NAX-S-Cb** (10 μM) to diverse analytes: Control (blank), $ZnSO_4$ (1 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), $FeCl_3$ (1 mM), serine (1 mM), lactose (1 mM), tyrosine (1 mM), arginine (1 mM), H_2O_2 (100 μM), •OH (100 μM), $ONOO^-$ (100 μM), ClO^- (100 μM), Vc (1 mM), $FeCl_2$ (1 mM), DTT (1 mM), Cys (1 mM), Hcy (1 mM) and GSH (2.4 mM). The results are the mean ± standard deviation of three separate measurements. λ_{ex}=670 nm.

8. Cytotoxicity assay

Standard MTT assay was performed to determine the cytotoxicity of **NAX-S-Cb**, **AX-S-Cb** and Cb towards HeLa, HepG-2 cells and HUVEC cells respectively.[4]

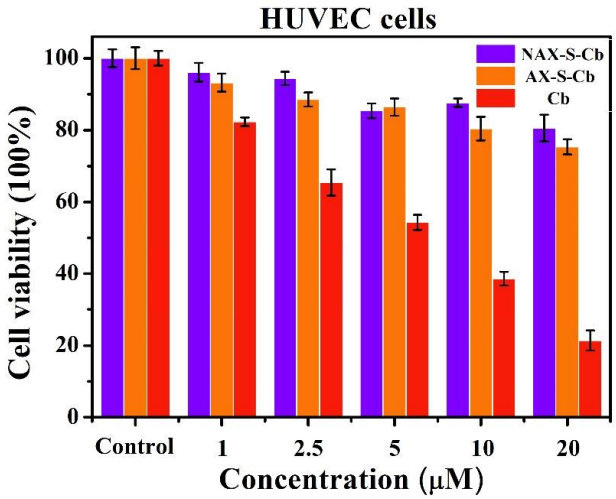

Fig. S13 Effects of Cb, **AX-S-Cb** and **NAX-S-Cb** with varied concentrations (1-20 μM) on the viability of HUVEC cells. The viability of the cells without respective analyses is defined as 100%. The results are the mean ± standard deviation of six separate measurements.

9. Fluorescence Imaging in Cells

HeLa and HUVEC cells were provided by key laboratory of analytical chemistry for life science of Shaanxi Province, Shaanxi normal University, China. HeLa cells and HUVEC cells were cultured in DMEM media, supported with 10% FBS, 100 U/mL penicillin, and 1% L-glutamine in a humidified 5% CO_2/95% air incubator at 37 ℃. Prior to imaging, the medium was removed, the cells were incubated with 10 μM **NAX-S-Cb** in DMEM solution for 30 min at 37 ℃. After washing with PBS solution, the imaging was performed with confocal laser scanning microscope (Leica, Germany) with 635 nm excitation. Image J software was used to analyze the pixel intensity of the cells. In doing so, the pixel intensity of 10 cells at least was averaged. All experiments were done in triplicate and the error bars refer to the standard error of mean of the triplicate experiments.

10. Fluorescence imaging in mice

BALB/c nu/nu mice (6 wk, 20-25 g) were obtained from Wuhan servicebio technology Co. Ltd. During procedure, they were kept under SPF conditions with free access to standard food and water. For tumor imaging experiments, HeLa tumor-bearing mice preparation procedures were in accordance with guidelines for Care and Use of Laboratory Animals of Shaanxi Normal University and approved by the Animal Ethics Committee of Shaanxi Normal University. Mice used in this study were established by orthotopically injection into the right quadrant of the abdomen of mice with HeLa cells (10^6 cells/0.1 mL/flank) suspension. After tumor size approximately 7-10 mm in the greater diameter, **NAX-S-Cb** was injected into tumor bearing mice through tail vein and the mice were imaged after injection at 15 min, 30 min, 1 h, 2 h, 4 h and 8 h by IVIS Lumina LT Series imaging system. The mice were kept on the imaging stage under anesthetized condition with isoflurane gas in oxygen flow during the imaging process.

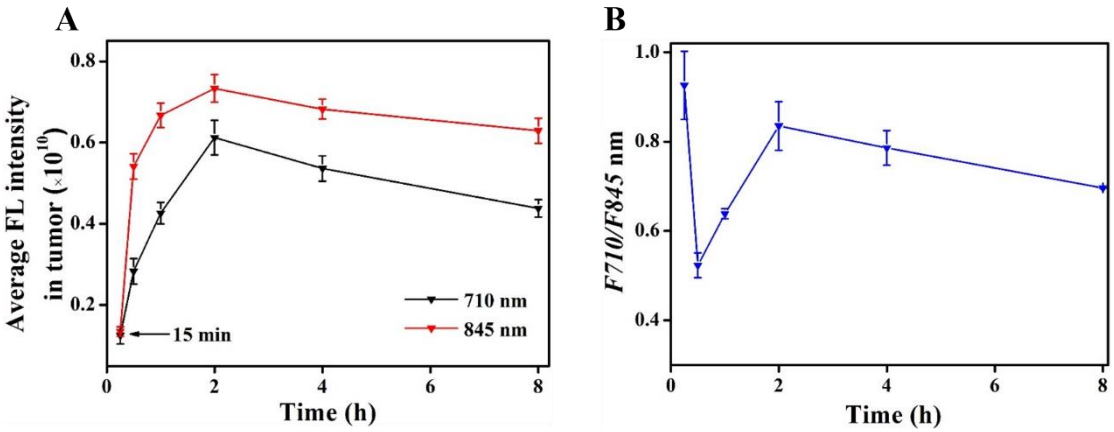

Fig. S14 (A) Fluorescence intensity increase in the tumor region at 710 nm and 845 nm as a function of postinjection time of **NAX-S-Cb**. (B) Fluorescence ratio (F_{710}/F_{845} nm) as a function of postinjection time.

11. Histochemical analysis

Similar to the procedures reported in literatures.[5] The mice were sacrificed after 20 days of treatment. The heart, liver, spleen, lung and kidney were fixed in 4% paraformaldehyde, and then paraffin embedded sectioning was conducted for hematoxylin and eosin (H&E) staining under standard protocols, and observation by optical microscope.

References

(1) W. Chen, T. Matsunaga, D. L. Neill, C. T. Yang, T. Akaike, M. Xian, *Angew. Chem. Int. Ed.*, 2019, **58**, 16067-16070.

(2) X. Y. He, Y. Hu, W. Shi, X. H. Li and H. M. Ma, *Chem. Commun.*, 2017, **53**, 9438-9441.

(3) S. Yuan, F. Wang, G. Yang, C. Lu, J. Nie, Z. Chen, J. Ren, Y. Qiu, Q. Sun, C. Zhao and W. H. Zhu, *Anal. Chem.*, 2018, **90**. 3914-3919.

(4) Z. Li, X. He, Z. Wang, R. Yang, W. Shi and H. Ma, *Biosens. Bioelectron.*, 2015, **63**, 112-116.

(5) L. Lin, X. Liang, Y. Xu, Y. Yang, X. Li and Z. Dai, *Bioconjugate Chem.*, 2017, **28**, 2410-2419.