Supporting Information

A nitroreductase-activatable near-infrared theranostic photosensitizer under mild hypoxia

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1、Materials and Methods

Materials. 4-Hydrazinobenzenesulfonic acid (98%), 3-methyl-2-butanone (98%), 4-bromomethylbenzoic acid (97%), iodoethane (98%), 4 -amino-TEMPO (98%), 4-Nitrobenzyl bromide (98%), cyclohexanone (99%), K_2CO_3 (99%) and phosphorus oxychloride (99.5%) were purchased from Energy Chemical. All reactions were carried out under a nitrogen atmosphere unless otherwise noted. N, N-Dimethylformamide (DMF) was dried before use. Ultrapure water used for synthesis, analysis, separation and purification steps was deionized from a Milli-Q ultrapure system.

Methods. ^{1}H NMR spectra were obtained from a VARIAN INOVA-400 spectrometer at room temperature. The spectrometer was with chemical shifts reported as ppm (in dimethyl sulfoxide (DMSO), tetramethylsilane (TMS) as internal standard. Mass spectrometry were recorded using LTQ Orbitrap (LTQ Orbitrap XL) from Thermo Scientific. The crude products of CYNT, CYNT-1, CYNT-OH and CYNT-1-OH were purified by FLEXA Purification System from Agela Technologies using methanol and deionized water as mobile phase.

2、Experimental procedures

Preparation of Stock Solutions

CYNT-1 (6.237 mg, 5 mmol) and CYNT (4.887 mg, 5 mmol) were respectively dissolved in DMSO (5 mL) and stored in the dark at 4 °C.

Absorption and Fluorescence Spectroscopy

The absorption spectroscopy was obtained from a UV-Visible spectrometer named Cary 60. The fluorescence spectroscopy was recorded with a fluorometer named Cary Eclipse. UV-Visible spectrometer and fluorometer were both purchased from Agilent Tech. All texts were carried at room temperature.

Methods for fluorescence spectroscopy.

Nitroreductase (NTR) were purchased from Sigma-Aldrich in powder. The stock solution of NTR was dissolved in ultrapure water and preserved at -20 °C in refrigerator. Nicotinamide adenine dinucleotide (NADH) was purchased from J&K Scientific Company. The NADH was prepared before use. The photosensitizer CYNT-1 (10 μM) and NADH (500 μM) were added into pH 7.4 PBS buffer (2914 μL). Then NTR was added to the above mixture. The above mixture was incubated at 37 °C for certain time and other conditions were same.

Cell and Culture Conditions.

HeLa cell line was purchased from the Chenyu biological company (Dalian, China). The culture medium of HeLa cells consisted of fetal bovine serum (FBS; Sijiqing, Zhejiang, China) and Dulbecco's modified Eagle medium (DMEM; Kaiji, Nanjing, China) (FBS: DMEM=1: 9, v/v, pH 7.2). HeLa cells were incubated at 37 °C in an incubator of 5% CO_2. HeLa cells were incubated in a 20-mm glass bottom dish (NEST Company) for 24 h. Hypoxia conditions (1% and 10% oxygen levels) was established by mixed solvent Anaero Pack-Anaero and Anaero Pack-Micro Aero (Mitsubishi Gas Chemical Company, Japan).

Confocal fluorescence imaging of cells.

The HeLa cells were inoculated in a glass bottom dish and cultured for 24 h under normoxia condition (oxygen concentrations 21%). Then the cells were incubated under normoxia or hypoxia for farther 6 h at 37 °C. Under the normoxia or hypoxia (oxygen concentrations 10% and 0.1%) atmosphere, CYNT-1 (10 μM) was added and incubated for farther 24 h. The blocking experiment

was performed by pre-incubating the cells with 0.1 mM dicoumarin for 30 min, then incubated with CYNT-1 for another 24 h under hypoxia (1% O_2). Before imaging, PBS buffer (pH 7.4) was used to wash the cells for three times. An OLYMPUS FV-1000 inverted fluorescence microscope with a 60× objective lens was used to Fluorescence imaging collected at 655-755 nm upon excitation at 635 nm.

3D Spheroidal Models (3DSMs) of HeLa cells

The tumor 3DSMs were formed by aggregation of HeLa cells using the hanging droplet technique (culture medium pH 7.2). Briefly, after trypsinization resuspended in cell culture media at a density of $1.0*10^4$ cells/mL, HeLa cells was obtained. For the hanging droplets, 2 mL ultrapure water were added into 35 mm dish dishes to afford humidification. The dispersed cell suspension (30 μL) was seeded on the lid. Then, the dispersed cell suspension was inverted over the dishes and allowed to incubate for 3~6 days (10 μL of fresh medium was replaced every day) to establish the tumor spheroid.

Singlet Oxygen Generation Measurements

1, 3-diphenylisobenzofuran (DPBF) as capture agent in absolute ethanol was added to detect singlet-oxygen generation measurements, which is a typical procedure. Before the measurement, CYNT-1 and CYNT were respectively dissolved in absolute acetonitrile (almost the same optical density (~0.1) at 660 nm) and mixed with DPBF at 50 μM. Similarly, the concentration of CYNT and CYNT-1 were adjusted to same (a control group, 3 μM). A near infrared LED array as excitated light source was used at power density of 3 mW/cm^2, λ_{ex} = 660 nm. At different intervals, the absorbance spectra of DPBF were recorded at 410 nm.

Intracellular Singlet Oxygen Detection

The generation of $^1O_2$ in HeLa cells was detected by 2, 7-Dichlorofluorescein diacetate (DCFH-DA). The HeLa cells were pre-incubated under hypoxia (oxygen concentrations 10 or 1%) or normoxia conditions for 6 h, then stained with CYNT-1 (10 μM) for 24 h at the same conditions. Then the cells were incubated with DCFH-DA (10 μM) in the same conditions as before for 20 min. After that, the cells were irradiated by 660 nm LED laser (50 mW/cm^2) 15 min. λ_{ex} = 488nm, λ_{em} = 500-540 nm.

Calcein AM/PI Co-stained HeLa Cells

After an overnight incubation, the HeLa cells were cultured under normoxia or hypoxia (oxygen concentration 10%) for 6 h at 37 °C. Then the HeLa cells were treated with CYNT-1 (10 μM) under the same condition incubated for 24 h. The cells were washed with PBS three times and then stained with a mixture of Calcein AM (2 μM) and PI (4 μM) for 20 min. λ_{ex} = 488nm, λ_{em} = 500-540 nm. The AM channel was collected at 500−540 nm, and the PI channel was collected at 560-600 nm. Scale bar: 200 μm.

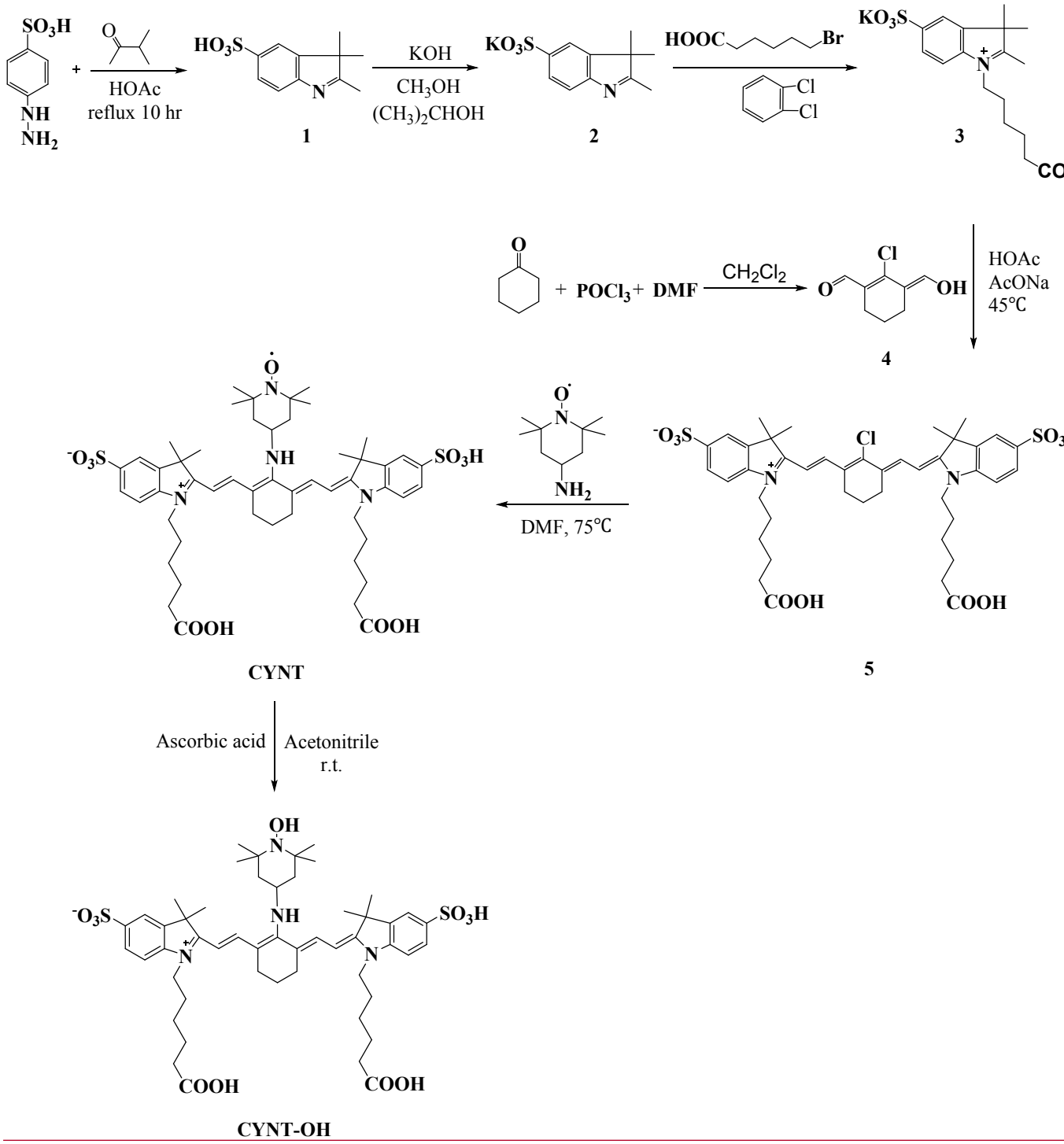
AM Test on 3DSMs of HeLa Cells

The **3DSMs** of HeLa cells were incubated with probe CYNT-1(10 μM) for 24 h. After changing to fresh medium, the cells were treated with or without a 660 nm LED laser (50 mW/ cm^2) for 15 min. Then AM (4 μM) was added to the system for 20 minutes. The green channel was excited at 488 nm and collected at 500−540 nm. The red channel was excited at 635 nm and collected at 655-755 nm. Scale bar: 200 μm.

3、Synthesis and Characterization of Compounds

Scheme S1 Synthetic strategy of heptamethine cyanine CYNT, CYNT-1, CYNT-OH and CYNT-

1-OH.


SO3H
NH
NH2
HOAc
reflux 10 hr
HO3S
1
KOH
CH3OH
(CH3)2CHOH
KO3S
2
HOOC
Br
Cl
Cl
3
CO
O
+ POCl3 + DMF
CH2Cl2
Cl
O
OH
4
HOAc
AcONa
45℃
5
-O3S
Cl
SO3
COOH
COOH
DMF, 75℃
NH2
CYNT
-O3S
NH
SO3H
COOH
COOH
Ascorbic acid
Acetonitrile
r.t.
CYNT-OH
OH
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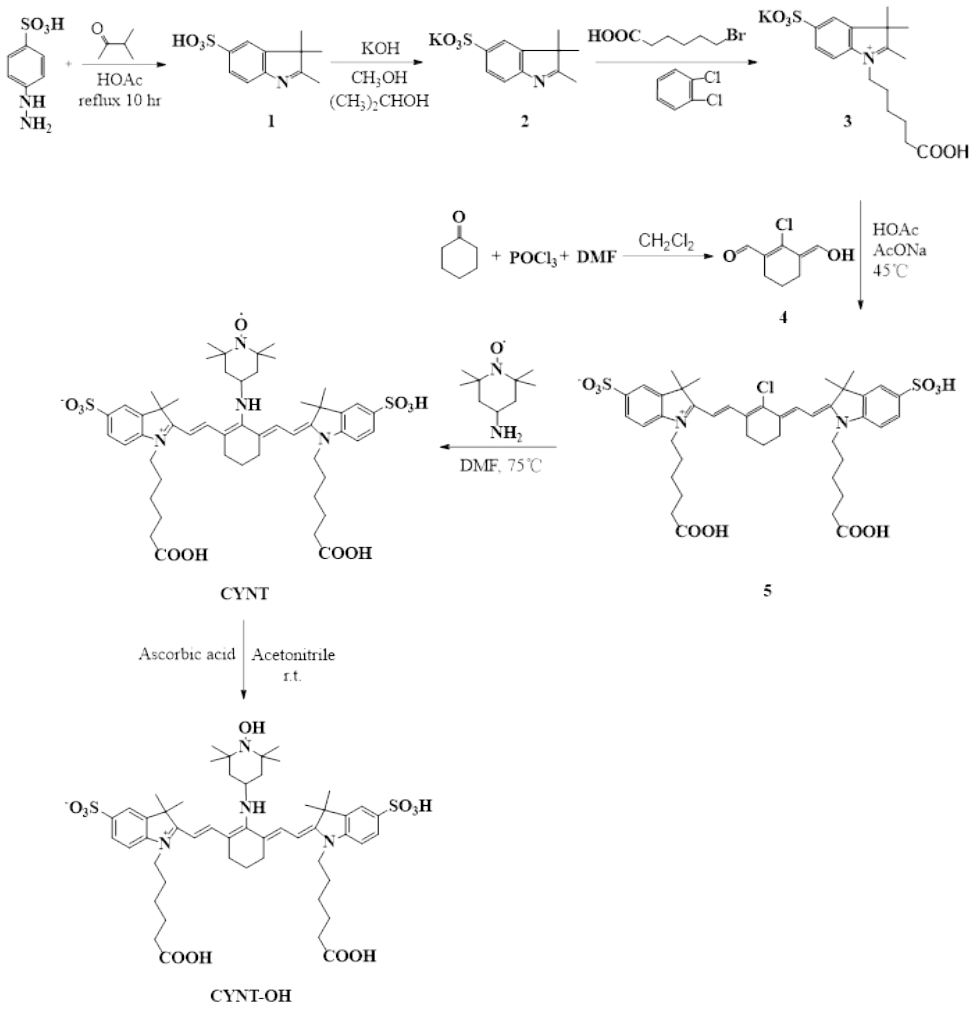
NH
SO3H
COOH
COOH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reflux 10 hr
KOH
CH3OH
(CH3)2CHOH
1
2
3
+ POCl3+ DMF
CH2Cl2
4
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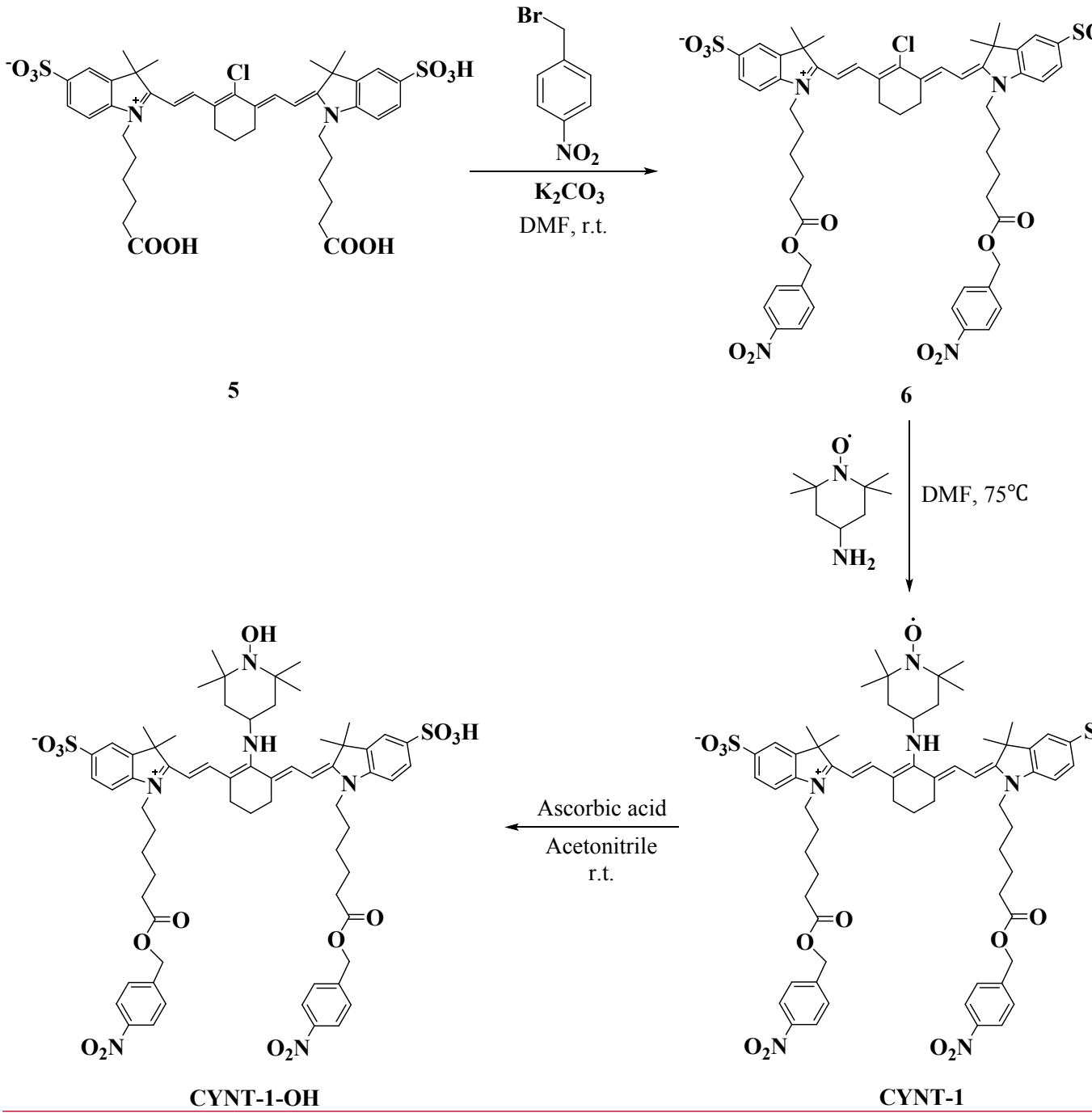
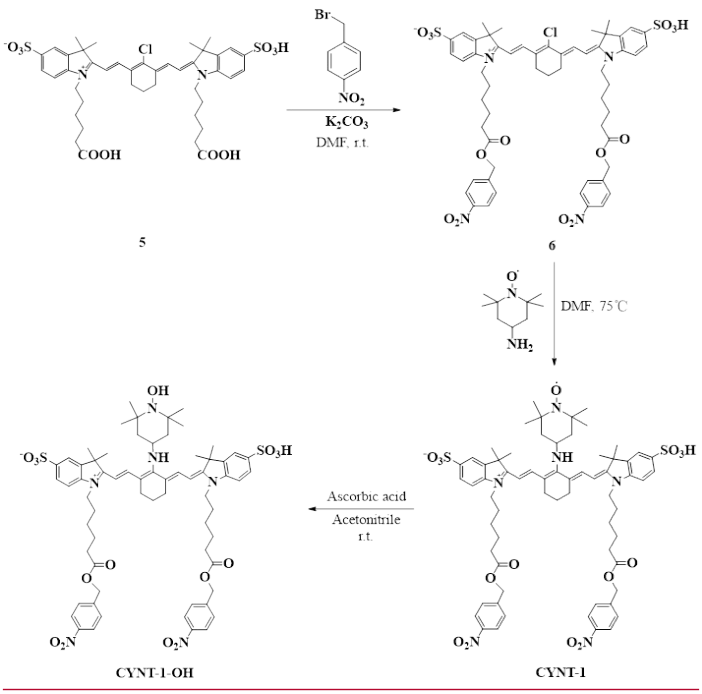
AcONa
45℃
DMF, 75℃
CYNT
5
Ascorbic acid
Acetonitrile
r.t.
CYNT-OH


Br
NO2
K2CO3
DMF, r.t.
5
6
DMF, 75℃
Ascorbic acid
Acetonitrile
r.t.
CYNT-1-OH
CYNT-1



Synthesis of probe CYNT. Intermediates **1**, **2**, **3**, **4**, **5** were synthesized according to our reported work[1, 2]. Intermediate **5** (200 mg, 0.3 mmol, 1.0 eq.) and 4-Amino-Tempo (153.33 mg, 0.9 mmol, 3.0 eq.) were mixed in anhydrous DMF (20 mL) under nitrogen and stirred at 75°C for 10 hours[3]. Then the crude product was purified by HPLC using methanol and deionized water as mobile phase. Pure compound **CYNT** was obtained as purple amorphous powder. MS (ESI): m/z calcd for $C_{51}H_{69}N_4O_{11}S_2^-$ $[M-H]^-$: 976.44, found: 976.64. Due to the paramagnetic property, satisfactory NMR spectra of **CYNT** can't be recorded. Therefore, **CYNT-OH** was synthesized from **CYNT** to support the structure characterization.

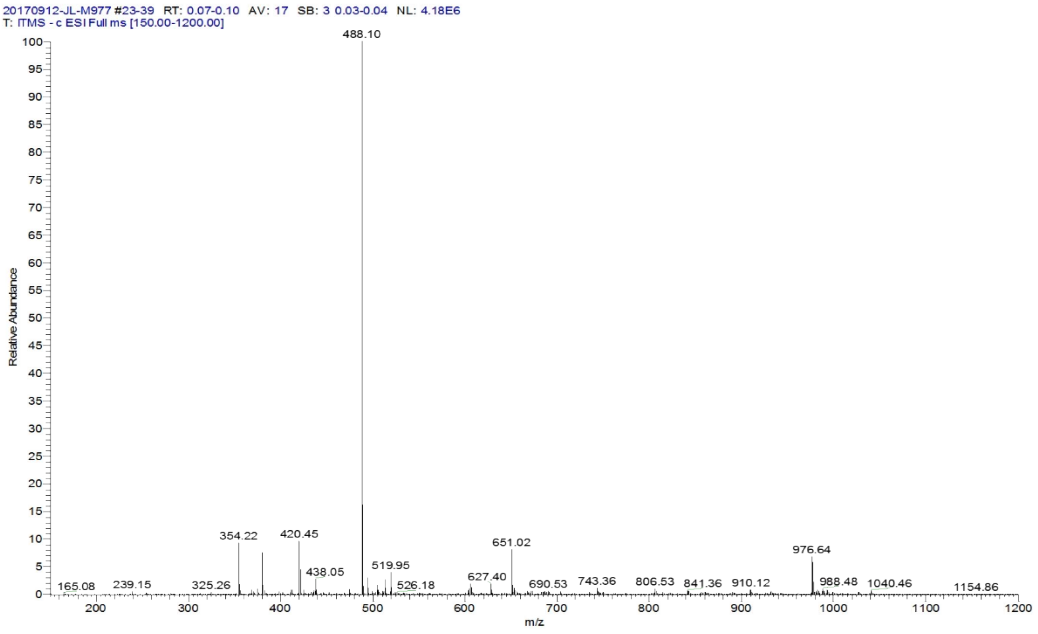


Figure S1. MS of compound CYNT

Synthesis of CYNT-OH. CYNT (50 mg, 0.05 mmol, 1.0 eq) was dissolved in 10 mL acetonitrile, and ascorbic acid (47.73 mg, 0.25 mmol, 5.0 eq) was added. The mixture was stirred at room temperature for 30 min under nitrogen. The solvent is evaporated until dry. Subsequently, the crude product was purified by HPLC using methanol, deionized water as mobile phase.

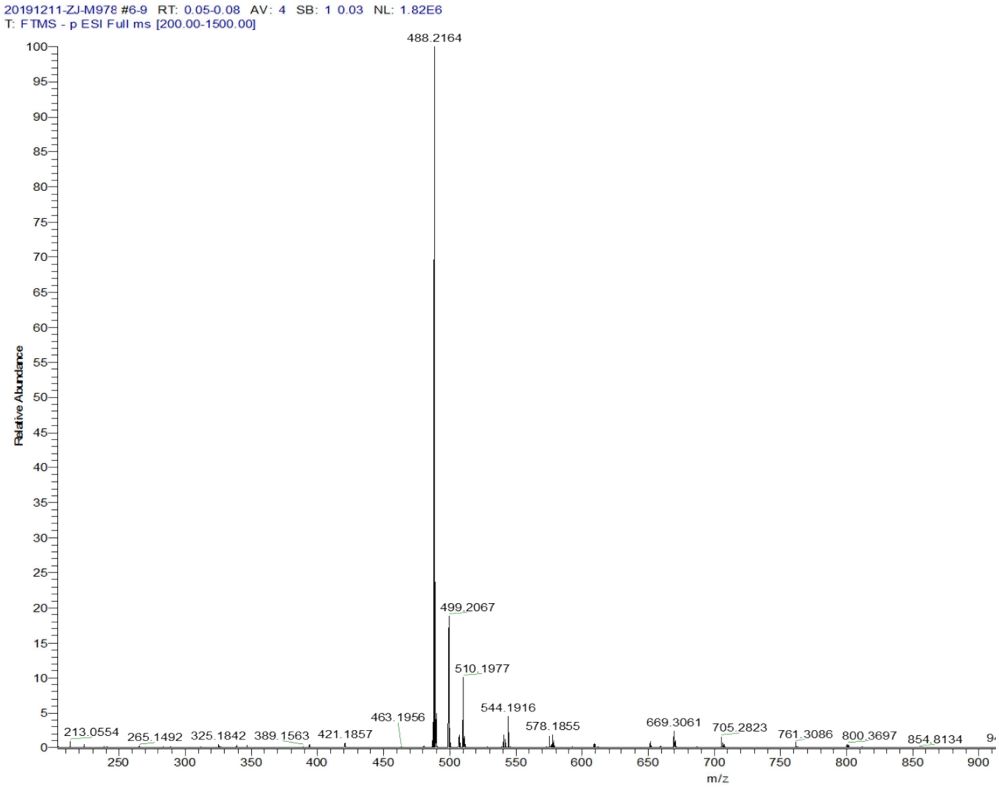


Figure S2. HRMS (ESI-) of CYNT-OH

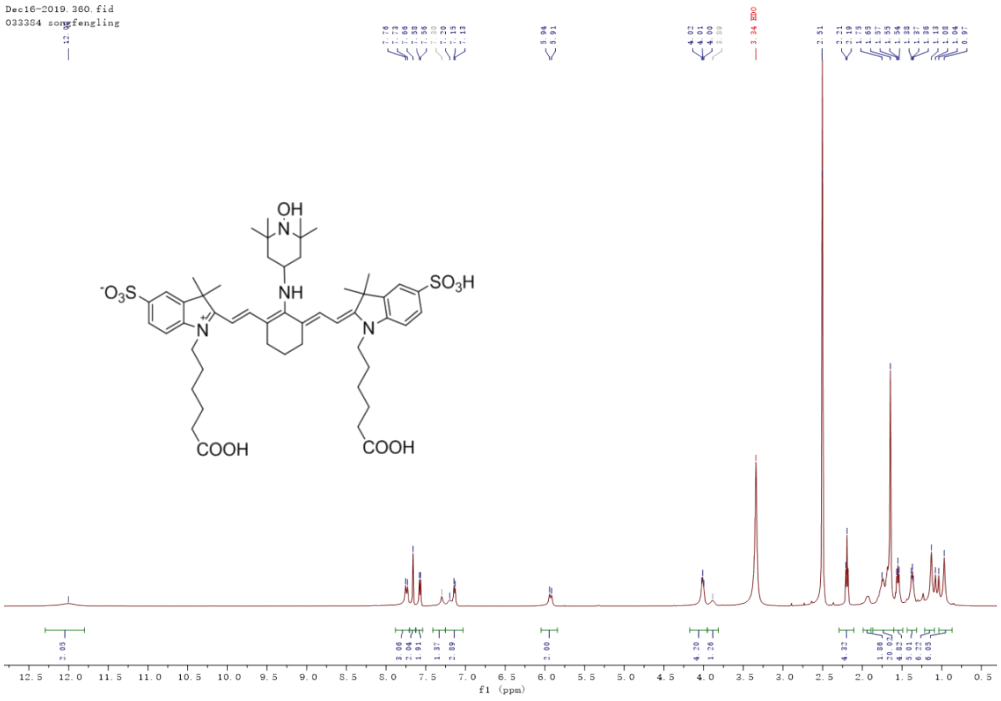


Figure S3. ^{1}H NMR spectrum of CYNT-OH

Synthesis of intermediate 6. Intermediate **5** (50 mg, 0.05 mmol, 1.0 eq.), 4-Nitrobenzyl bromide (44.20 mg, 0.2 mmol, 4.0 eq.) and K_2CO_3 (21.18mg, 0.15 mmol, 3.0 eq.) were mixed in anhydrous DMF (20 mL) under nitrogen and stirred for 24 hours[4]. Then the crude product was purified by HPLC using methanol and deionized water as mobile phase. Pure intermediate **6** was obtained as green amorphous powder.

Synthesis of probe CYNT-1. Intermediate **6** (50 mg, 0.045 mmol, 1.0 eq.) and 4-Amino-Tempo (68.18 mg, 0.135 mmol, 3.0 eq.) were mixed in anhydrous DMF (20 mL) under nitrogen and stirred at 75°C for 10 hours. Then the crude product was purified by HPLC using methanol and deionized water as mobile phase. Pure compound CYNT-1 was obtained as purple amorphous powder. MS (ESI): m/z calcd. for $C_{65}H_{79}N_6O_{15}S_2^-$ $[M-H]^-$: 1246.50, found: 1246.4971. Due to the paramagnetic property, satisfactory NMR spectra of CYNT can't be recorded. Therefore, CYNT-1-OH was synthesized from CYNT-1 to support the structure characterization.

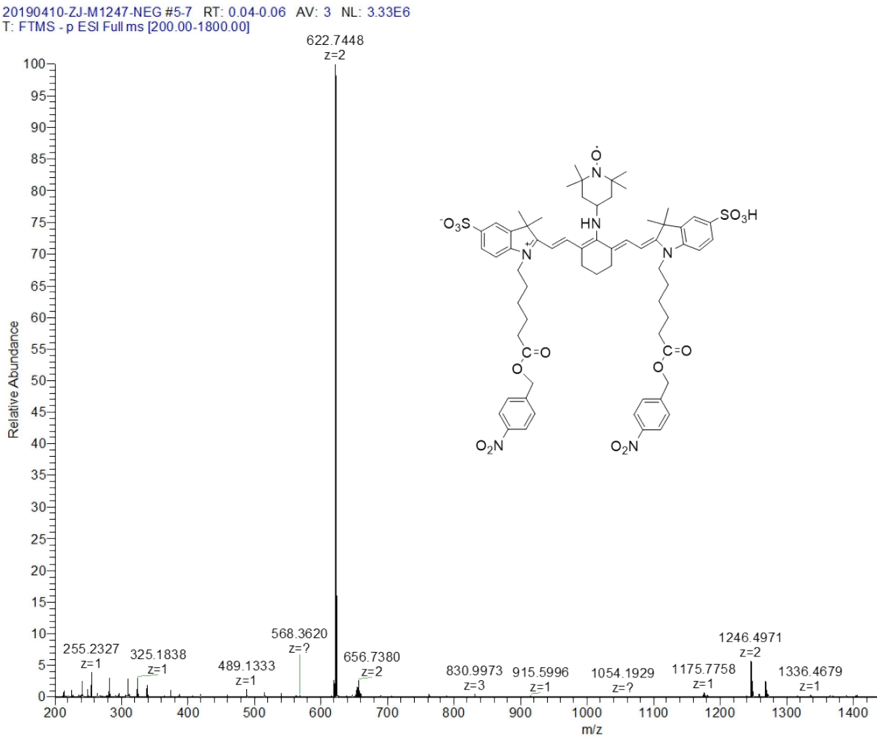


Figure S4. HRMS (ESI-) of CYNT-1

Synthesis of CYNT-OH. CYNT (50 mg, 0.04 mmol, 1.0 eq) was dissolved in 10 mL acetonitrile, and ascorbic acid (88.06 mg, 0.2 mmol, 5.0 eq) was added. The mixture was stirred at room temperature for 30 min under nitrogen. The solvent is evaporated until dry. Subsequently, the crude product was purified by HPLC using methanol, deionized water as mobile phase.

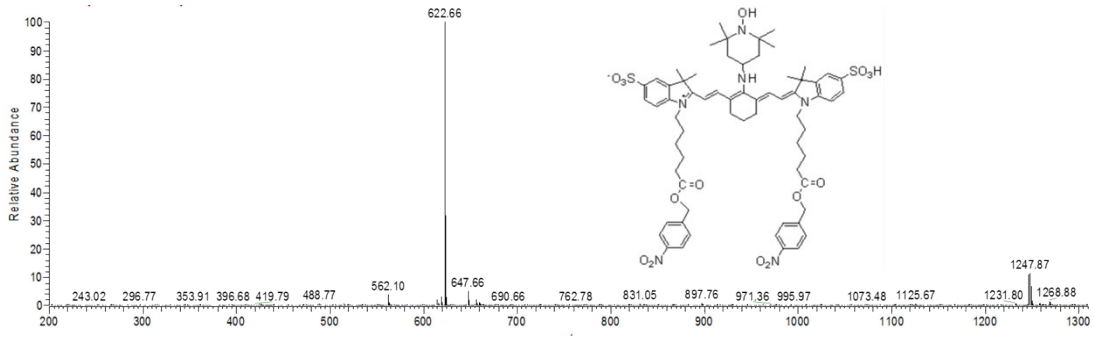


Figure S5. MS (ESI-) of CYNT-1-OH

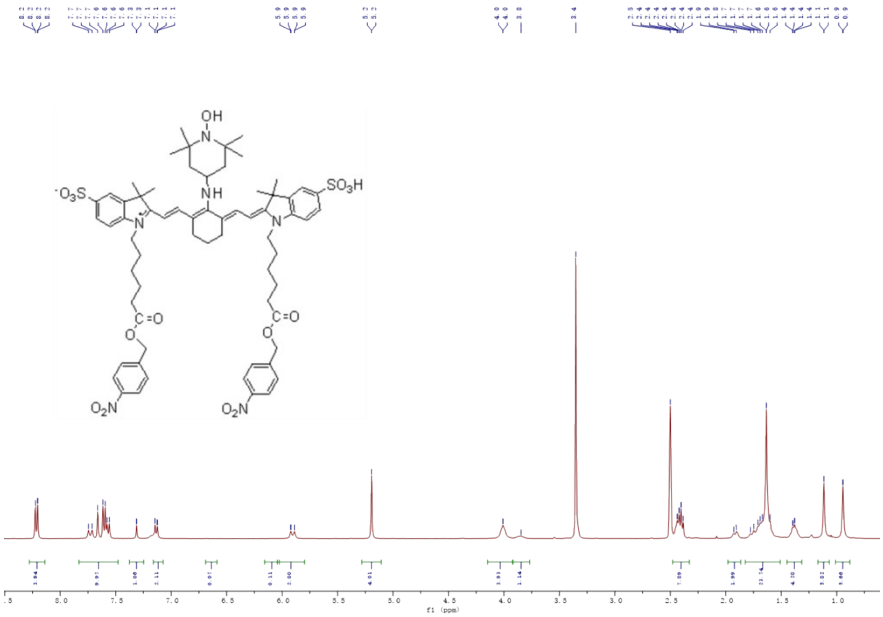


Figure S6. ^{1}H NMR spectrum of CYNT-1-OH

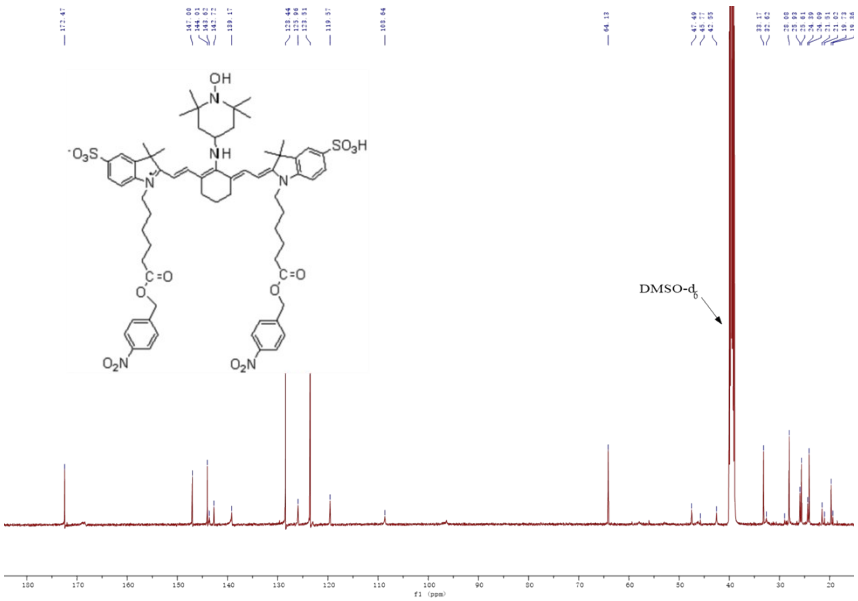


Figure S7. ^{13}C NMR spectrum of CYNT-1-OH

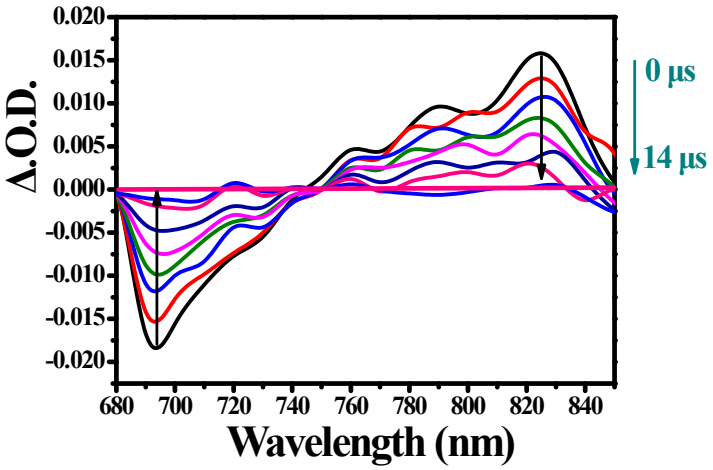
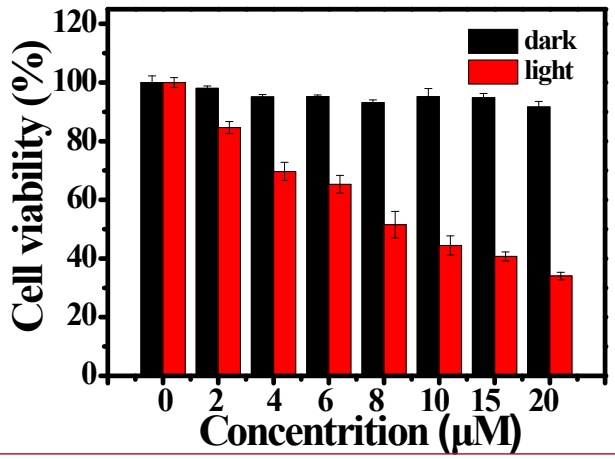


Figure S8. (a) Nanosecond time-resolved transient difference absorption spectra of CYNT (10. μM in deaerated ethanol). 532 nm laser pulse, decay times as indicated.



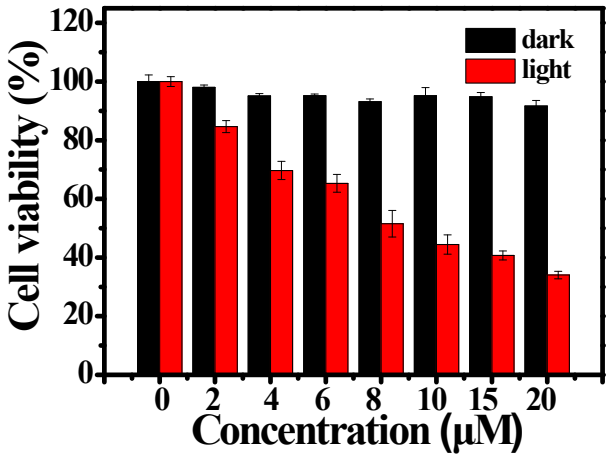


Figure S9. Cell viabilities of HeLa cells based on the MTT assay of CYNT (0-20 μM) under 660

nm LED light irradiation (50 mW/cm^2 for 15 minutes) or dark.

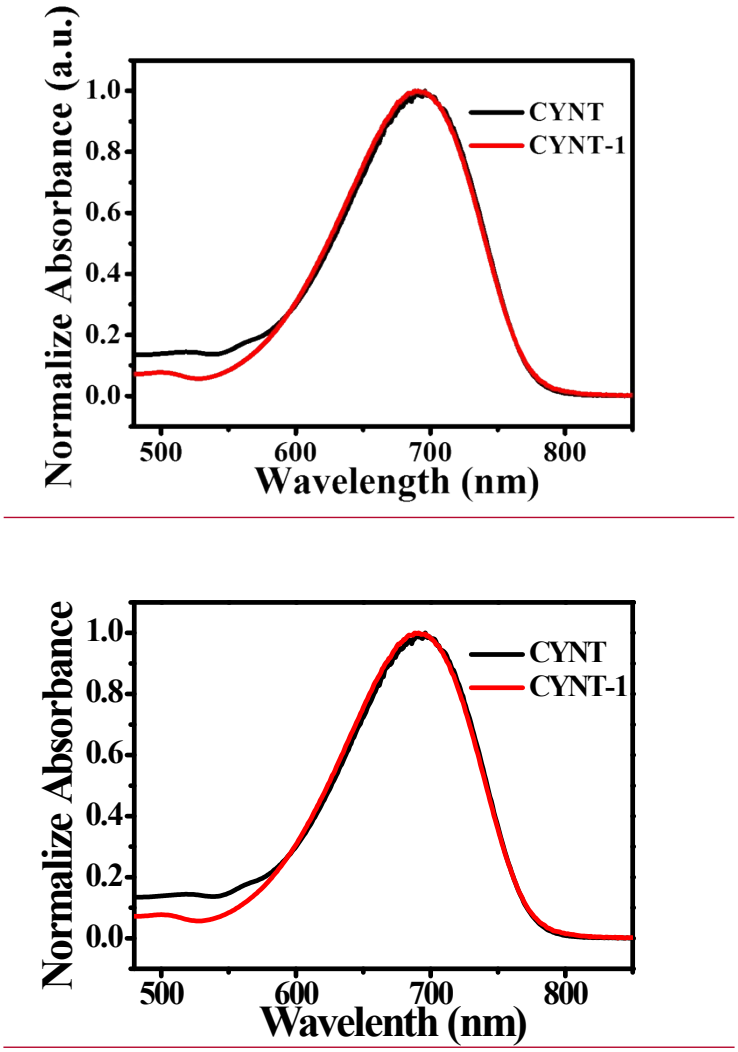


Figure S10. Normalize absorption spectra of compounds CYNT-1 (10 μM) and CYNT (10 μM) in ethanol.

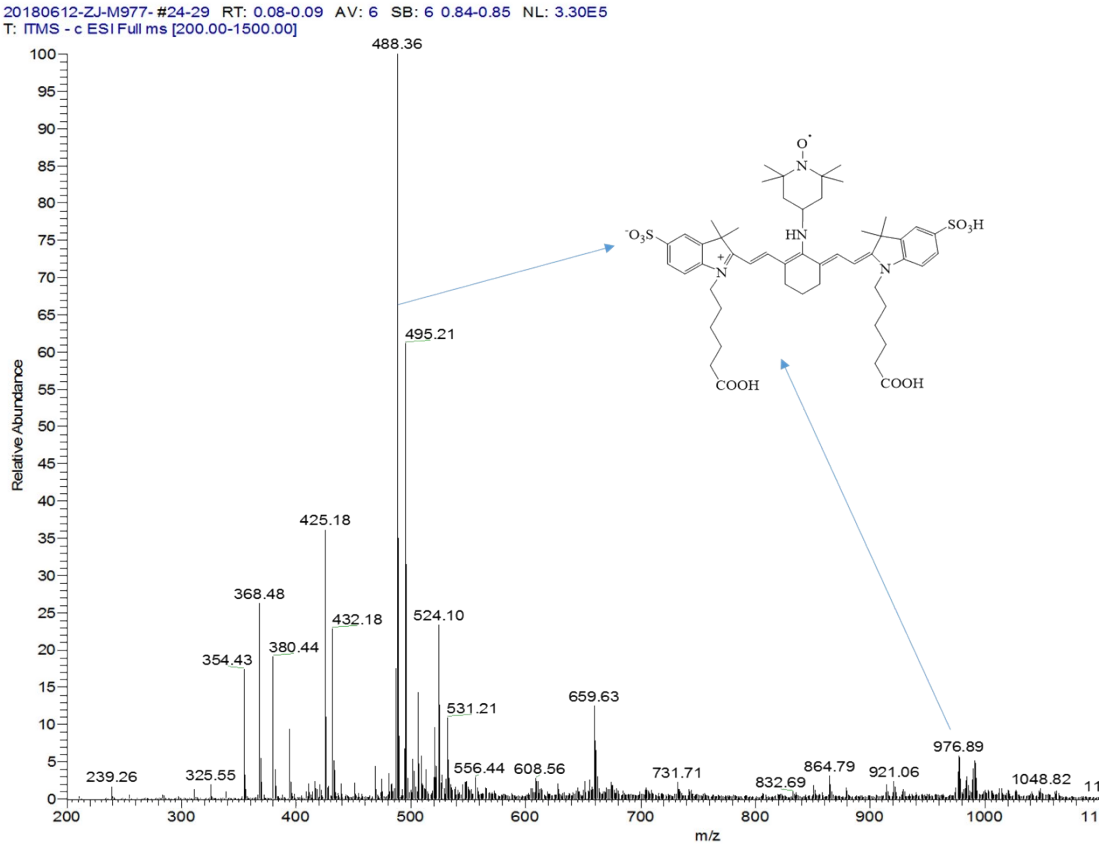


Figure S11. MS spectrum of the reaction mixture of CYNT-1 (10 μM) reacted with NTR (1 μg/mL).

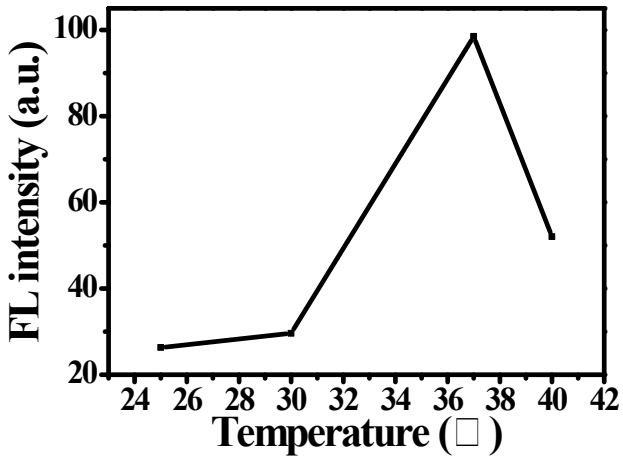


Figure S12. Effect of temperature on the fluorescence intensity of CYNT-1 (10 μM) reacted with NTR (1 μg/mL) in the presence of NADH (500 μM). The fluorescence spectra were measured at 15 min after the NTR was added to the mixture (λ_{ex} = 635nm).

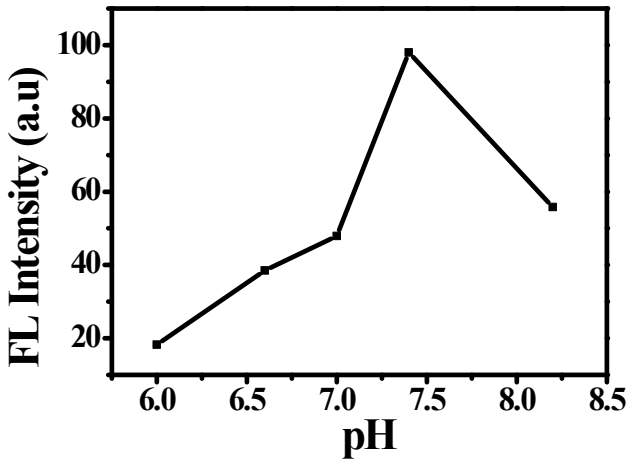


Figure S13. Effect of PH on the fluorescence intensity of CYNT-1 (10 μM) reacted with NTR (1 μg/mL) in the presence of NADH (500 μM). The fluorescence spectra were measured at 15 minutes after the NTR was added to the mixture (λ_{ex} = 635nm).

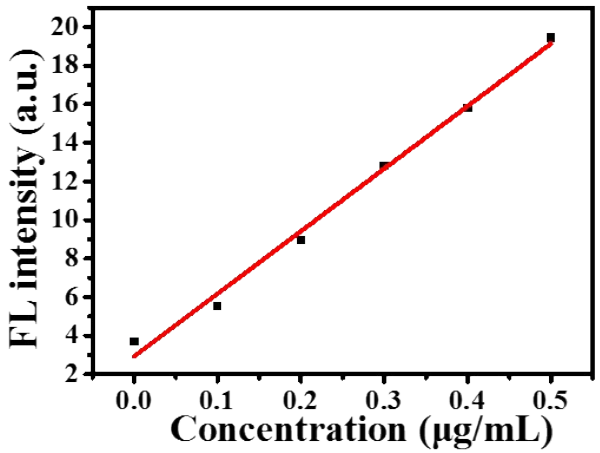


Figure S14. The fitting linear relationship between the fluorescence intensity and NTR concentration (0.1 μg/mL, 0.2 μg/mL, 0.3 μg/mL, 0.4 μg/mL, 0.5 μg/mL). The detection limit of CYNT-1 (10 μM) for NTR in the presence of NADH (500 μM) in PBS at 37 °C. The fluorescence spectra were measured at after the NTR added to the mixture for 15 minutes (λ_{ex} = 635 nm)

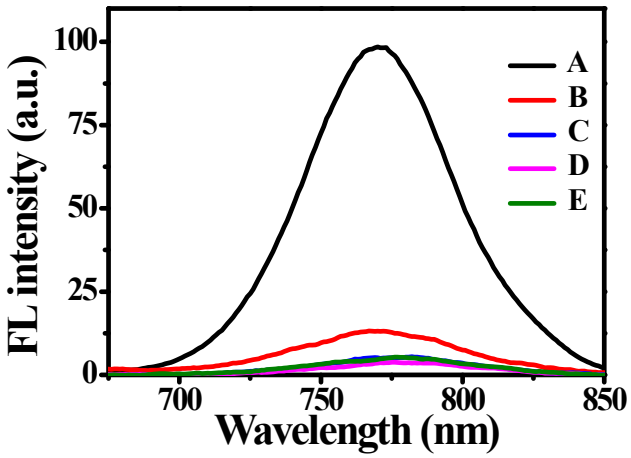


Figure S15. The fluorescence emission spectra of CYNT-1 (10 μM) in the different reaction systems. (A): treated with 1 μg/mL NTR and 500 μM NADH at 37 °C; (B): A system treated with 0.1 mM dicoumarin; (C): A system at 4 °C. (D): treated with 500 μM NADH; (E): treated with 1 μg/mL NTR. The fluorescence intensities were measured in PBS buffer (PH=7.4) after a 15-min response time. (λ_{ex} = 635 nm.)

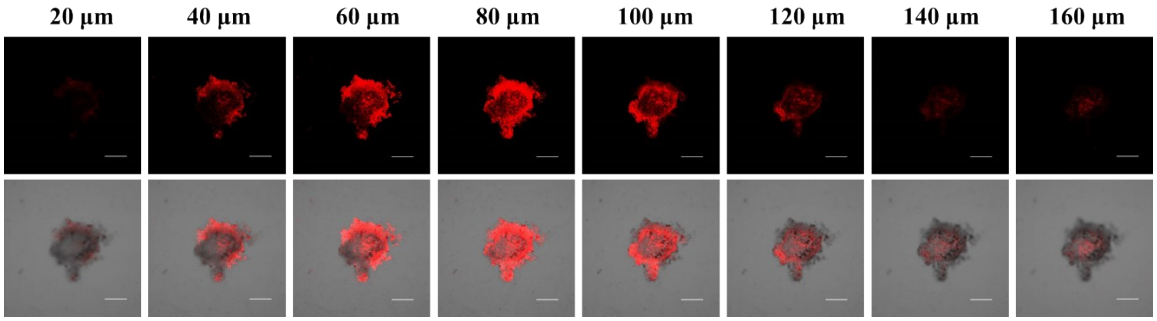


Figure S16. Z-scanned confocal fluorescence images of 3DSMs of HeLa cells following incubation with probe CYNT-1 (10 μM) for 24 h. λ_{ex} = 635nm, λ_{em} = 655-755 nm). Scale bar: 200 μm.

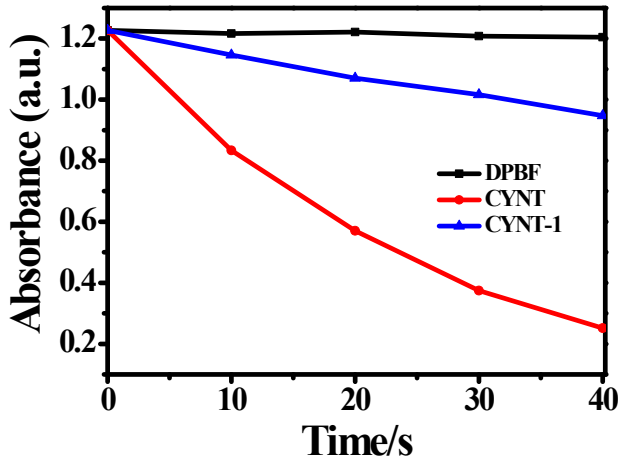


Figure S17. The bleaching rate comparison of DPBF at 410 nm. NIR LED array irradiation (λ_{ex} =

660 nm; 3 mW/cm^2) for different times. CYNT-1 and CYNT were respectively dissolved in absolute ethanol to a final concentration with almost the same absorbance (~0.1) at 660 nm and mixed with DPBF (50 μM).

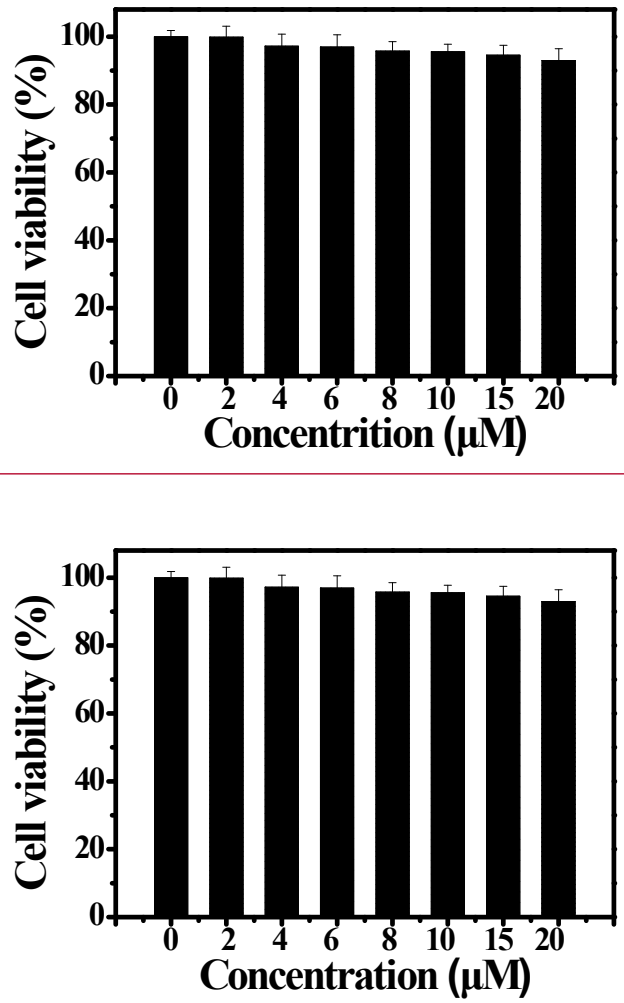


Figure S18. Cell viabilities of HeLa cells based on the MTT assay of CYNT-1(10 μM) under normoxia in dark.

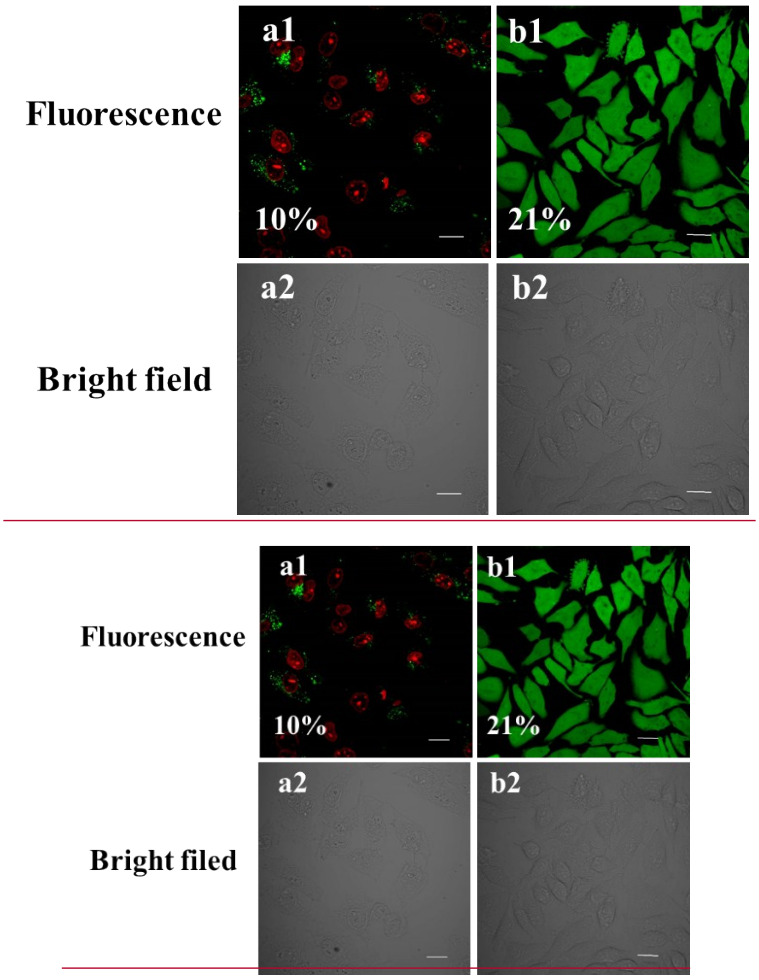


Figure S19. AM and PI co-staining of CYNT-1-treated HeLa cells under different oxygen concentration conditions ((a) 10%; (b) 21%) with 660nm laser irradiation (50 mW/cm^2 for 15 minutes). Scale bar: 20 μm.

References:

1. L. Jiao, F. Song, B. Zhang, H. Ning, J. Cui and X. Peng, *J. Mater. Chem. B.*, 2017, **5**, 5278-5283.
2. X. J. Peng, F. L. Song, E. Lu, Y. N. Wang, W. Zhou, J. L. Fan and Y. L. Gao, *J. Am. Chem. Soc.*, 2005, **127**, 4170-4171.
3. L. Jiao, F. Song, J. Cui and X. Peng, *Chem. Commun.*, 2018, **54**, 9198-9201.
4. Z. Liu, F. Song, W. Shi, G. Gurzadyan, H. Yin, B. Song, R. Liang and X. Peng, *Appl. Mater. Inter.*, 2019, **11**, 15426-15435.