Switching between type I/II photosensitization processes for of an unconventional dye as modulated by the tumor microenvironment for inducing immunogenic death

Lei Hao,[a,b,#] Yumei Wang,[a,#] Shuang Zeng,[a,b] Zhuo Yang,[c] Saran Long,[a] Wen Sun,[a] Jingyun Wang,*[a,b] Xiaojun Peng[a] and Haidong Li*[a,b]

[a] State Key Laboratory of Fine Chemicals, Frontiers Science Center for Smart Materials Oriented Chemical Engineering, Dalian University of Technology, 2 Linggong Road, Dalian 116024, China. *E-mail: (H. Li) lihd@dlut.edu.cn; (J. Wang) wangjinyun67@dlut.edu.cn.

[b.] MOE Key Laboratory of Bio-Intelligent Manufacturing, School of Bioengineering, Dalian University of Technology, 2 Linggong Road, Dalian 116024, China.

[c.] Department of Gynecology, Cancer Hospital of Dalian University of Technology (Liaoning Province Cancer Hospital & Institute), 44 Xiaoheyan Road, Shenyang 110042, China.

# These authors contributed equally.

1.Materials

All chemical reagents were purchased directly from company and can be used without further purification. Nitroreductase (NTR) from Escherichia coli and NADH were purchased from Sigma-Aldrich. ^{1}H NMR spectra were characterized by Bruker Avance 600 MHz spectrometer, electrospray ionization mass spectra (ESI-MS) were recorded in LTQ mass spectrometry. UV-visible absorption and fluorescence emission spectra were measured with an Agilent (USA) spectrophotometer and a spectrofluorophotometer, respectively. Confocal laser scanning microscopy (CLSM) images were collected on a Zeiss LSM 980 (Germany) microscope. Absorbance measurements were carried out on a SpectraMax M2e (USA) plate reader.

2. Syntheses and characterizations

Synthesis of HCySe-OH.

Compound HCySe-OH was synthesized as described in the literature procedure[1].

Synthesis of HCySe-O-NO_2.

HCySe-OH (100 mg, 0.17 mmol), 4-Nitrobenzyl bromide (183.4 mg, 0.85 mmol), K_2CO_3 (117.3 mg, 0.85 mmol), and anhydrous acetonitrile (5 mL) were combined. The mixture was heated to 80 °C and stirred for 4 h. The solvent was then evaporated under reduced pressure. The crude product was purified by silica gel chromatography (CH_2Cl_2/ MeOH = 100:3), HCySe-O-NO_2 was received as blue-green solid (76 mg,0.127 mmol), yield: 62 % ^{1}H NMR (600 MHz, Methanol-d_4) δ 8.28 (d, J = 8.6 Hz, 3H), 7.73 (d, J = 8.8 Hz, 5H), 7.61-7.50 (m, 3H), 7.47 (d, J = 2.4 Hz, 1H), 7.14 (s, 2H), 6.83 (d, J = 14.6 Hz, 1H), 5.34 (s, 2H), 4.49 (q, J = 7.3 Hz, 2H), 2.85 – 2.81 (m, 2H), 2.71 (t, J = 6.2 Hz, 2H), 1.82 (s, 6H), 1.52 (t, J = 7.3 Hz, 3H). HRMS (ESI, m/z): $[M]^+$ calcd. for $C_{31}H_{30}NO_4^+$: 597.1651, found: 597.1660.

3. ROS generating ability test

Singlet oxygen ($^1O_2$): 1,3-Diphenylisobenzofuran (DPBF) was added to a sample solution (PBS) containing HCyX-OH or HCyX-O-NO_2 (10 μM) as a $^1O_2$ indicator (X = O/S/Se). The fluorescence spectra of DPBF were then measured after the mixed solution was exposed to 760 nm laser irradiation (20 mW/cm^2) at various intervals.

Hydroxyl radical (•OH): Hydroxyphenyl Fluorescein (HPF) was added to a sample

solution (PBS) containing HCyX-OH or HCyX-O-NO_2 (10 μM) as an indicator for hydroxyl radicals (X = O/S/Se). The fluorescence spectra of HPF were then measured after the mixed solution was exposed to 760 nm laser irradiation (20 mW/cm^2) for varying intervals.

4. Selective test

The PS was incubated with the following different substances at 37 °C for one hour, and the fluorescence intensity at 790 nm was measured: NaCl (500 μM), Ammonium chloride (500 μM), Sodium sulfate (500 μM), Zinc chloride (500 μM), Iron(III) chloride hexahydrate (500 μM), L-Ascorbic acid (500 μM), L-Glutamic acid (500 μM), L-Cystine (500 μM), L-Cysteine (500 μM), Glutathione (500 μM), Sodium acetate (500 μM), Sodium carbonate (500 μM), Sodium chloride (500 μM), Potassium chloride (500 μM), Glucose (500 μM), Citric acid (500 μM), L-Isoleucine (500 μM), L-Histidine (500 μM), L-Arginine (500 μM), L-Phenylalanine (500 μM), L-Threonine (500 μM) L-Aspartic acid (500 μM), and NADH (500 μM) + NTR (10 μg/mL).

5. Cell culture

The Human breast cancer cells (MCF-7) were acquired from the Institute of Basic Medical Sciences of the Chinese Academy of Science. DMEM was used to cultivate MCF-7 cells. This cancer cells were cultured in the above medium containing 10% fetal bovine serum and 1% antibiotics (penicillin/ streptomycin; 100 U mL^{-1}) at 37 °C in a 5% CO_2 atmosphere.

6. Confocal fluorescence imaging for cells

MCF-7 cells were plated at 1×10^5 cells per 35mm confocal dishes and incubated with HCySe-O-NO_2 (5 μM) for 0-1 h in the 2% O_2 and 21% O_2 respectively. Subsequently, confocal fluorescence imaging was conducted and corresponding images were acquired.

7. Cytotoxicity assay

MCF-7 cells were used in the experiments for cytotoxicity testing. The 96-well plates were inoculated with MCF-7, and the cells were incubated for 24 h. DMEM medium containing HCySe-O-NO_2 (0-5 μM) was added and incubated for a total of 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) medium (0.5 mg/mL)

was added to the 96-well plates containing MCF-7 cells and incubated for a total of 4 h. Then, the medium was removed. Dimethyl sulfoxide (DMSO, 100 μL) was added and the OD 570 nm and 630 nm reading was determined using a SpectraMax M2e (multifunctional plate reader), and cell viability was calculated according to the following formula:

$$\text{Cell viability (\%)} = (OD_{PS}\text{-}OD_{blank})/ (OD_{control}\text{-}OD_{blank}) \times 100$$

8. Intracellular ROS imaging

The production of •OH, and total ROS in MCF-7 cells was demonstrated using HPF, and DCFH-DA, respectively. MCF-7 cells were plated on 35 mm confocal dishes, incubated with DCFH-DA (10 μM) for 30 min, and then stained with HCySe-O-NO_2 (5 μM) for 30 min, using DCFH-DA as an example. The light group was exposed to 760 nm laser (50 mW/cm^2, 10 min) following washing with serum-free medium. Subsequently, confocal fluorescence imaging was conducted and the corresponding images were acquired. Conditions: excitation wavelength for DCFH-DA was 488 nm, and the emission wavelength ranged from 500–530 nm. For other ROS evaluations, the same procedure as for DCFH-DA measurement was followed. In addition, to simulate a hypoxic tumor environment, cells were cultured in an incubator chamber (MIC-101, Billups–Rothenberg) at 37 °C in a humidified, 2% O_2 and 5% CO_2 atmosphere while the oxygen content of the chamber was monitored using an oxygen detector (Nuvair, O_2 Qucikstick). Other operations were identical to those conducted in a normoxic environment.

9. LDH and ATP release assays

MCF-7 cells were plated at 1×10^5 cells per well in a 96-well cell-culture plate, followed by incubation at 37 °C for 24 h. Then the cells were incubated with 5 μM HCySe-O-NO_2 for 1h and washed with 100 μL fresh medium. The light groups were irradiated with 760 nm laser at a power density of 50 mW/cm^2 for 10 min and continued growing for 12 h. The LDH release were detected according to the instruction manual. MCF-7 cells were plated at 1×10^5 cells per well in a 96-well cell-culture plate, followed by incubation at 37 °C for 24 h. Then the cells were incubated with 5 μM HCySe-O-NO_2 for 1 h and washed with 100 μL fresh medium. The light groups were irradiated with

760 nm laser at a power density of 50 mW/cm^2 for 10 min and continued growing for 2 h. The ATP release was detected by Enhanced ATP Assay Kit (Beyotime).

10. CRT and HMGB1 staining in MCF-7 cancer cells

MCF-7 cells were plated onto 35 mm confocal dishes and incubated with 5 μM HCySe-O-NO_2 for 1h, then the cells were washed and irradiated by 760 nm laser irradiation (50 mW/cm^2) for 10 min. After 12 h, the cells were washed with pre-cooled 1×PBS, fixed with 4% paraformaldehyde on the ice for 1 min, and further incubated with Alexa Fluor 488 labelled CRT antibody or Alexa Fluor 488 labelled HMGB1 antibody for 1 h. The CRT and HMGB1 expression fluorescence images collected by CLSM with the excitation at 488 nm and signal acquisition in the range from 500 to 540 nm.

11. Supporting figures

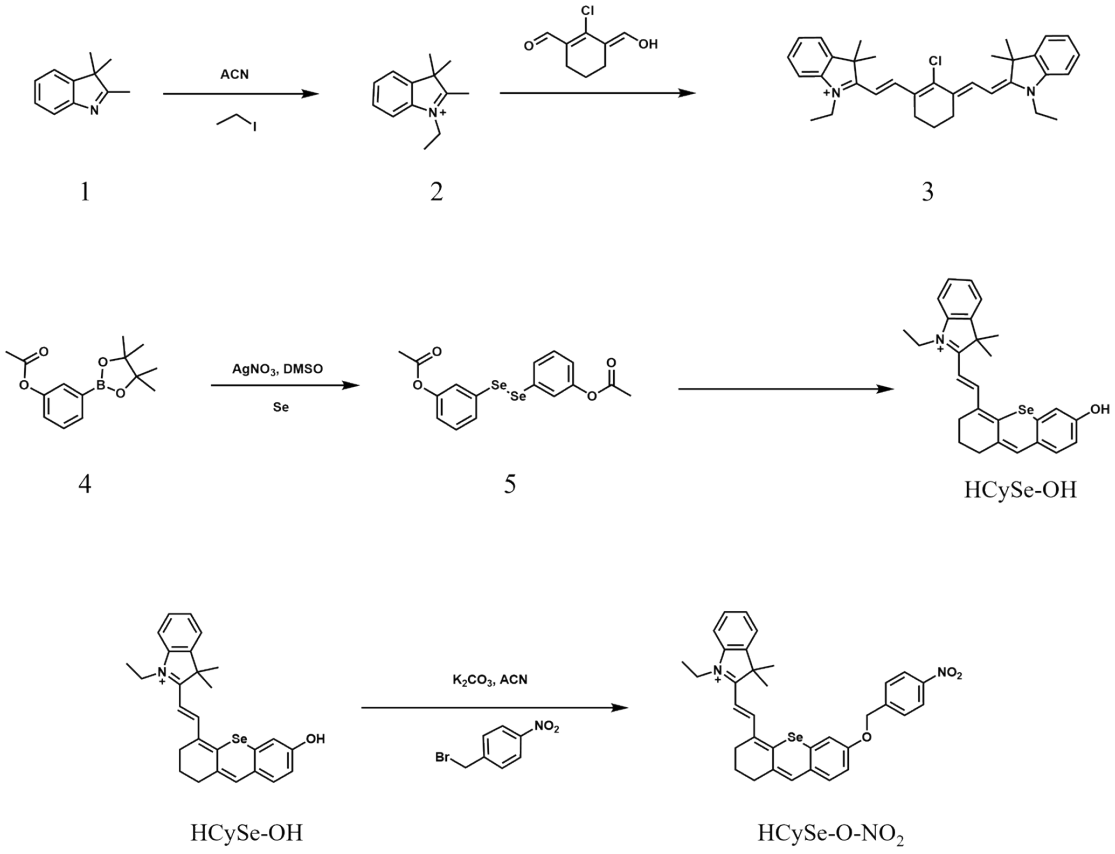

Figure S1. Synthetic routes of HCySe-O-NO_2.

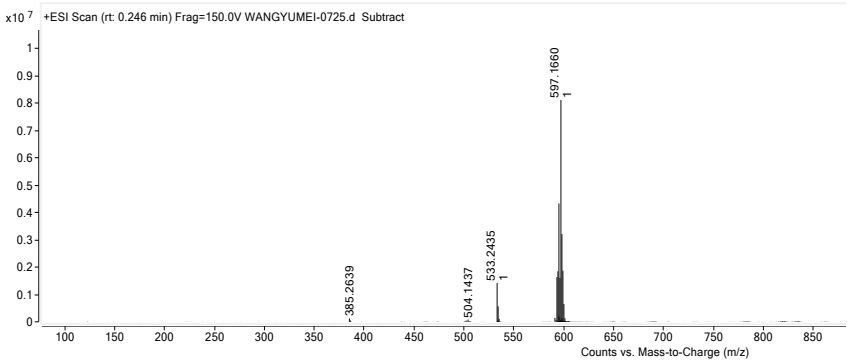

Figure S2. HRMS spectrum of HCySe-O-NO_2.

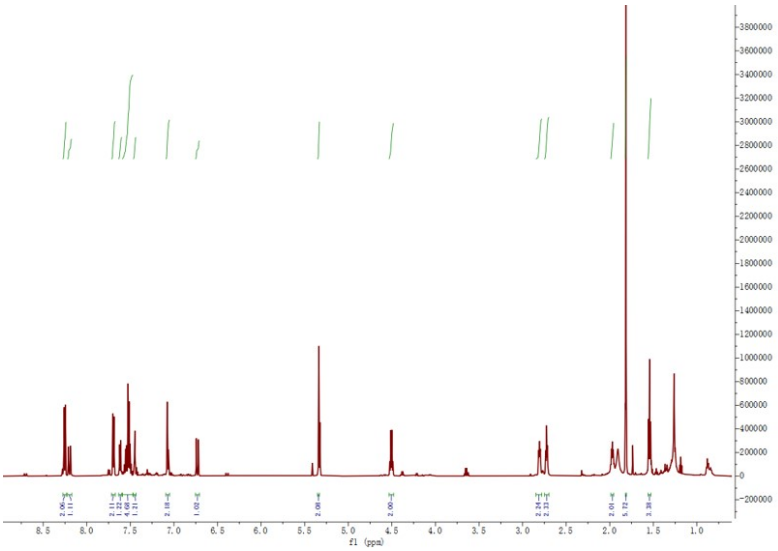

Figure S3. ^{1}HNMR spectrum of HCySe-O-NO_2.

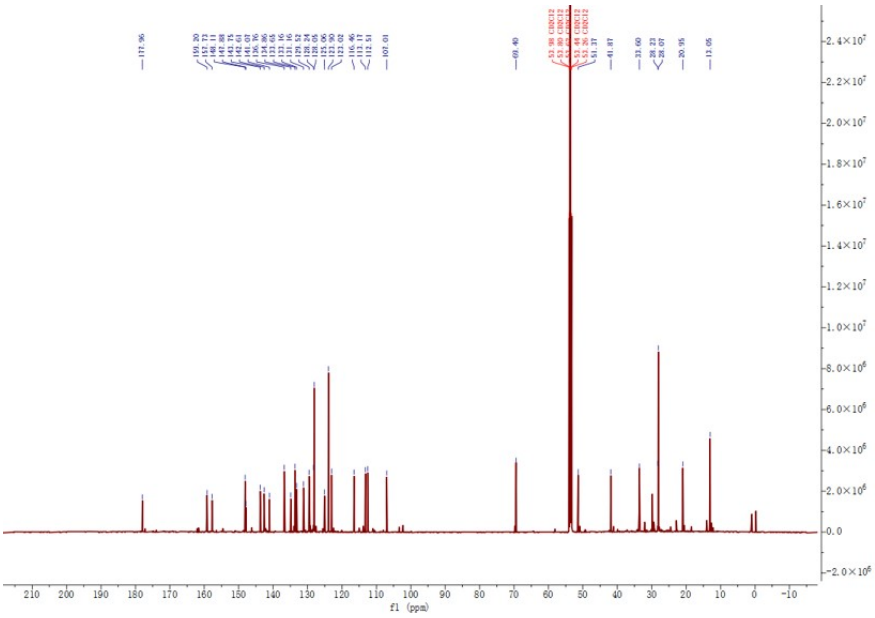

Figure S4. ^{13}C NMR spectrum of HCySe-O-NO_2.

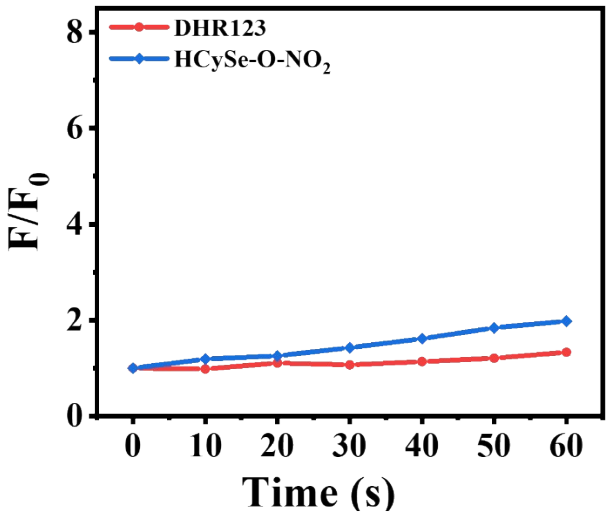

Figure S5. Fluorescence intensity changes at 525 nm of DHR123 co-incubated with dyes HCySe-O-NO_2 plus irradiation (760 nm laser, 5 mW/cm^2).

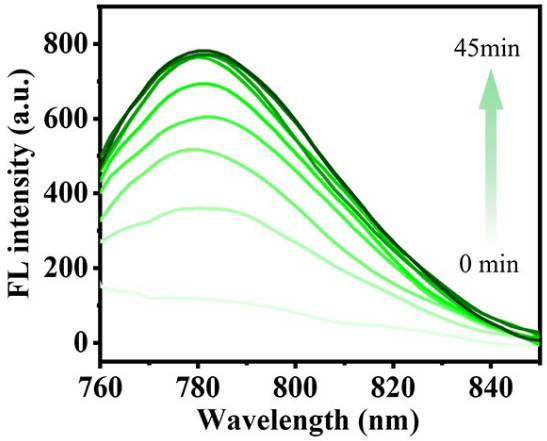

Figure S6. Time-dependent changes in the fluorescence emission of HCySe-O-NO_2 in the presence of NTR (10 μg mL^{-1}).

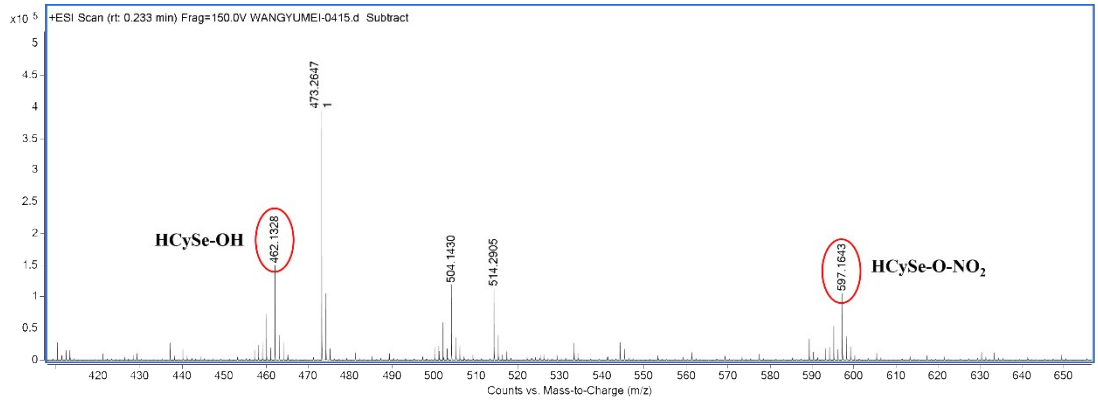

Figure S7. HRMS spectrum of HCySe-O-NO_2 after reaction with NTR in presence of NADH.

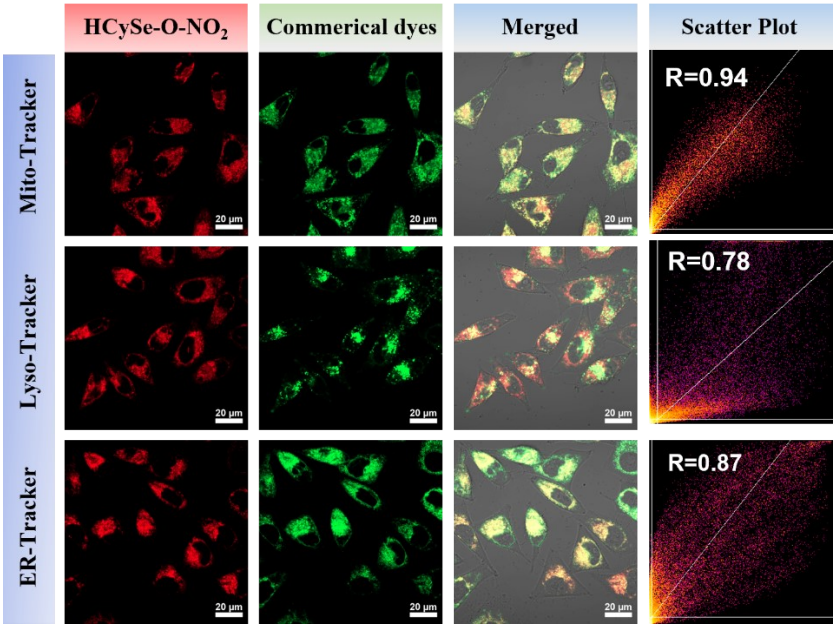

Figure S8. Co-localization imaging of HCySe-O-NO_2 (red, λ_{ex} = 639 nm, λ_{em} = 700-730 nm) and commercial suborganelles-tracker dyes (green, λ_{ex} = 488 nm, λ_{em} = 510–530 nm) in MCF-7 cells.

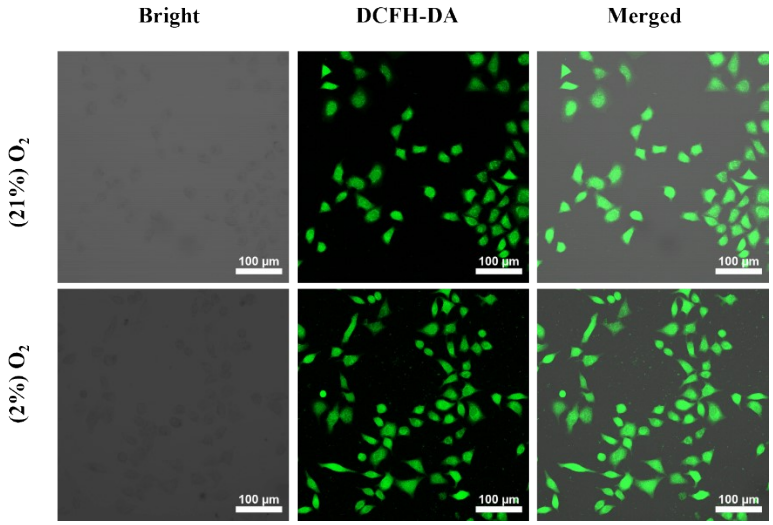

Figure S9. ROS production in MCF-7 cells (2% and 21%) treated with HCySe-O-NO_2 (5 μM) and measured using DCFH-DA as a fluorescence indicator (λ_{ex} = 488 nm, λ_{em} = 510–530 nm), respectively. The light group was exposed to 760 nm laser (50 mW/cm^2, 10 min). Scale bars = 100 μm.

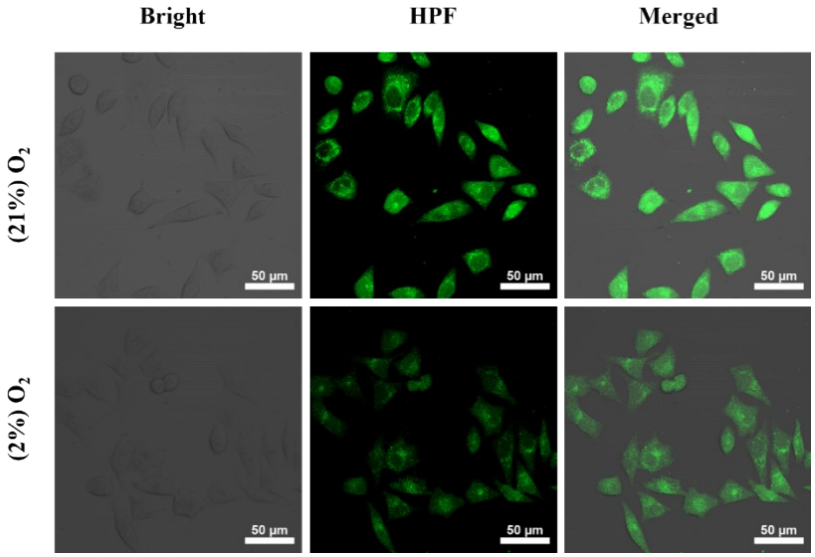

Figure S10. ROS production in MCF-7 cells using HPF as the •OH fluorescence indicators (λ_{ex} = 488 nm, λ_{em} = 510–530 nm). The light group was exposed to 760 nm laser (50 mW/cm^2, 10 min). Scale bars = 50 μm.

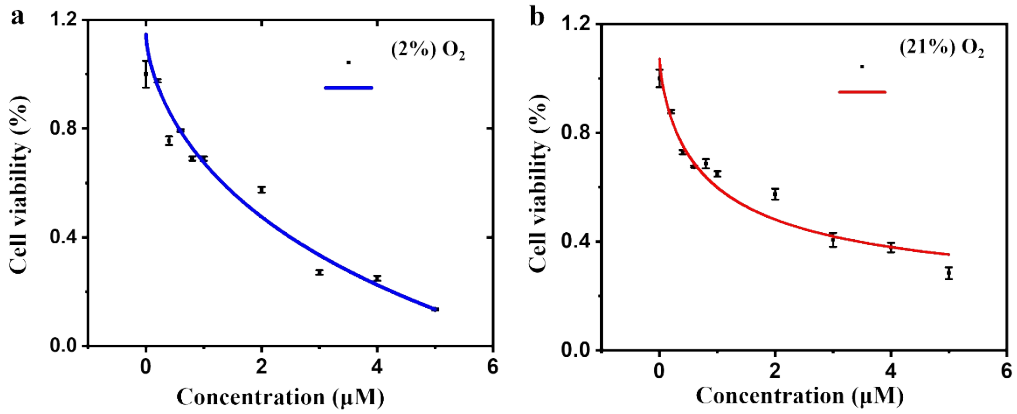

Figure S11. Quantitative analysis of IC_{50} values of MCF-7 cells based on nonlinear regression models under different conditions.

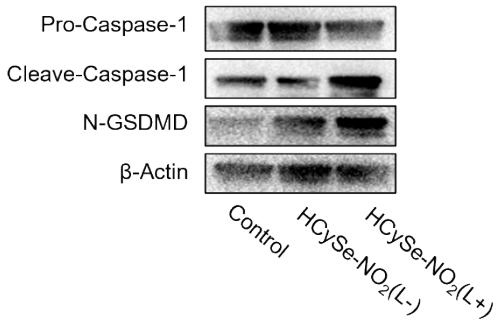

Figure S12. Western blot analysis of N-GSDMD and Caspase-1 in different treatment groups.

12. References

1. Y. Wang, C. Zhang, L. Hao, X. Huo, L. Dou, Y. Xie, J. Wang, X. Peng and H. Li, *Dyes Pigm.*, 2024, **224**, 11226.