Electronic Supplementary Information for

Two-step lighting DNA tetrahedral nanoprobe for precise imaging-guided photodynamic therapy of tumor

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1. Experimental Section

1.1 Materials and Instruments.

All oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Table S1) and purified by high-performance liquid chromatography (HPLC). Ultrapure water obtained from a Millipore water purification system (18.2 MΩ) used in all assays. 5,10,15,20-Tetrakis-(N-methyl-4-pyridyl) porphine (TMPyP4) was purchased from Sigma-Aldrich (MO, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Beyotime Institute of biotechnology (China), while rat liver microsomes were purchased from CHI Scientific, Inc. (China).

1.2 Assembly of the Tetrahedral DNA Nanostructures

Four single-stranded DNAs (S1-S4) were assembled in PBS buffer (20 mM PBS, 10 mM $MgCl_2$, 100 mM KCl (pH=7.4) at the same molar ratio) and placed at 95°C for 5 min, then quickly cooled to 4°C in ice, and keep at 4°C for at least 2 h for further use. The final concentration of DNA tetrahedral nanostructures was 1 μM.

1.3 Preparation of TAH and TAH@TMPyP4

L1, L2 and Apt strands were modified onto the assembled DNA tetrahedra in buffer (20 mM PBS, 10 mM $MgCl_2$, 100 mM KCl. pH = 7.4) at appropriate molar ratios (DNA tetrahedra: L1: L2: Apt = 1: 1: 1: 1), and place at 25°C for more than 3 h to prepare TAH. The prepared TAH nanoprobes was stored at 4°C in the dark until use. The TAH@TMPyP4 was prepared by successively adding the TAH to TMPyP4 in the PBS buffer. The mixture was shaken at room temperature for 6 h, and the unloaded TMPyP4 was removed by ultrafiltration for three times. According to the standard curve, we obtained the load amount of TMPyP4 in TAH@TMPyP4.

1.4 Gel Electrophoresis Analysis

1.5% agarose gel electrophoresis was used to verify the assembly of DNA tetrahedral nanostructures and TAH nanoprobes. The agarose gel was carried out in

1×TBE buffer at room temperature. The electrophoresis was performed at 100 V for 45 min after loading 10 μL samples (1 μM) into each lane. The gel was stained with SYBR Gold and scanned *via* Bio-Rad gel imaging system.

1.5 Responsiveness of Constructed Nanoprobes to MUC1 and Azoreductase in Buffer Solution

The prepared TAH nanoprobes were added to PBS buffer solutions containing different concentrations of MUC1 (0-200 nM), and the mixed solutions were incubated at room temperature for 1 h. The fluorescence intensity of Cy5.5 was recorded at the excitation wavelength of 673 nm. For the selectivity, different proteins: MUC1 (active or inactive, 100 nM), GOX (100 μM), RNase H (1 U/mL), RNase A (10 ng/μL), APE1 (1 U/mL) were added to the reaction buffer TAH (100 nM) for 1 h at 37°C. The fluorescence emission intensity of each sample was measured.

The prepared TAH nanoprobes were incubated with different concentrations (0, 25, 50, 100, 125, 150, 175, 200 μg/mL) of rat liver microsomes and 50 μM NADPH at 37°C for 6 h under hypoxic conditions (1% O_2). The fluorescence intensity of TAMRA was recorded every 1 h using the maximal excitation wavelength at 542 nm and collected between 560 and 650 nm. For the selectivity study, different metal ions or bioactive small molecules: rat liver microsomes (active or inactive, 100 μg/mL), Na^+ (10 mM), K^+ (10 mM), RNase A (10 ng/μL), RNase H (1 U/mL), GSH (10 mM) were added to the reaction buffer and reacted with TAH (100 nM) for 6 h at 37°C. The fluorescence emission intensity of each sample was measured.

1.6 $^1O_2$ Production of TAH@TMPyP4 in Buffer Solution

A commercial reagent (1, 3-Diphenylisobenzofuran, DPBF) was chose to evaluate the generation of $^1O_2$ in buffer solution. The TMPyP4, TAH@TMPyP4 and UB-TAH@TMPyP4 were dissolved in PBS buffer containing 10 μg/mL DBPF, separately. Then, the mixed solution was irradiated with 660 nm light (2.2 $mW \cdot cm^{-2}$) for different time periods. The results were recorded with a UV-vis spectrophotometer.

1.7 Probe Stability Investigation

TAH (1 μM) was incubated in fresh DMEM medium plus 10% FBS at 37℃ for different time spans (0, 3, 6, 9, 12, 24 h). Then, 10 μL of the sample was mixed with glycerin (2 μL) and stained with SYBR Gold. Electrophoresis was performed in 1×TBE buffer at 100 V for 30 min. Finally, gels were imaged on a Bio-Rad molecular imager.

1.8 Confocal Fluorescence Imaging of TAH in Living Cells

Activatable hypoxia imaging in living cells: First, human breast adenocarcinoma (MCF-7) cells were seeded in confocal culture dishes (1×10^5 cells per well) for 24 h and incubated with the TAH for 6 h under different O_2 concentrations (21%, 10%, and 1% O_2) and then washed three times with PBS buffer to remove the free TAH. Next, the TAH-incubated cells were subjected for imaging using laser scanning confocal microscope.

Imaging of cell membrane surface glycoprotein MUC1: The Apt strand labeled with Cy5.5 was incubated with each of these three kinds of cells (including MCF-7 cells, HepG2 cells, NIH/3T3 cells) for 30 min and then imaged them with laser scanning confocal microscope.

Generation of activatable formula $^1O_2$: First, MCF-7 cells were seeded in culture dishes and then placed in a cell incubator for 24 h. The cells were incubated with TAH@TMPyP4 at different O_2 concentrations for 4 h then incubated with 2',7'-Dichlorofluorescein diacetate (DCFH-DA, 10 μM) in DMEM solution for 30 min, following by washing with PBS three times. For the cells that require light irradiation, 660 nm light at a density of 2.2 $mW \cdot cm^{-2}$ was applied for 30 min. Fluorescence images of MCF-7 cells were immediately recorded using laser scanning confocal microscope.

1.9 Cytotoxicity Investigation

Cell viability was tested *via* 3-(4,5-dimethylthiazol-2-yr)-2,5- diphenyltetrazolium bromide (MTT) assay. Specifically, MCF-7 cells were seeded into 96-well plates for

48 h. Then, the cells were treated with TAH@TMPyP4 under different O_2 concentrations for 4 h following by irradiation with 660 nm light at 2.2 $mW \cdot cm^{-2}$ for 30 min. After incubation for another 24 h, the cells were washed with PBS three times and 100 μL of MTT solution (0.5 mg/mL) was added to incubate together for 4 h. The supernatant was removed and 100 μL of dimethyl sulfoxide (DMSO) was then added to dissolve the formazan. The absorbance (490 nm) of the resulting solution was recorded using a microplate reader.

1.10 *In Vivo* Fluorescence Imaging

All animal operations were performed according to the protocol No. SYXK (Xiang) 2015-0017, and met the requirements with animal use and care regulations. 4T1 cells were injected into the subcutaneous tissue on the right side of the back of 4-week-old female nude mice to establish a tumor-bearing mouse model. When the tumors of the mice grew to around 6 mm, we performed live imaging of the mice. The tumor-bearing mice were divided into three groups: PBS group, UB-TAH group and TAH group. Three groups of mice were injected with PBS, 1 μM UB-TAH and 1 μM TAH in the tumor area and non-tumor area, respectively. The mice were anesthetized by air anesthesia, and then the mice were imaged by fluorescence using an intravital imaging system.

1.11 *In Vivo* Photodynamic Therapy Research

The MCF-7 tumor-bearing mouse model was established by injecting MCF-7 cells into the subcutaneous tissue of the right back of female nude mice. When the tumor volume reached approximately 60 mm^3, the mice were randomly divided into four groups of four mice each. Mice were injected with PBS, TAH, and TAH@TMPyP4 every other day at a dose of 1 μM TMPyP4. The tumor volume and body weight of each mouse were measured every 2 days and monitored continuously for 15 days.

2. Experimental Data

Name	Sequence (5'-3')
S1	5'-TAT CAC CAG GCA GTT GAC AGT GTA GCA AGC TGT AAT AGA TGC GAG GGT CCA ATA CTT-3'
S2	5'-TCA ACT GCC TGG TGA TAA AAC GAC ACT ACG TGG GAA TCT ACT ATG GCG GCT CTT CTT-3'
S3	5'-TTC AGA CTT AGG AAT GTG CTT CCC ACG TAG TGT CGT TTG TAT TGG ACC CTC GCA TTT-3'
S4	5'-GAG CCC AGG TTC TCT TTTTTT ACA TTC CTA AGT CTG AAA CAT TAC AGC TTG CTA CAC GAG AAG AGC CGC CAT AGT A-3'
L1	5'-GGT GGT GGT GGT TGT GGT GGT GGT GG AGA GAA CCT GGG CTC TT AAA CTC TAA TTG TA CCA GGG TAT CC-3'
L2	5'-TAMRA- TA CAA TTA GAG TTT AA-BHQ2-3'
L2'	5'-TAMRA- TA CAA TTA GAG TTT AA-3'
Apt	5'-GCA GTT GAT CCT TTG GAT ACC CTG G-Cy5.5-3'

Table S1 Oligonucleotides used in this work. The underlined part indicates the G-tetramer fragment.

Name	Modification group	Purity value (%)
L2	5'TAMRA,3'BHQ2	99.07
L2'	5'TAMRA	99.09
Apt	3'Cy5.5	96.71

Table S2 HPLC purity values of fluorophore-labeled strands used in this work.

These data are provided by Sangon Bioengineering Co., Ltd (Shanghai, China).

HPLC spectrum of L2 strand:

Empower™ 3 SOFTWARE GB138

样品信息

样品名称:	1931672647	采集者:	System
样品类型:	未知	样品组名称:	230411
瓶号:	1	采集方法组:	GB15
进样次数:	1	处理方法:	gb14
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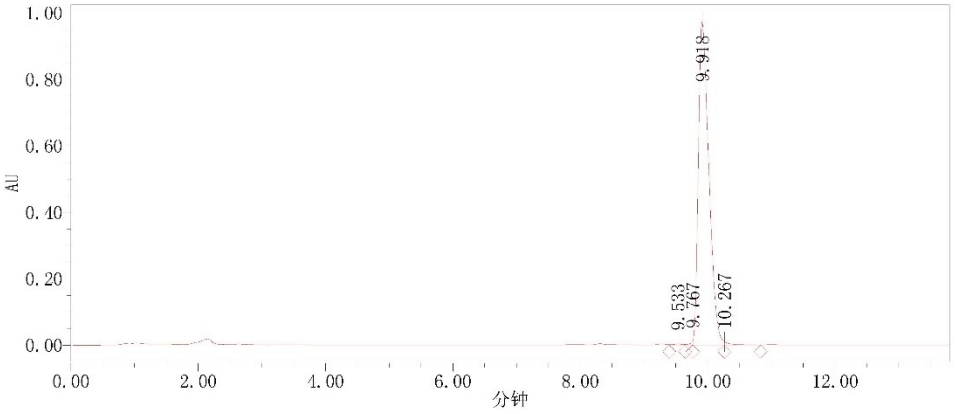
自动标尺色谱图



峰结果

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1		9.533	28055	3020	0.25
2		9.767	22188	9159	0.20
3		9.918	11082541	974193	99.07
4		10.267	53726	8814	0.48

报告用户: System
报告方法: GB138
报告方法 ID: 1377
页码: 1 (共计 1)

项目名称: HPLC检测1
打印日期:
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11:52:50 PRC

HPLC spectrum of L2' strand:

Empower 3 SOFTWARE GB15

样品信息

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样品类型:	未知	样品组名称:	230411
瓶号:	2	采集方法组:	GB
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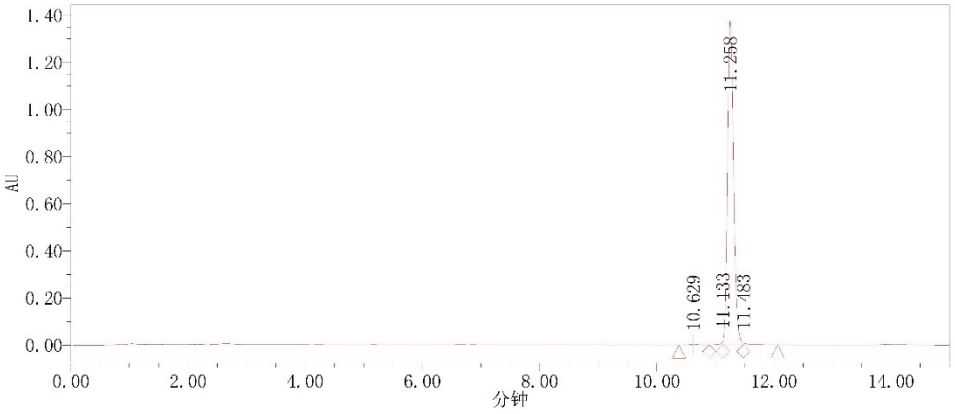

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1		10.629	19251	2901	0.19
2		11.133	29305	13150	0.29
3		11.258	9962977	1384021	99.09
4		11.483	43273	7573	0.43

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报告方法 ID: 1378
页码: 1 (共计 1)

项目名称: HPLC检测1
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11:57:30 PRC

HPLC spectrum of Apt strand:

Empower™ 3 SOFTWARE

GB15

样品信息

样品名称:	1932204337	采集者:	System
样品类型:	未知	样品组名称:	230411
瓶号:	3	采集方法组:	GB
进样次数:	1	处理方法:	GB15
进样体积:	70.00 ul	通道名称:	W2489 ChA
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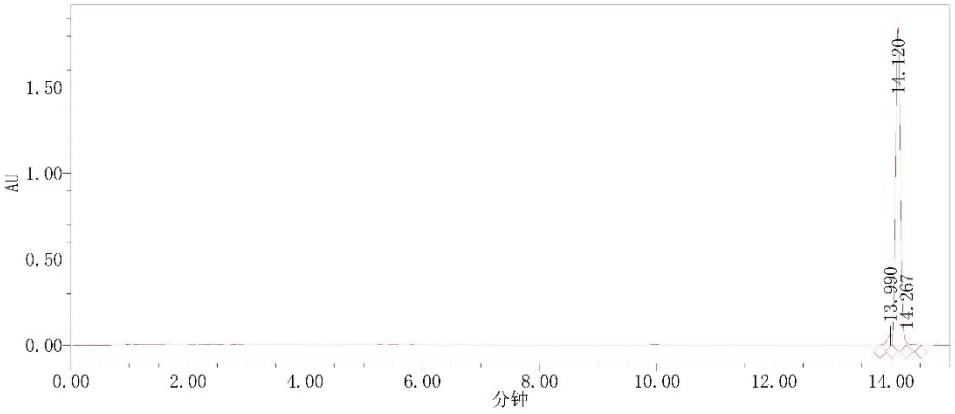

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2		14.120	10314461	1873717	96.71
3		14.267	53782	13070	0.50

报告用户: System
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报告方法 ID: 1378
页码: 1 (共计 1)

项目名称: HPLC检测1
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These HPLC spectrograms are provided by Sangon Bioengineering Co., Ltd (Shanghai, China).

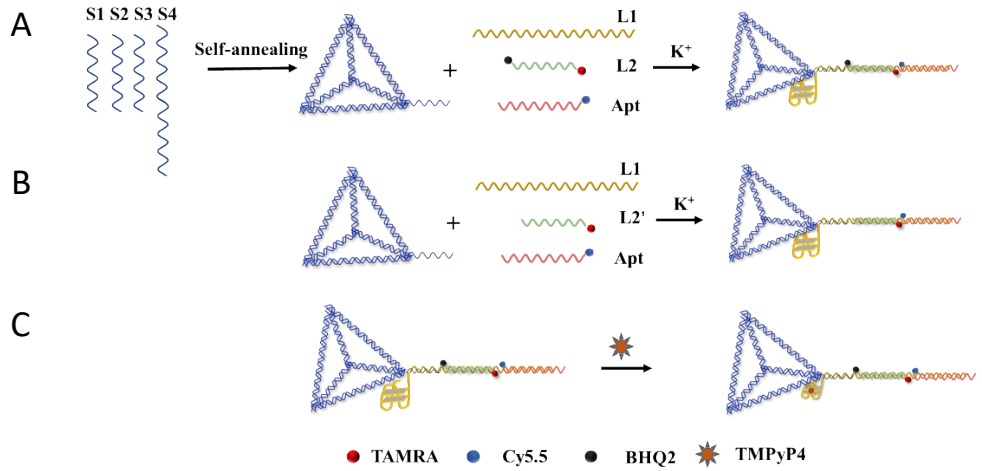

Fig. S1 Schematic diagrams of the assembly of TAH (A), UB-TAH (B), TAH@TMPyP4(C).

Fig. S2 1.5% agarose gel electrophoresis showed self-assembly of TDN (at a concentration of 1 μM). Lane 1: S1; Lane 2: S1 + S2; Lane 3: S1 + S2 + S3; Lane 4: S1 + S2 + S3 + S4. Stained with SYBR Gold.

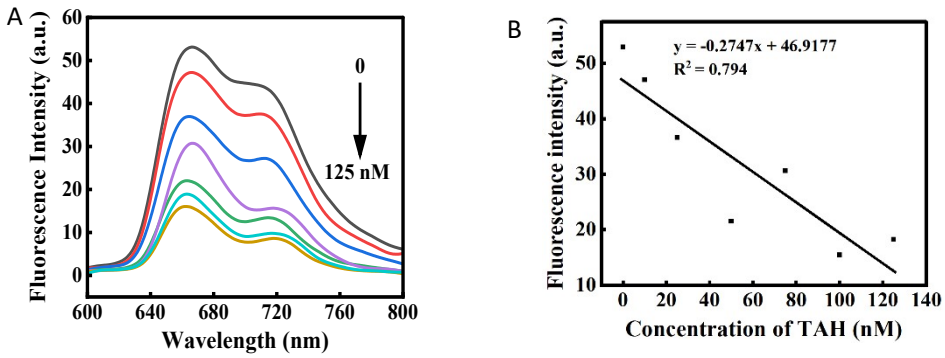

Fig. S3 (A) Fluorescence emission spectra of TMPyP4 (1 μM) upon addition of different concentrations (0, 10, 25, 50, 75, 100 nM and 125 nM) of L1, λex = 435 nm. (B) Fluorescence quantitative curve of TMPyP4 with addition of L1. λem = 668 nm.

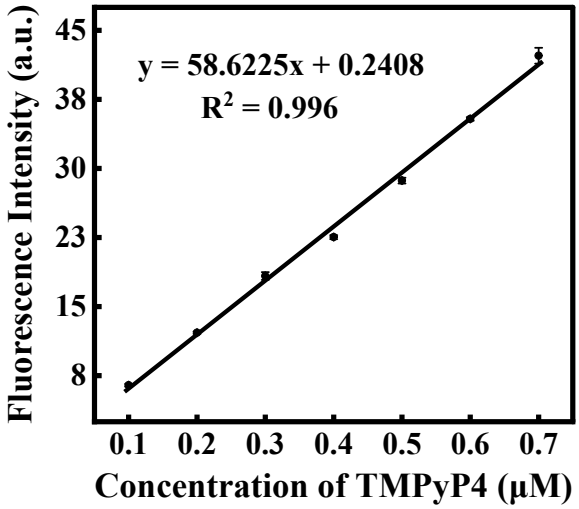

Fig. S4 Standard curve of fluorescence intensity of TMPyP4 (0.1-0.7 μM). λex = 435 nm, λem = 668 nm. Error bars represent standard deviations from three repeated experiments.

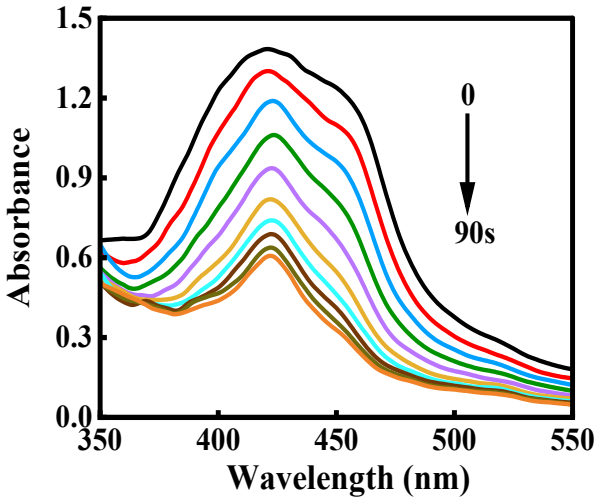

Fig. S5 UV-vis absorption spectra of mixed solutions containing TMPyP4 and DPBF irradiated for different time. The concentration of DPBF is 10 μg/mL.

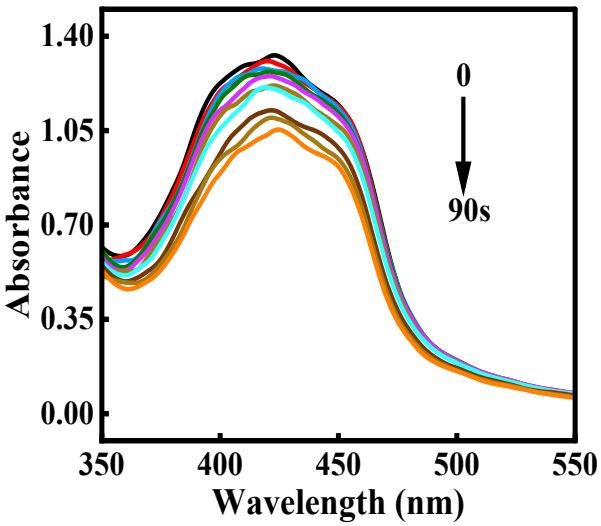

Fig. S6 UV-vis absorption spectra of mixed solutions containing TDN@TMPyP4 and DPBF irradiated for different time. The concentration of DPBF is 10 μg/mL.

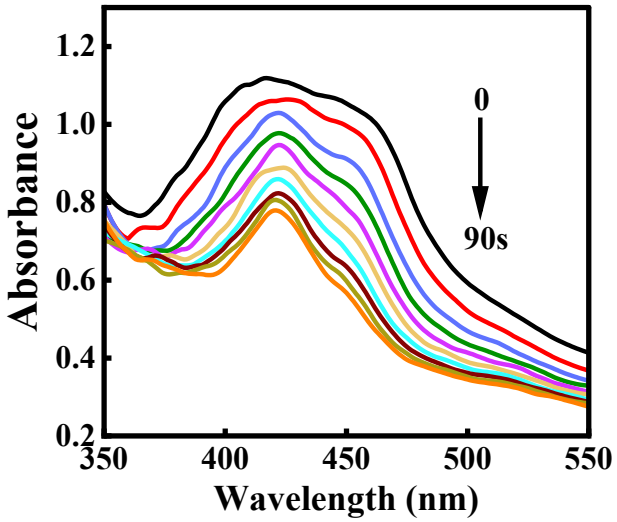

Fig. S7 UV-vis absorption spectra of mixed solutions containing UB-TDN@TMPyP4 and DPBF irradiated for different time. The concentration of DPBF is 10 μg/mL.

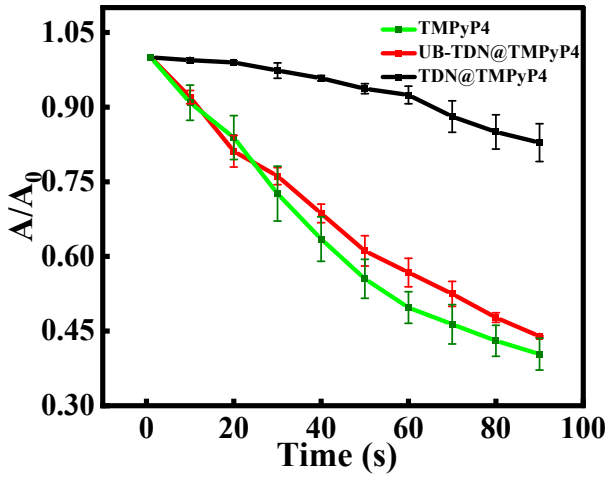

Fig. S8 Normalized intensity of UV-vis absorption of DPBF in different mixed solutions irradiated for different time. Error bars represent standard deviations from three repeated experiments.

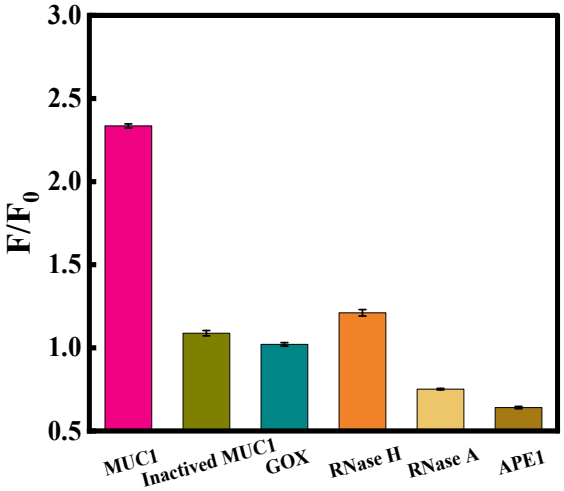

Fig. S9 Fluorescence emission intensity changes of 100 nM TAH in the presence of different proteins: MUC1 (active or inactive, 100 nM), GOX (100 μM), RNase H (1 U/mL), RNase A (10 ng/μL), APE1 (1 U/mL) (F/F_0, F and F_0 represent the Cy5.5 fluorescence emission intensity of TAH nanoprobes before and after adding MUC1 or other substances, respectively). λex = 673 nm, λem = 710 nm. Error bars represent standard deviations from three repeated experiments.

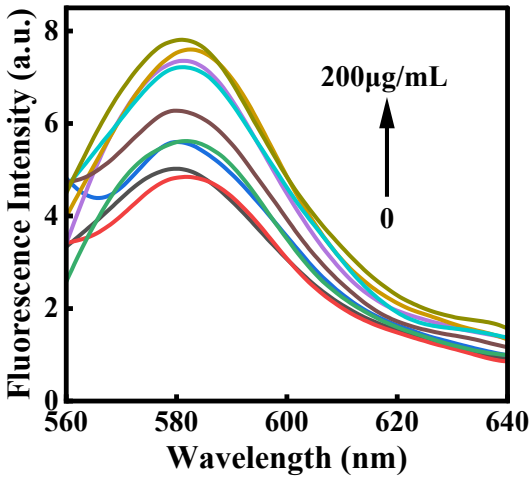

Fig. S10 Fluorescence emission spectrum of TAH upon incubated with different concentrations (0, 25, 50, 100, 125, 150, 175, 200 μg/mL) of inactivated liver microsomes and NADPH (50 μM) under 1% O_2 concentration. λex = 542 nm.

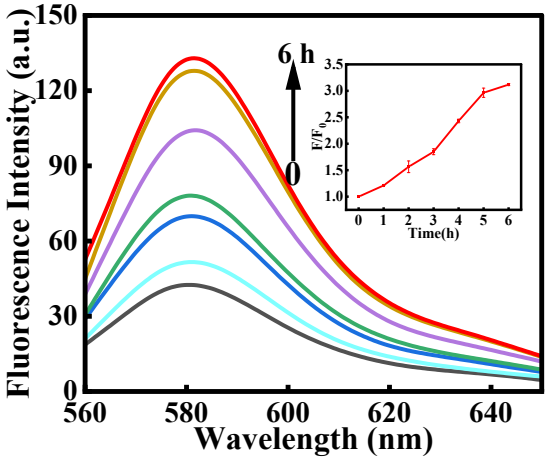

Fig. S11 Fluorescence emission spectra of TAH upon incubated with liver microsomes (100 μg/mL) and NADPH (50 μM) at 1% O_2 for different time. Inset: The TAMRA fluorescence enhancement of TAH after incubated with liver microsomes (100 μg/mL) and NADPH (50 μM) at 1% O_2 for different time (F/F_0, F and F_0 represent the fluorescence intensity of TAMRA before and after incubation of TAH with liver microsomes and NADPH for different time). λex = 542 nm, λem = 580 nm. Error bars represent standard deviations from three repeated experiments.

Fig. S12 Electropherograms of TAH which were incubated in fresh medium containing 10% fetal bovine serum for different time. Stained with SYBR Gold. The concentration of TAH is 1 μM.

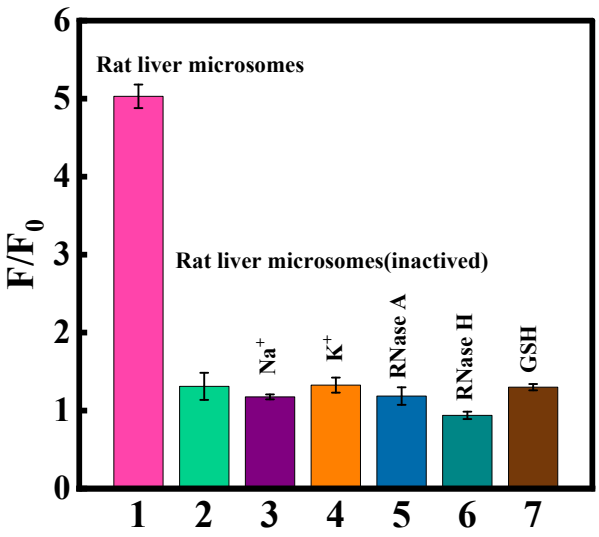

Fig. S13 Fluorescence emission intensity changes of 100 nM TAH in the presence of different metal ions or bioactive small molecules: rat liver microsomes (active or inactive, 100 μg/mL), Na^+ (10 mM), K^+ (10 mM), RNase A (10 ng/μL), RNase H (1 U/mL), GSH (10 mM) (F/F_0, F and F_0 represent the TAMRA fluorescence emission intensity of TAH nanoprobes before and after adding rat liver microsomes or other interfering substances, respectively). λex = 542 nm, λem = 580 nm. Error bars represent standard deviations from three repeated experiments.

Fig. S14 Confocal fluorescence images of MCF-7, HepG2 and NIH/3T3 cells upon incubated with Apt strand labeled by Cy5.5 at 21% O_2 . Scale bar: 20 μm. λex = 640 nm.

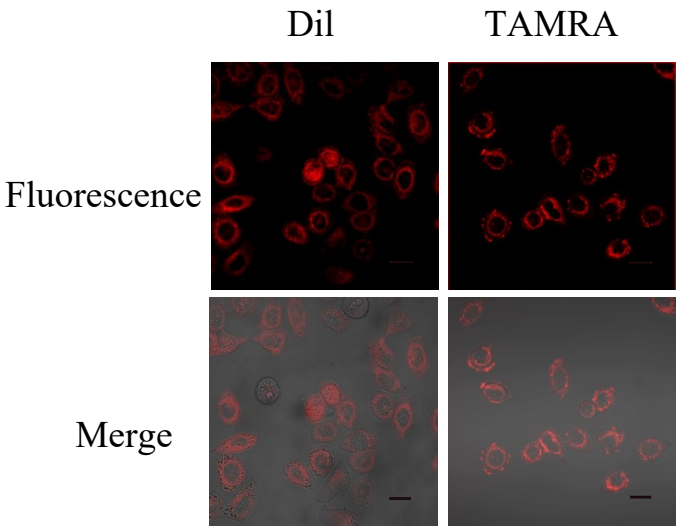

Fig. S15 Confocal fluorescence images of MCF-7 cells incubated with membrane dye (Dil) and TAH respectively. Scale bar: 20 μm. λex = 561 nm.

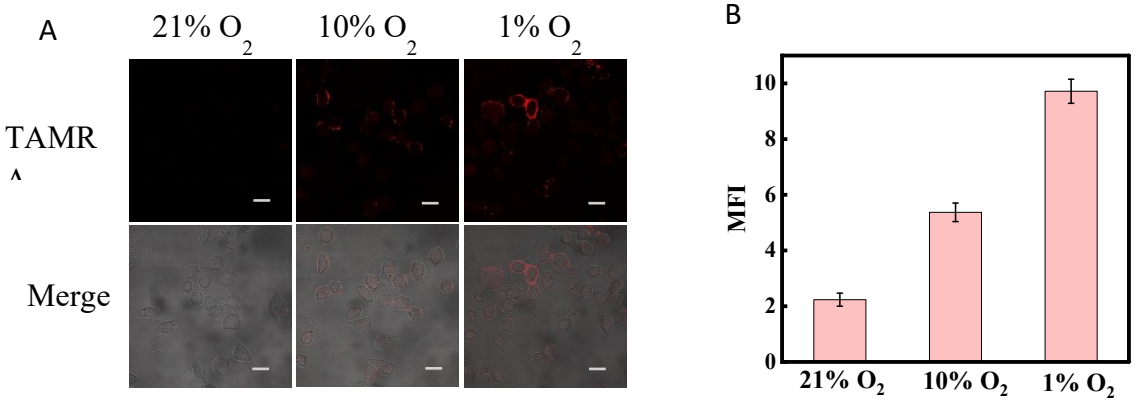

Fig. S16 (A) Confocal fluorescence images of MCF-7 cells upon incubated with TAH under different O_2 concentrations (21%, 10%, and 1% O_2). Scale bar: 20 μm. (B) The relative mean fluorescence intensities of TAMRA under different conditions. λex = 561 nm. Error bars represent standard deviations from three repeated experiments.

Fig. S17 Confocal fluorescence images of MCF-7 cells upon incubated with TAH at different O_2 concentrations (21%, 10%, and 1% O_2). Scale bar: 50 μm. TAMRA channel: λex = 561 nm, Cy5.5 channel: λex = 640 nm.

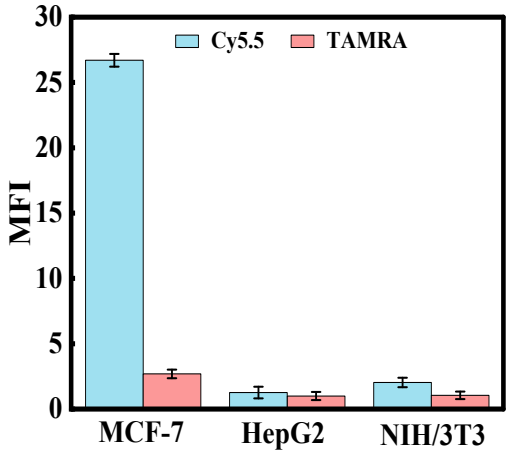

Fig. S18 The relative mean fluorescence intensities of Cy5.5 and TAMRA collected from different cells (MCF-7 cells, HepG2 cells, and NIH/3T3 cells) in Fig. 3B.

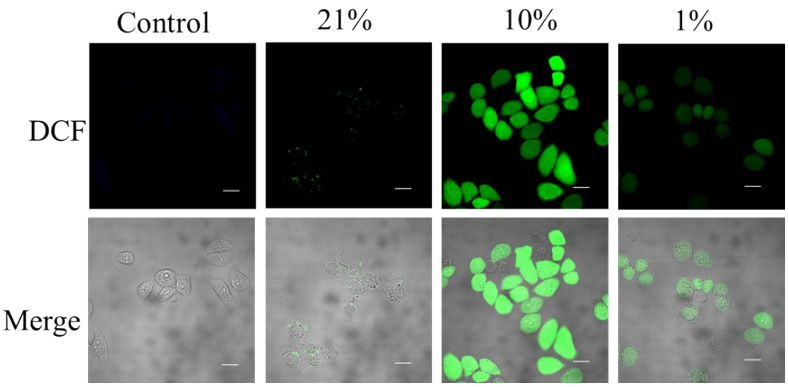

Fig. S19 Confocal fluorescence images of THA@TMPyP4 pre-treated MCF-7 cells at different O_2 concentrations (21% O_2, 10% O_2 and 1% O_2) followed by illumination and DCFH-DA treatment. The cells without any treatment were set as the control group. Scale bar: 20 μm. λex = 488 nm.

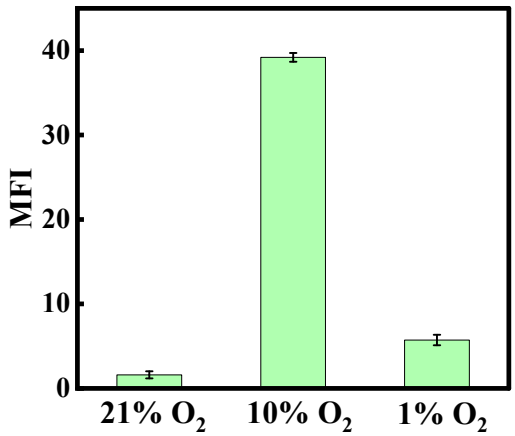

Fig. S20 Fluorescence quantification of DCF collected from the MCF-7 cells upon incubated with THA@TMPyP4 and DCFH-DA probes at different O_2 concentrations (21%, 10% and 1% O_2). λex = 488 nm. Error bars represent standard deviations from three repeated experiments.

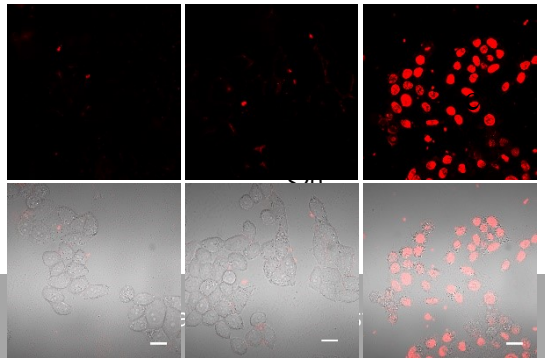

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Fig. S21 Confocal fluorescence imaging images of pyridium iodide stained MCF-7 cells which treated with different conditions (a: Control, b: TAH@TMPyP4, c: TAH@TMPyP4 under 1% O_2). Scale bar: 20 μm. λex = 561 nm.

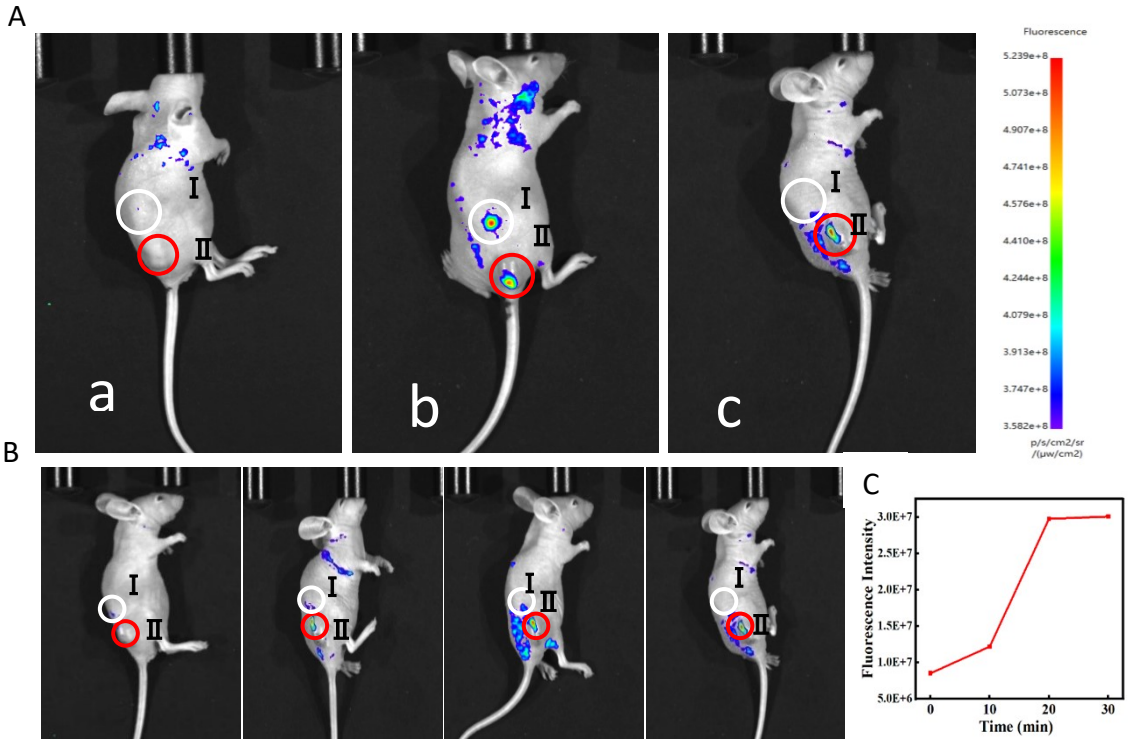

Fig. S22 (A) Schematic diagram of *in vivo* fluorescence imaging of mice injected with PBS (a), UB-TAH (b) and TAH (c) into normal and tumor sites, respectively. Circle I is the normal area, and circle II is the tumor area. (B) *In vivo* fluorescence imaging of mice after injection of TAH into the tumor area over time. (C) Quantitative fluorescence analysis of Fig. S22B.

Fig. S23 Levels of urea (a marker of renal function) (A), alanine aminotransferase (GPT, a marker of liver function) (B) and lactate dehydrogenase (LDH, a marker of lung function) (C) in serum of mice after different treatments. The different treatment groups are consistent with Figure 4B.