Supporting Information

A hydrogen peroxide activated near-infrared ratiometric fluorescent probe for ratio imaging *in vivo* †

Bing Zheng,[ab] Shulong Wang,[*ab] Lixian Huang,[a] Jiayao Xu,[ab] Yanni Luo,[a] Shulin Zhao[*a]

[a] State Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Pharmaceutical Sciences, Guangxi Normal University, Guilin 541004, China. E-mail: zhaoshulin001@163.com

[b] Guangxi Key Laboratory of Agricultural Resources Chemistry and Biotechnology, College of Chemistry and Food Science, Yulin Normal University, Yulin 537000, China. E-mail: shulonghs@163.com

Materials and reagents

Dichloromethane ($C_2H_2Cl_2$), methanol (CH_3OH), ethyl acetate, anhydrous sodium sulfate, ethanol, acetone, trichloromethane, N,N'-dimethylformamide (DMF), trichloromethane, cyclohexanone, phosphorous tribromide, boron tribromide, 2-hydroxy-4-methoxybenzaldehyde, 4-bromomethylphenylboronic acid pinacol ester were purchased from Aladdin (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were obtained from ExCell Biology Co., Ltd. (Shanghai, China). 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2- H-tetrazolium bromide (MTT) was purchased from Biyuntian Biotechnology Co., Ltd. (Shanghai, China). All chemicals were used without further purification.

Instruments & equipment

The Cary Eclipse fluorescence spectrometer (Agilent Technologies, USA) was used to conduct fluorescence spectroscopy. A UV-vis spectrophotometer (Agilent Technologies) was used to conduct absorption spectroscopy. Powder X-ray diffraction (PXRD) was performed with a D8 ADVANCE diffractometer. XPS analysis was performed with an ESCALAB 250Xi X-ray photoelectron spectrometer (Thermo Fisher Scientific). Proton (^1H) or ^{13}C nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker Avance III HD 400 MHz NMR spectrometer (Bruker BioSpin GmbH, Ettlingen, Germany). MS analysis was performed on an Exactive liquid chromatography-mass spectrometry (LC-MS) system (Thermo Fisher Scientific. Inc., Waltham, MA, USA). Cellular fluorescence imaging was observed with an inverted optical microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Synthesis of carbon dots

A precise amount of 0.6854 g of glutathione was meticulously weighed and subsequently introduced into a high-pressure reactor that already contained 40 mL of formamide. The reactor was then positioned inside an oven and subjected to a reaction

temperature of 160 °C for 3 h. Following the completion of the reaction, the reactor was allowed to cool down to room temperature. Subsequently, the reaction mixture was centrifuged using a centrifuge to separate the components. The supernatant was collected and further purified by passing it through a 0.22 μm filter membrane to eliminate larger particles. To further refine the product, dialysis was performed using a dialysis bag with a molecular weight cut-off (MWCO) of 1000 Da in ultra-pure water for a period of one week, until the water became essentially colorless. Ultimately, green carbon dots (GCDs) were successfully obtained.

Synthesis of organic small-molecule probe (HCy-H_2O_2)

The synthetic route for the organic small molecule probes was shown in Fig. S1.

The synthesis of the compound Y-COOH was initiated by adding 2,3,3-trimethyl-3H-indole (0.5 M, 1.6 g) and 6-bromoacetic acid (0.6 M, 2.34 g) to a 50 mL round-bottomed flask. Subsequently, 20 mL of o-dichlorobenzene was introduced into the flask. The reaction mixture was then heated to 100 °C under a nitrogen atmosphere and maintained at this temperature for 24 h. Upon completion of the reaction, the mixture was cooled to room temperature, allowing solid precipitation to occur. The solids were collected by filtration and washed with ether to remove any impurities. The precipitate was then spun dry to yield a purple viscous solid. Appropriate amount of acetone was added, ultrasonic treatment, and then filtered and washed to obtain a pure white solid. No further purification is required for the next step.

The synthesis of the compound HDXC-B was carried out by placing HDXC (228 mg) and 4-bromomethylphenylborate pinacol ester (445.5 mg) in a 50 mL single-neck round-bottom flask. To this mixture, 25 mL of anhydrous acetone was added. Subsequently, K_2CO_3 (138 mg) and a suitable amount of potassium iodide were introduced into the flask. The reaction mixture was then refluxed under the protection of N_2 for 6 h. The completion of the reaction was monitored using thin-layer chromatography (TLC) spot plate analysis. Once the reaction was deemed complete, the mixture was cooled to room temperature. Insoluble impurities were removed by filtration, and the solvent was evaporated under reduced pressure to obtain a yellow crude product. To purify the crude product, a silica gel chromatography column was

employed. The purified product was collected and resulted in a yellow solid with a yield of approximately 43%. ^{1}H NMR (400 MHz, Chloroform-d) δ 10.27 (s, 1H), 7.86–7.78 (m, 2H), 7.39 (dd, J = 20.1, 7.9 Hz, 3H), 7.09–7.04 (m, 1H), 6.75–6.68 (m, 2H), 6.63 (s, 1H), 5.28 (s, 1H), 5.10 (s, 2H), 2.59–2.50 (m, 2H), 2.43 (t, J = 6.0 Hz, 2H), 1.70 (p, J = 6.0 Hz, 2H), 1.34 (d, J = 1.7 Hz, 16H), 1.28 (s, 2H), 1.24 (d, J = 6.0 Hz, 2H) (Fig. S2). ^{13}C NMR (101 MHz, Chloroform-d) δ 161.03, 160.49, 153.39, 139.32, 127.61, 127.15, 126.79, 126.57, 115.03, 112.63, 111.72, 101.68, 83.97, 70.31, 65.24, 31.53, 21.59, 20.46. HRMS (ESI) m/z calcd for $C_{27}H_{29}BO_5$ (M+H): 444.2108, Found: 444.2134 (Fig. S3).

The synthesis of the compound HCy-H_2O_2 was conducted by placing HDXC-B (222 mg) and Y-COOH (274 mg) in a 50 mL single-neck round-bottom flask. To this mixture, 20 mL of anhydrous ethanol and 2 drops of piperidine were added. The reaction solution was then refluxed under N_2 protection at 80 °C for 10 h. The completion of the reaction was monitored using thin-layer chromatography (TLC) spot plate analysis. Upon completion of the reaction, the mixture was cooled to room temperature. To purify the crude product, a silica gel chromatography column was employed. The purified product was obtained as a blue solid in a yield of approximately 60%. HRMS (ESI) m/z calcd for $C_{44}H_{51}BNO_6^+$ $(M+H)^+$: 700.3839, Found: 700.3804 (Fig. S4).

The synthesis of the nanoprobe GCDs-HCy-H_2O_2 began by dissolving 7 mg of the compound HCy-H_2O_2 in 1.0 mL of DMSO to prepare a 5 mM stock solution. Subsequently, a specific quantity of the HCy-H_2O_2 stock solution was dissolved in water. To this solution, 0.1 g of EDC and 0.1 g of NHS were added, and the mixture was stirred for 1 h. Next, 100 μL of a GCDs solution was introduced into the reaction mixture and stirred overnight to ensure complete conjugation. Following the reaction, the product was dialyzed for several hours to remove any unreacted molecules and impurities, ultimately yielding the nanoprobe GCDs-HCy-H_2O_2.

Characterization of GCDs-HCy-H_2O_2

The optical properties of GCDs-HCy-H_2O_2 were measured by UV-vis spectrophotometer and fluorescence spectrophotometer. The morphology, surface

structure, particle size of GCDs and GCDs-H_2O_2 were characterized through TEM, XPS, XRD, FTIR, etc.

Spectral determination of H_2O_2

The absorption and fluorescence spectra of GCDs-HCy-H_2O_2 solution after reacting with different concentrations of H_2O_2 were measured using a UV-vis spectrophotometer and fluorescence spectrophotometer. To investigate the specificity of H_2O_2, other common ions, reactive oxygen and reactive sulphur were added under the same conditions and their spectral data were determined after 10 min of reaction.

Cytotoxicity of GCDs and GCDs-HCy-H_2O_2

The toxicity of GCDs and GCDs-HCy-H_2O_2 was determined by MTT method. After digesting the RAW24.7 cells, the cells were inoculated into 96-well plate and cultured in an incubator (37 °C, 5% CO_2). When the cell density reached about 90%, different concentrations of GCDs and GCDs-HCy-H_2O_2 were added to continue the culture for 24 h. Then, 20 μL of MTT solution was added to each well, respectively, and the incubation was continued for 4 h. The medium was discarded, and 100 μL of DMSO was added to each well, the well plates were shaken for 10 min. The well plates were put into an enzyme labeller to determine the absorbance at 490 nm.

Cell imaging

RAW246.7 cells were digested and inoculated into a confocal petri dish and cultured in an incubator (37 °C, 5% CO_2). When the cell density reached about 60%, the first group was incubated with nano-probe GCDs-HCy-H_2O_2 (10 μg/mL) for 30 min. The second group: H_2O_2 (150 μM) was added and cultured for 0.5 h, then washed with PBS for three times, and was incubated for 30 min with GCDs-HCy-H_2O_2 (10 μg/mL). The third group: PMA (1.0 mM) was added and cultured for 0.5 h, then washed with PBS for three times, then was incubated for 30 min with GCDs-HCy-H_2O_2 (10 μg/mL). The fourth group: PMA (1.0 mM) was added and cultured for 0.5 h, then treated with NAC (1.0 mM), finally incubated for 30 min with GCDs-

HCy-H_2O_2 (10 μg/mL). At the end of the incubation, the cells were washed three times with PBS and imaged using Zeiss LSM710 laser scanning confocal microscope system.

Fluorescence imaging of GCDs-H_2O_2 *in vivo*

Female BALB/c mice (6-8 weeks) were provided by Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). The animal handling procedures were approved by the Animal Ethics Committee of Guangxi Normal University (No. 202309-004). Ten mice were randomly divided into two groups of five. The first group received a subcutaneous injection of 100 μL of GCDs-HCy-H_2O_2 solution (20 μg/mL) and was subsequently placed into a small animal live imager for imaging. In the second group, LPS (100 μL, 2.5 mg/mL) was first injected to construct an LPS-induced acute inflammation mice model. After 4.0 h, GCDs-HCy-H_2O_2 solution (100 μL, 20 μg/mL) was injected intraperitoneally, and then imaging was performed. The fluorescence of GCDs was collected using 700 nm filter, while the fluorescence of dye HCy-OH was collected using 750 nm filter.

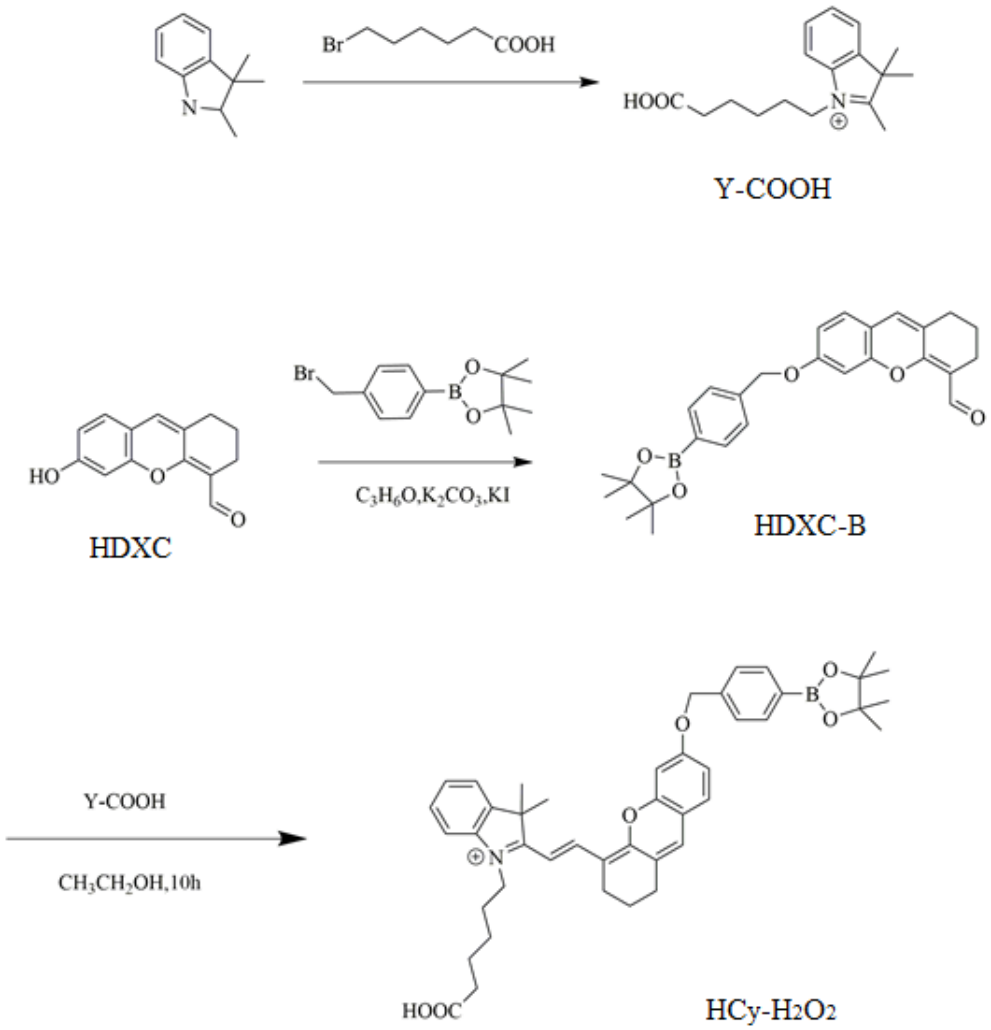

Fig. S1. Synthesis route of HCy-H_2O_2.

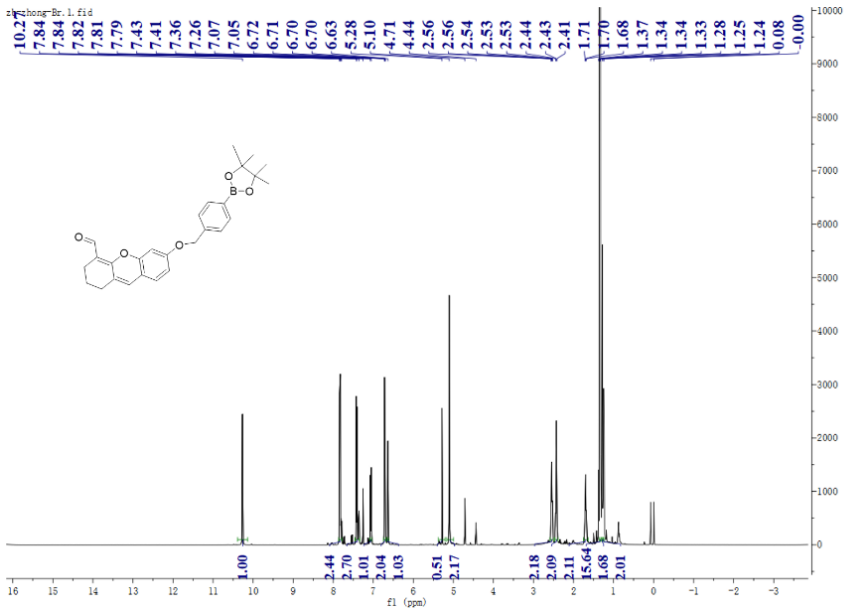

Fig. S2. [1] H NMR spectrum of HDXC-B.

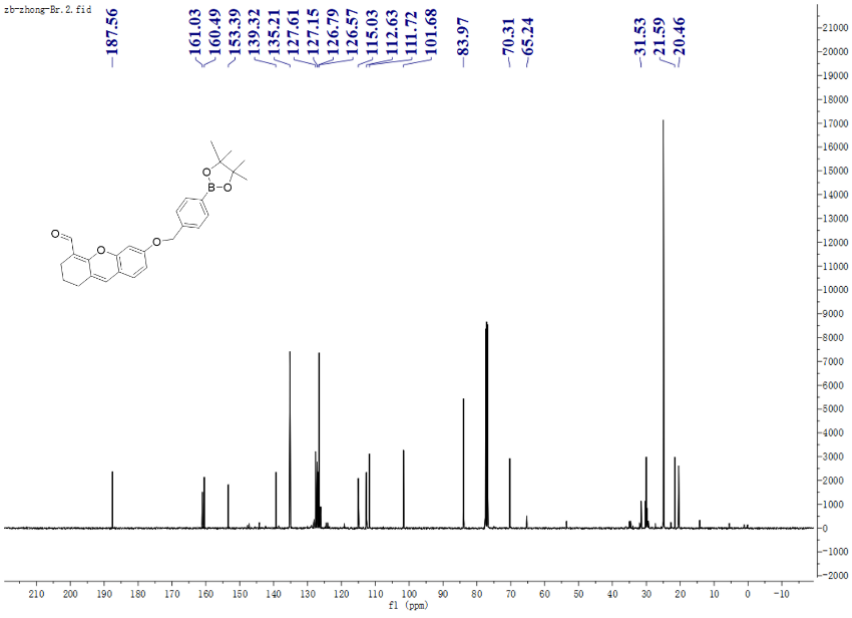

Fig. S3. [13] C NMR spectrum for HDXC-B.

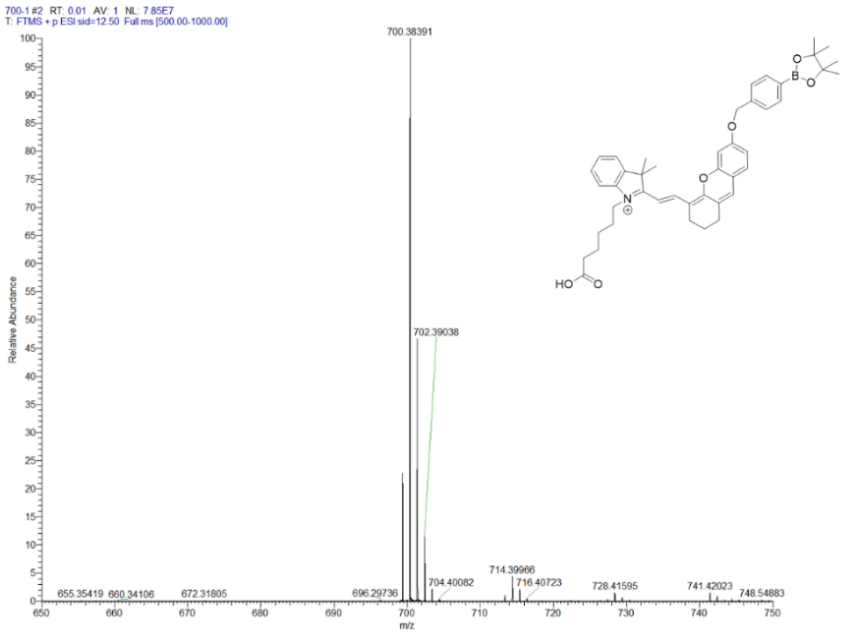

Fig. S4. HRMS of HCy-H_2O_2.

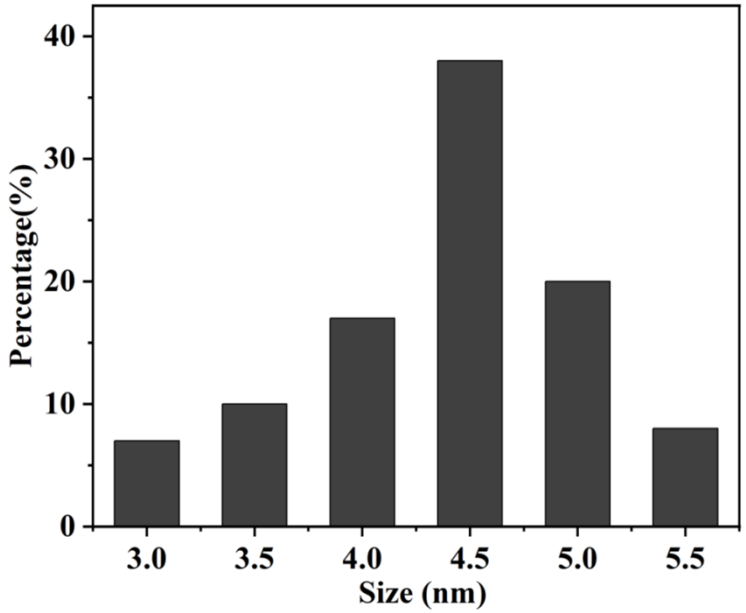

Fig. S5. The size of GCDs.

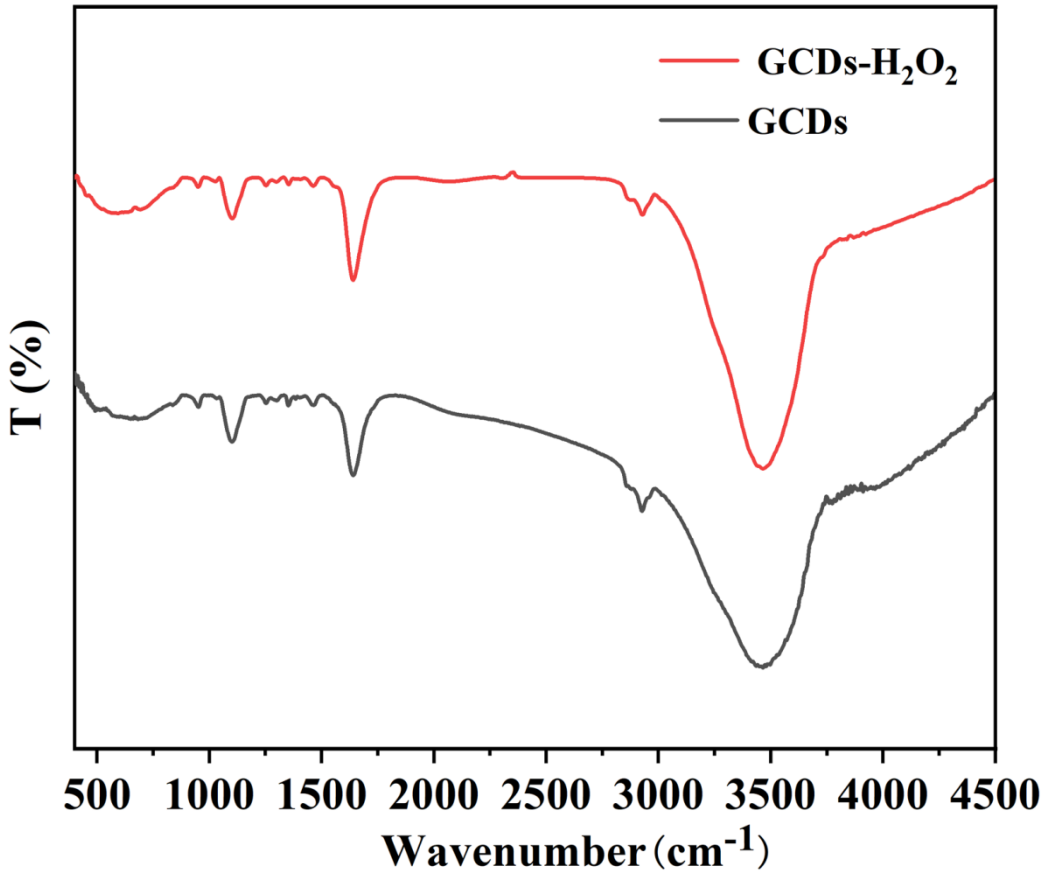

Fig. S6. FT-IR spectra of GCDs and GCDs-H_2O_2.

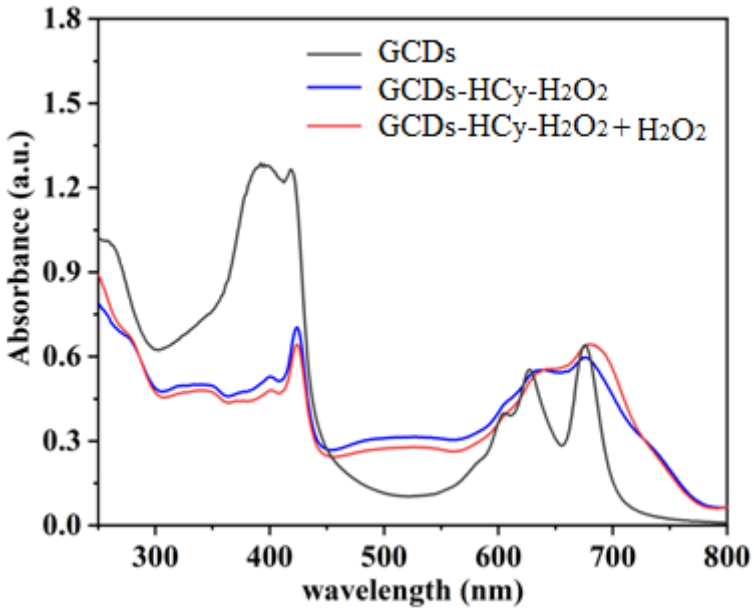

Fig. S7. Absorption spectra of GCDs, GCDs-HCy-H_2O_2 and GCDs-HCy-H_2O_2 reaction with H_2O_2.

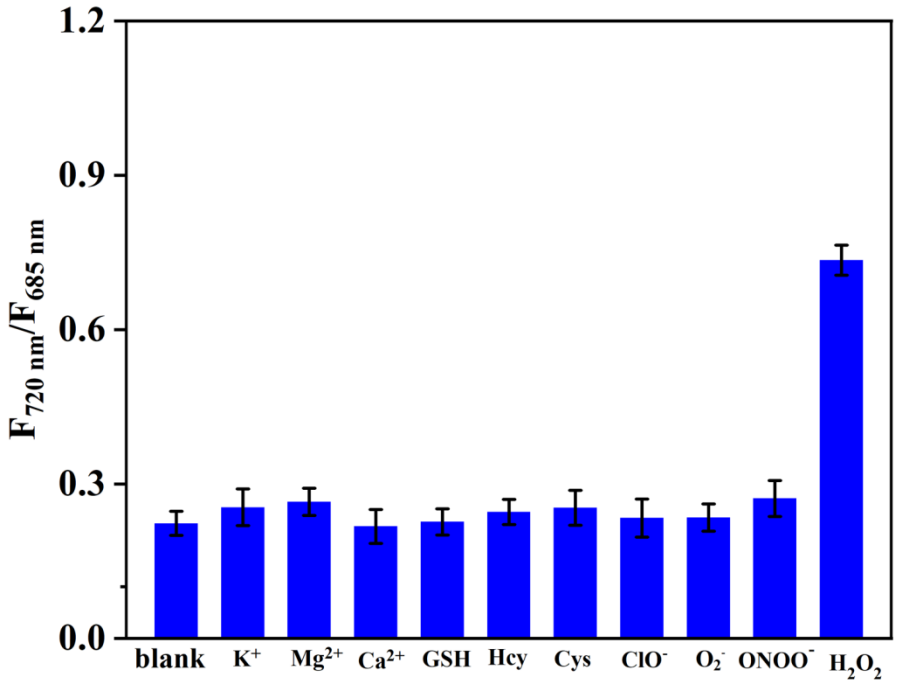

Fig. S8. The ratio of fluorescence intensities ($F_{720\ nm}/F_{685\ nm}$) of GCDs-HCy-H_2O_2 solution in the presence of different substance (200 μM each).

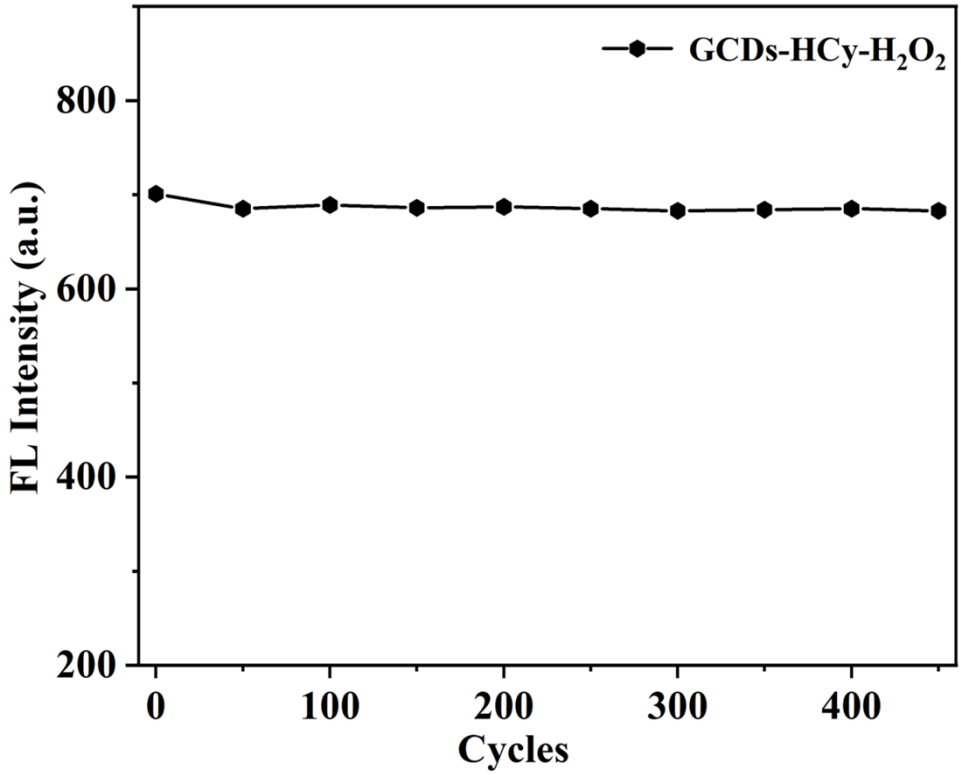

Fig. S9. Changes of Fluorescence (FL) signal intensity for GCDs-HCy-H_2O_2 solution (10 μg/mL, λ_{Ex} = 627 nm and λ_{Em} = 685 nm) under continuous cyclic scanning.

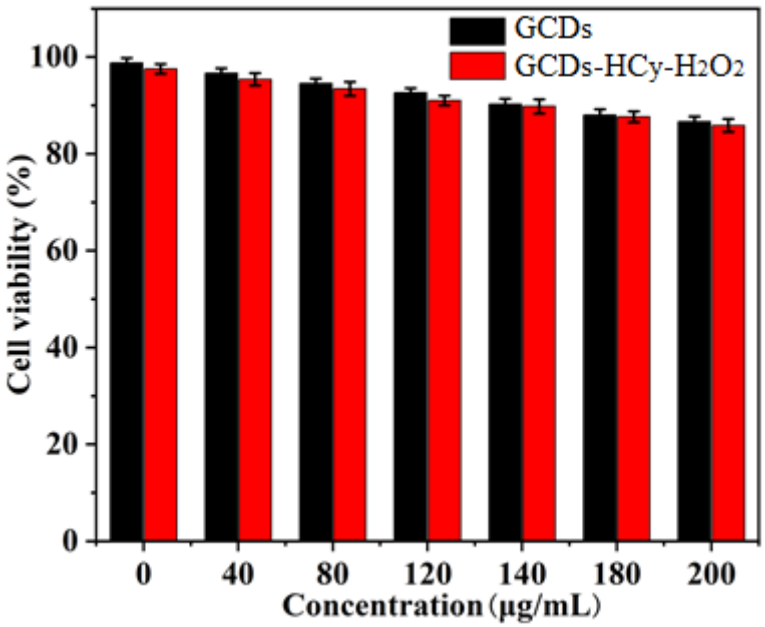

Fig. S10. Survival rate of cells under different concentrations of GCDs and GCDs-HCy-H_2O_2.