$ONOO^-$ generator constructed by a small molecule photosensitizer and photoinduced NO donor for tumor therapy

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Materials

Unless stated otherwise, all the chemical reagents and solvents were obtained commercially and used without further purification. 3-(trifluoromethyl)-4-nitrobenzenamine was purchased from Bide Inc. DMATPE-Br was synthesized via the previously reported method.[S1] WP5-PEG-OH was synthesized by our previously reported method.[S2] Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from PAA Laboratories (Austria). Other organic reagents were purchased from Meryer Chemical Inc and TCI Chemical Inc. Human cervical cancer cells (HeLa cells) were ordered from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The Balb/c nude mice were obtained from the Chinese Academy of Sciences (Shanghai, China).

Instruments

The ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker AVANCE 400 MHz spectrometer. Scanning electron microscopy (SEM) investigations were carried out on a HitachiS-3400 SEM instrument. Dynamic light scattering measurements were performed on a goniometer ALV/CGS-3 using a UNIPHASE He-Ne laser operating at 632.8 nm. UV-Vis spectra were recorded in a quartz cell (light path 10 mm) on a Shimadzu UV-3600 spectrophotometer. Fluorescence spectra were recorded in a conventional quartz cell (light path 10 mm) on a FL970 (Techcomp, China). The output power of the white light was controlled by a fiber coupled Xenon lamp. The intracellular fluorescence imaging was observed using a Nexcope NIB610-FL or a Nikon-A1R HD25 microscope.

Synthesis

Synthesis of NTA-CN: To a one-necked round bottom flask were added 3-(trifluoromethyl)-4-nitrobenzenamine (500 mg, 2.43 mmol), 5-bromopentanenitrile (1.95 g, 12 mmol), KI (2 g, 12 mmol), CH_3CN (20 mL). The mixture was refluxed for 3 days. After cooling down to room temperature, the mixture was poured into water. Then the organic layer was separated and the water layer was extracted with DCM. After removing the solvent, the residue was purified by column chromatography over silica gel to give a yellow solid (237 mg, 34%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, J = 9.0 Hz, 1H), 6.89 (s, 1H), 6.67 (d, J = 7.4 Hz, 1H), 3.31 (t, J = 6.4 Hz, 2H), 2.45 (t, J = 6.3 Hz, 2H), 1.82 – 1.90 (m, 4H).^{13}C NMR (101 MHz, $CDCl_3$) δ 151.5, 136.8, 129.2, 126.7 (q, J = 133.5 Hz), 123.6, 120.8, 119.1, 112.6 (s), 111.2 (q, J = 25.6 Hz), 42.6, 28.0, 22.9, 17.1.

Synthesis of DMATPE-CHO: To a three-necked round bottom flask were added DMATPE-Br (597 mg, 1.20 mmol), 5-formylthiophene-2-boronic acid (382 mg, 2.45 mmol), Pd(dppf)Cl_2 (45 mg, 0.06 mmol), K_2CO_3 (850 mg, 6.12 mmol) and MeOH/toluene (25 mL, *v*/*v*=1/1). The mixture was deoxygenated with argon for 30 min and then stirred at 75 °C for 16 h. After cooling down to room temperature, the mixture was filtered. After removing the solvent, the residue was purified by column chromatography over silica gel to give a yellow solid (450 mg, 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.85 (s, 1H), 7.69 (d, J = 3.9 Hz, 1H), 7.41 (d, J = 8.2 Hz, 2H), 7.33 (d, J = 3.9 Hz, 1H), 7.14 – 7.05 (m, 7H), 6.91 (dd, J = 13.6, 8.6 Hz, 4H), 6.46 (dd, J = 11.6, 8.7 Hz, 4H), 2.90 (d, J = 4.4 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.8, 154.9, 149.1, 148.9, 147.2, 144.9, 142.5, 141.7, 137.6, 135.7, 132.7, 132.6, 132.3, 131.9,

131.9, 131.6, 129.8, 127.8, 125.8, 125.6, 123.5, 111.4, 111.3, 40.37.

Synthesis of DMATPE-CN: To a one-necked round bottom flask were added DMATPE-CHO (106 mg, 0.20 mmol), malononitrile (26 mg, 0.40 mmol), Et_3N (two drops) and DCM (2 mL). The mixture was stirred at room temperature for 16 h. After removing the solvent, the residue was purified by column chromatography over silica gel to give a brown red solid (29 mg, 25%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.75 (s, 1H), 7.67 (d, *J* = 4.1 Hz, 1H), 7.42 (d, *J* = 8.4 Hz, 2H), 7.36 (d, *J* = 4.1 Hz, 1H), 7.15 – 7.05 (m, 7H), 6.91 (dd, *J* = 11.9, 8.8 Hz, 4H), 6.46 (dd, *J* = 11.8, 8.8 Hz, 4H), 2.91 (d, *J* = 7.5 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.3, 150.5, 149.2, 149.0, 149.0, 148.3, 144.7, 143.1, 140.2, 135.5, 133.6, 132.7, 132.7, 132.5, 131.7, 128.9, 127.8, 125.9, 125.8, 124.1, 114.5, 113.6, 111.4, 111.2, 75.7, 40.3.

Fluorescence titration experiments

Fluorescence titration experiments were conducted to estimate the association constant (*K*a) and the stoichiometry of the complexation. The fixed initial concentration of NTA-CN in CH_2Cl_2 is 100.0 μM. The fluorescence spectra of the NTA-CN solution mixed with WP5-PEG-OH of different concentrations were obtained. The fluorescence intensities at 447 nm were recorded respectively.

NO release assay

Different concentrations of $NaNO_2$ standard solution were prepared. Each quartz dish was added with $NaNO_2$ standard solution (3 mL), Griess reagent 1 (50 μL) and Griess reagent 2 (50 μL). After 10 minutes, the absorbance of each dish at 540 nm was measured. The relationship between the absorbance and the concentration of NO was

build up by drawing a standard curve. According to the standard curve, we can calculate the NO release efficiency.

Photodynamic experiments

$^1O_2$ detection: DMATPE-CN in CH_2Cl_2 (10 μM) in quartz cuvettes were mixed with ABDA (10 μM), which was then irradiated by white light (25 mW/cm^2) for a period of 6 min. The absorbance of the solution was recorded at the pre-set time points during the process.

•OH detection: DMATPE-CN in CH_2Cl_2 (10 μM) in quartz cuvettes were mixed with APF in DMF (200 μM), which was then irradiated by white light (25 mW/cm^2) for a period of 100 s. The fluorescence intensity at 515 nm of the solution was recorded at the pre-set time points during the process. (Excitation wavelength: 488 nm).

$O_2^{\bullet-}$ detection: DMATPE-CN in CH_2Cl_2 (10 μM) in quartz cuvettes were mixed with DHE in DMSO (40 μM) and ctDNA in H_2O (500 μg/mL), which was then irradiated by white light (25 mW/cm^2) for a period of time. The fluorescence intensity at 580 nm of the solution was recorded at the pre-set time points during the process. (Excitation wavelength: 510 nm).

$ONOO^-$ detection

LAP was employed to detect $ONOO^-$ generation of WP5-NTA/CN. WP5-NTA/CN aqueous solution (200 μg/mL) was mixed with LAP (10 μM) in PBS (20 mM, pH = 7.4) solution containing 1% DMSO, which was then irradiated by white light (25 mW/cm^2) for a period of time. The absorbance of the solution was recorded at the pre-set time points during the process.

Cell experiments

Intracellular NO detection: HeLa cells were incubated with WP5-NTA/CN (200 μg/mL) for 4 h followed by incubation with 5 μM DAF-FM DA for 30 min. After being washed by PBS buffer for three times, cells were irradiated with white light (25 mW/cm^2) for 15 min. Then, the fluorescence was immediately observed using fluorescence microscopy (λ_{ex}: 460 – 495 nm, $\lambda_{em:}$ > 510 nm).

Intracellular $O_2^{\cdot-}$ detection HeLa cells were incubated with WP5-NTA/CN (200 μg/mL) for 4 h followed by incubation with DHE for 30 min. After being washed by PBS buffer for three times, cells were irradiated with white light (25 mW/cm^2) for 15 min. Then, the fluorescence was immediately observed using fluorescence microscopy (λ_{ex}: 510 – 550 nm, λ_{em}: > 575 nm).

Intracellular $ONOO^-$ detection: HeLa cells were incubated with WP5-NTA/CN (200 μg/mL) for 4 h followed by incubation with LAP for 30 min. After being washed by PBS buffer for three times, cells were irradiated with white light (25 mW/cm^2) for 20 min. Then, the fluorescence was immediately observed using fluorescence microscopy (λ_{ex}: 510 – 550 nm, λ_{em}: > 575 nm).

Cytotoxicity experiments Cells were incubated in DMEM. The medium was supplemented with 10% FBS and 1% Penicillin-Streptomycin. HeLa cells were seeded in 96-well plates (5×10^4 cell mL^{-1}, 0.1 mL per well) for 24 h at 37 ^{o}C in 5% CO_2. Then DMEM containing different concentrations of WP5-NTA/CN was introduced to replace the original medium. Four hours later, the cells were treated with or without a white light (25 mW/cm^2). After 20 min irradiation, cells were cultured for the next 24

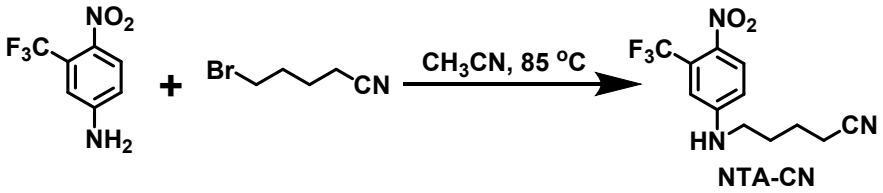
h. The relative cellular viability was determined by the MTT assay. For the experiment under hypoxic condition, HeLa cells were incubated in DMEM with 10% FBS and 1% Penicillin-Streptomycin. For hypoxia, the original culture medium was replaced by 1 mL fresh medium containing DFO (100 μM). Then the cells were returned to incubator (5% CO_2, 20% O_2 at 37 °C) and incubated for 8 h. Next, the cells were washed thoroughly with PBS three times. Other operations were same to that in normoxic environment. For the NO therapy alone, HeLa cells were incubated in DMEM with 10% FBS and 1% Penicillin-Streptomycin. the original culture medium was replaced by 1 mL fresh medium containing Vc (4 mM). other operations were same to that in normal therapy process. For the PDT alone, HeLa cells were incubated in DMEM with 10% FBS and 1% Penicillin-Streptomycin. the original culture medium was replaced by 1 mL fresh medium containing PTIO (5 mM). other operations were same to that in normal therapy process.

Live-Dead Cell Staining The same density of HeLa cells (3×10^5 cell mL^{-1}) were distributed into three confocal dishes (35 mm) for 12 h. Then the 2-plate cells were cultured with new DMEM containing WP5-NTA/CN (200 μg/mL). After 4 h, the cells were subjected to dark or a white light (25 mW/cm^2). After 48 h, the cells were stained with a calcein AM/propidium iodide mixture for 30 min and washed twice using PBS. The fluorescence images were eventually acquired via a confocal laser scanning microscope.

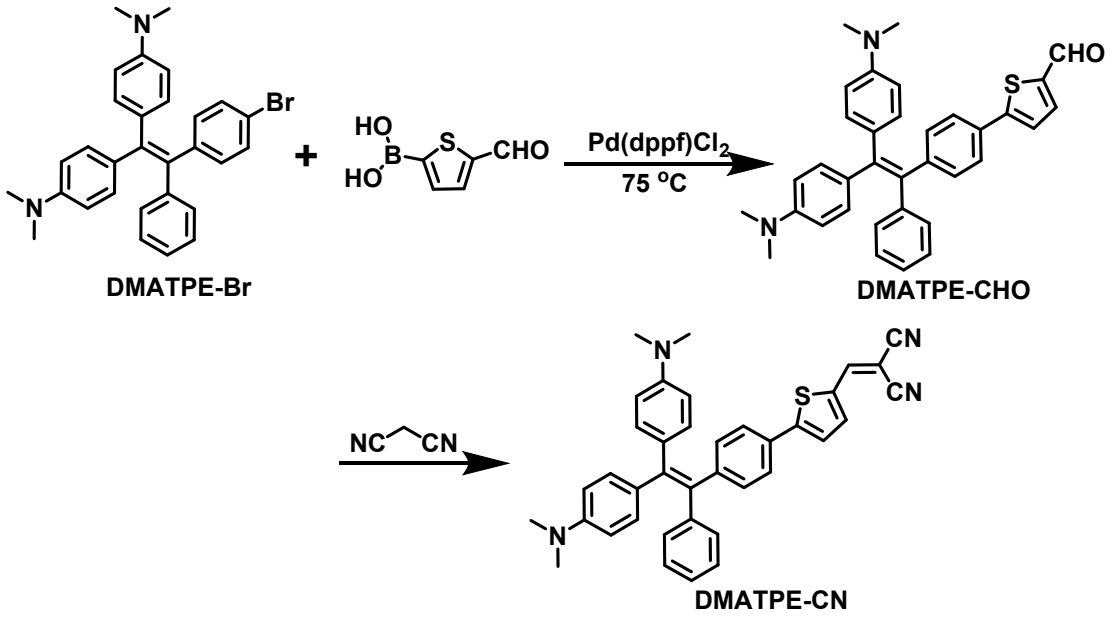
In vivo antitumor

All animal experiments were approved by the Laboratory Animal Ethics Committee of the Nantong University.

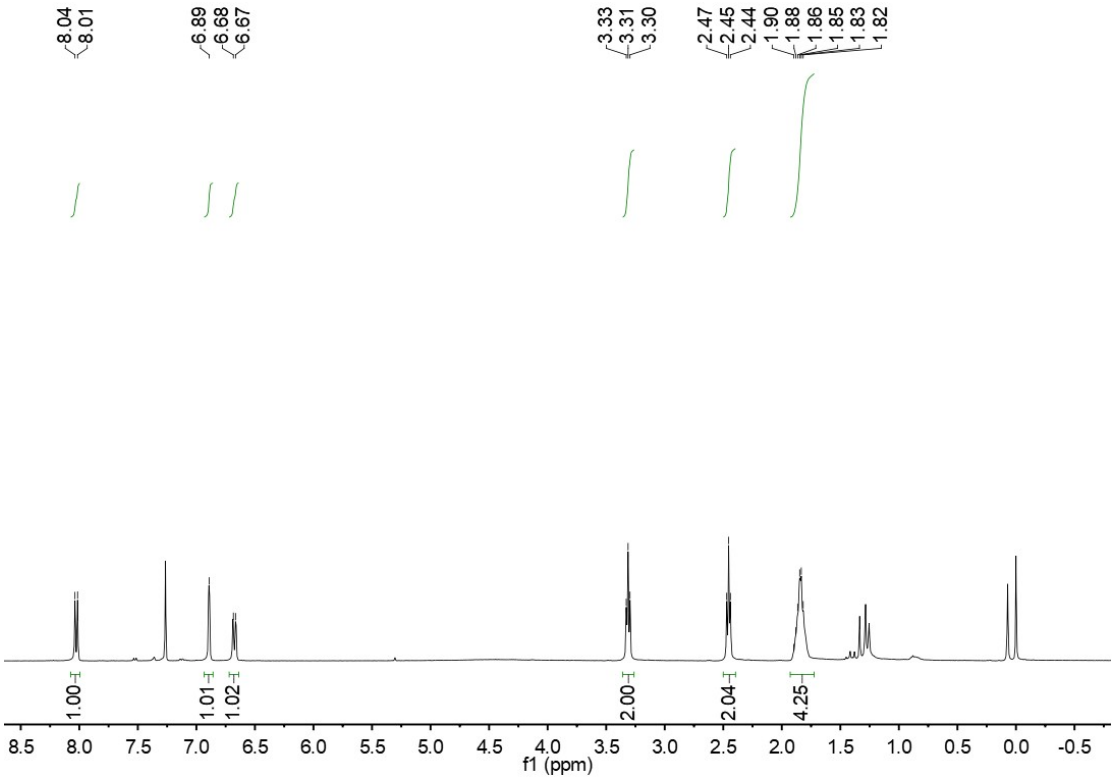
After being acclimated and tested for infectious diseases for 1 week, 4-week-old Balb/c mice were subcutaneously injected with HeLa cells (1×10^7 cells each mouse) at the flank region. When the tumor volume reached approximately 80 mm^3, the HeLa tumor-bearing Balb/c nude mice were stochastically assigned to four groups (n = 3) for different treatments. group I (PBS group), administration with PBS (200 μL) alone; group II (PBS + light group), PBS administration (200 μL) followed by white light irradiation (150 mW/cm^2, 20 min); group III (WP5-NTA/MN group), administration with WP5-NTA/MN (2 mg/mL, 200 μL) alone; group IV (WP5-NTA/MN + light group), administration with WP5-NTA/MN (2 mg/mL, 200 μL) and followed by light irradiation (150 mW/cm^2, 20 min). The body weight and tumor volumes of mice from different groups were continually measured and recorded up to 14 days. After 14 days, the tumors were dissected for H&E staining.



Scheme S1 The synthesis route for NTA-CN



Scheme S2 The synthesis route for NTA-CN.



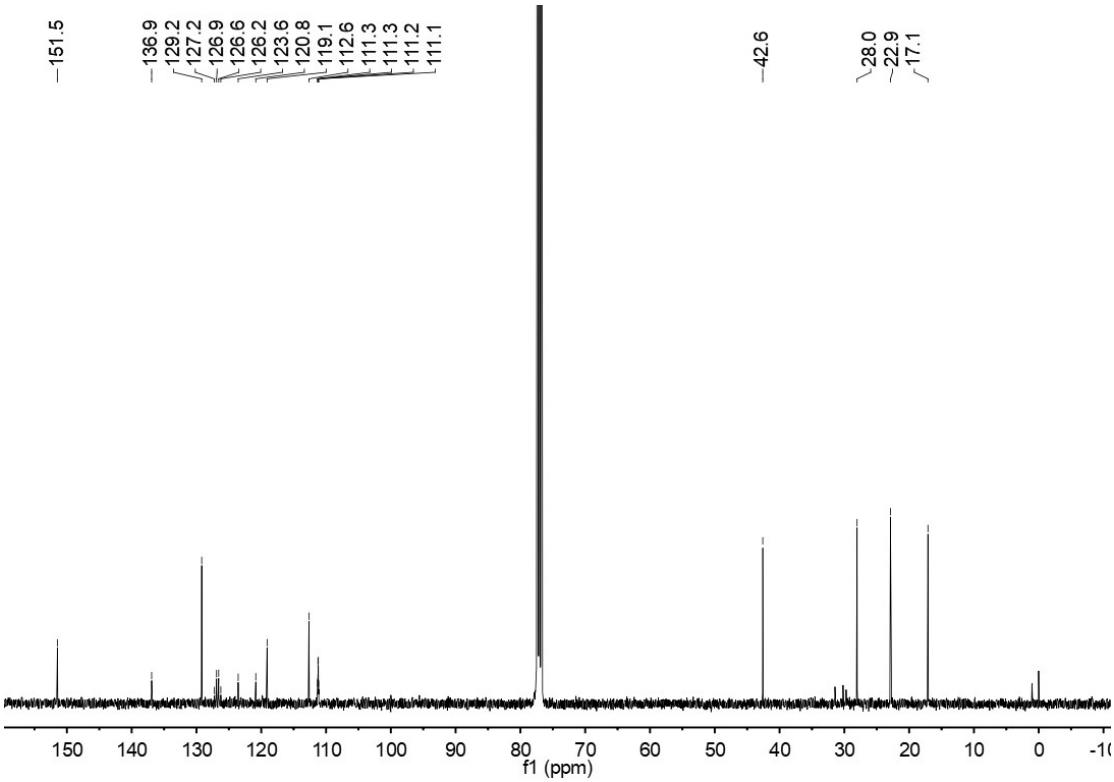
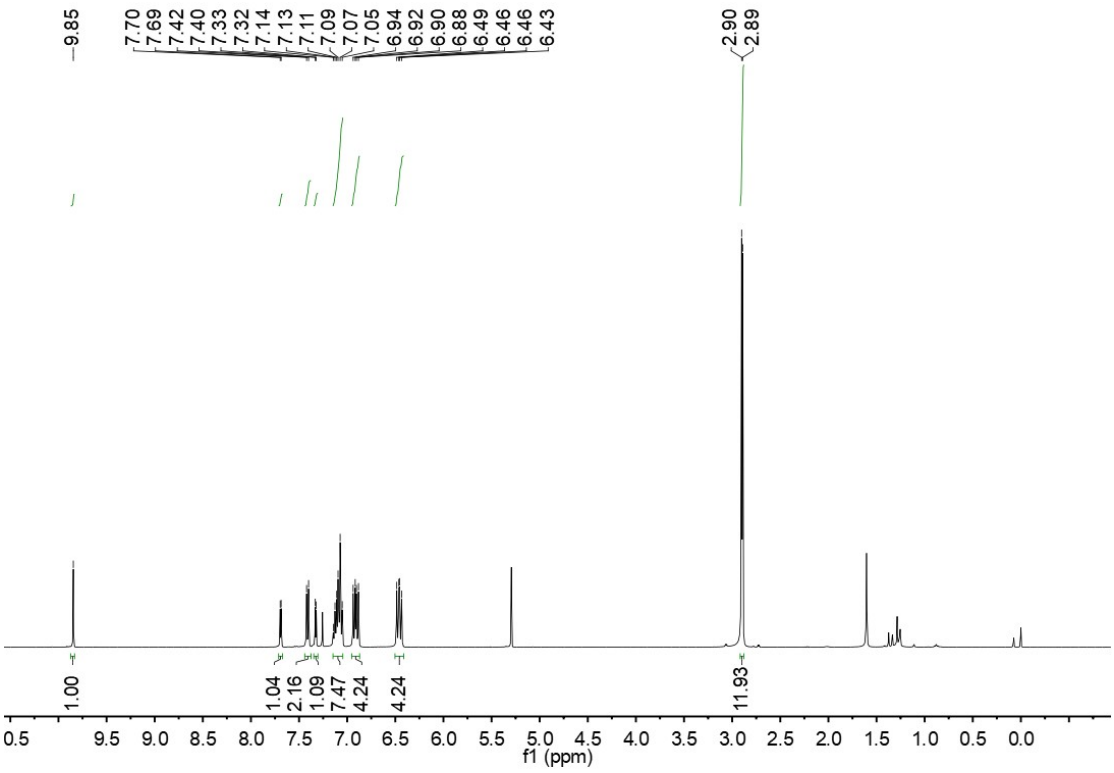


Fig. S1 The ^{1}H NMR and ^{13}C NMR spectra for NTA-CN



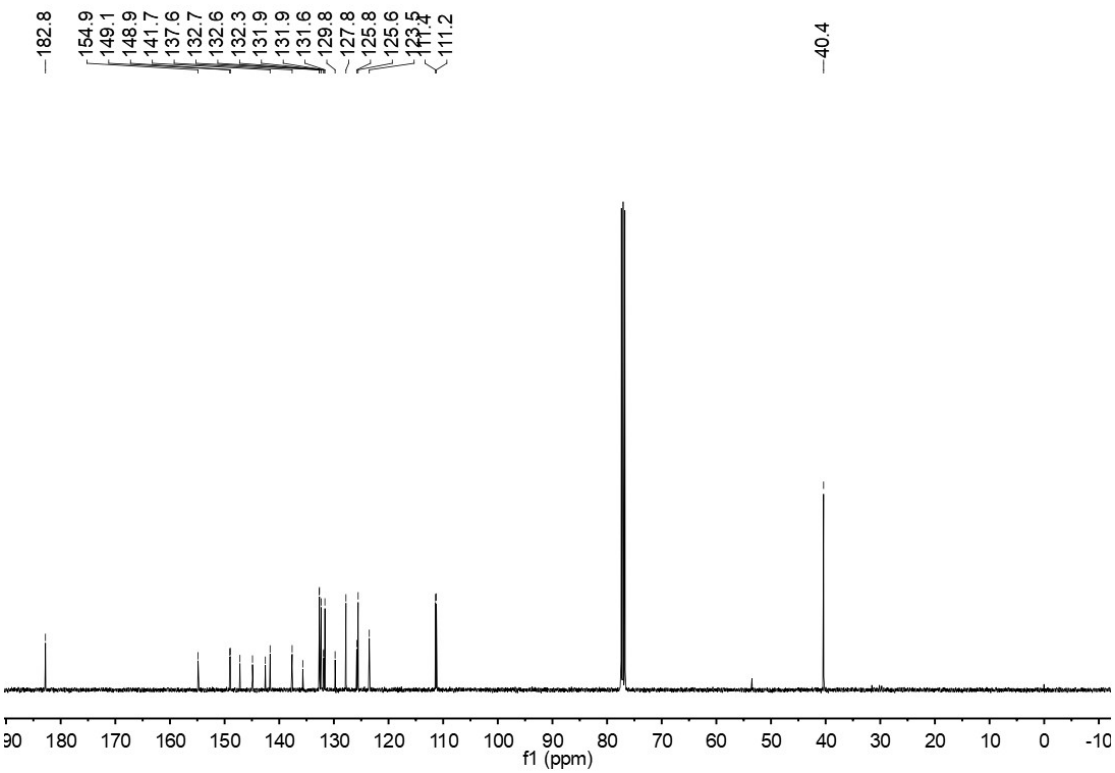
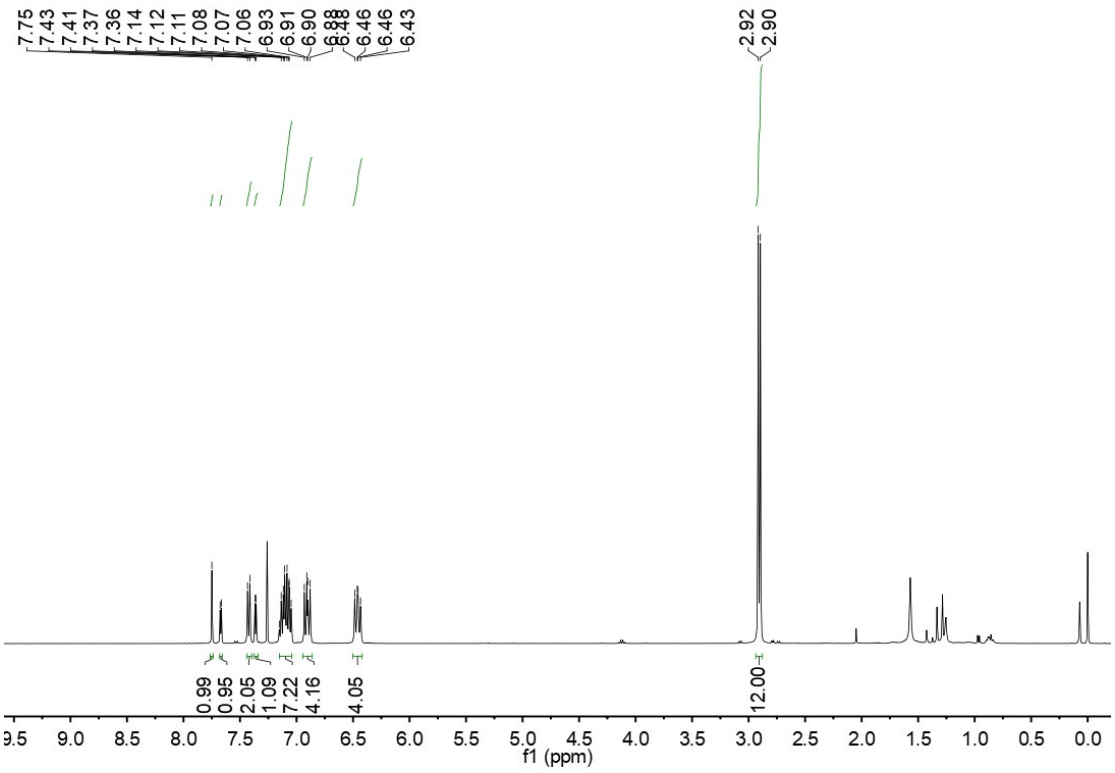


Fig. S2 The ^{1}H NMR and ^{13}C NMR spectra for DMATPE-CHO



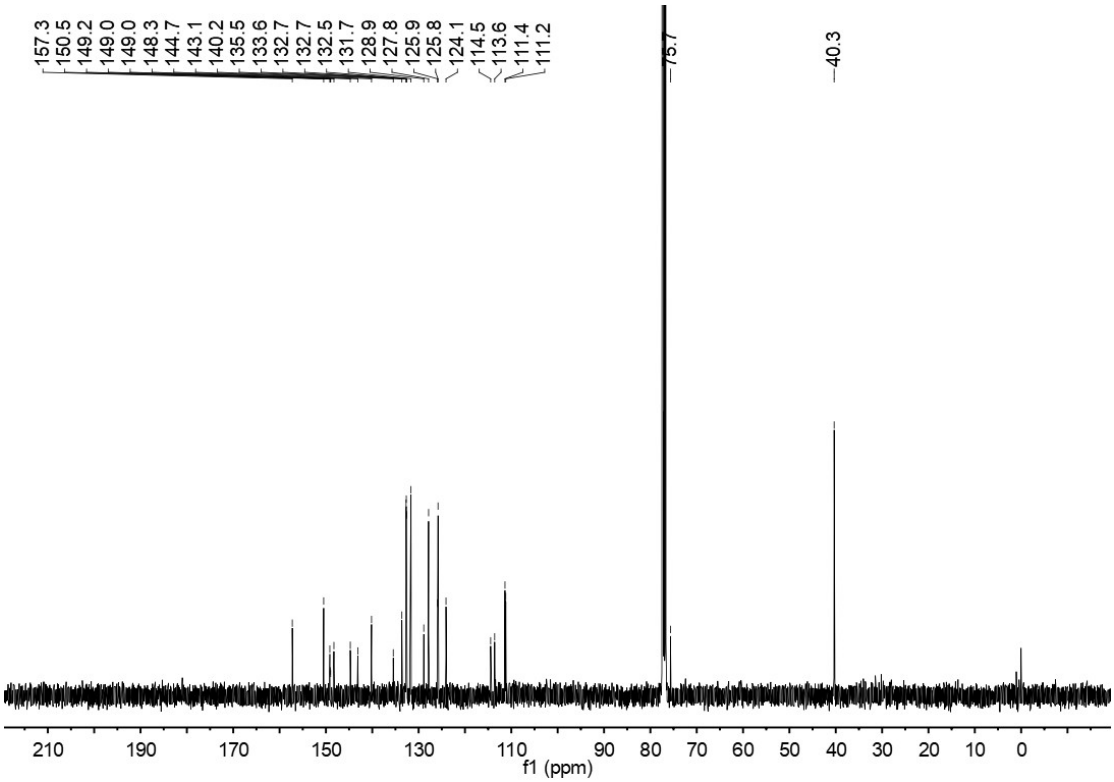


Fig. S2 The ^{1}H NMR and ^{13}C NMR spectra for DMATPE-MN

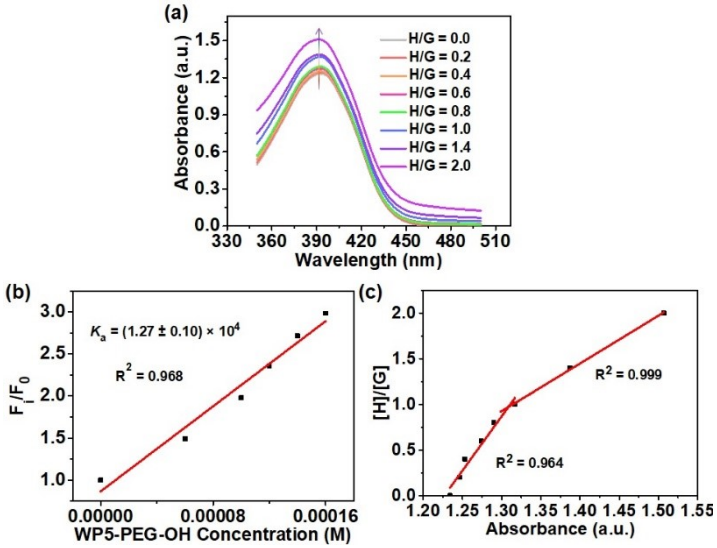


Fig. S4 (a) The fluorescence spectra of NTA-CN with the addition of different concentrations of WP5-PEG-OH. (b) The fitting lights of *ΔF* versus concentration of WP5-PEG-OH. (c) The stoichiometry of host and guest complex.

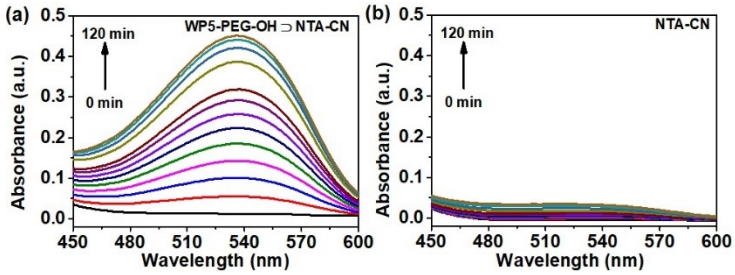


Fig. S5 (a) The absorption spectra of WP5-PEG-OH ⊃ NTA-CN complex solution (H_2O/DMF = 9/1) with the addition of Griess reagent after light irradiation. (b) The absorption spectra of NTA-CN solution (H_2O/DMF = 9/1) with the addition of Griess reagent after light irradiation.

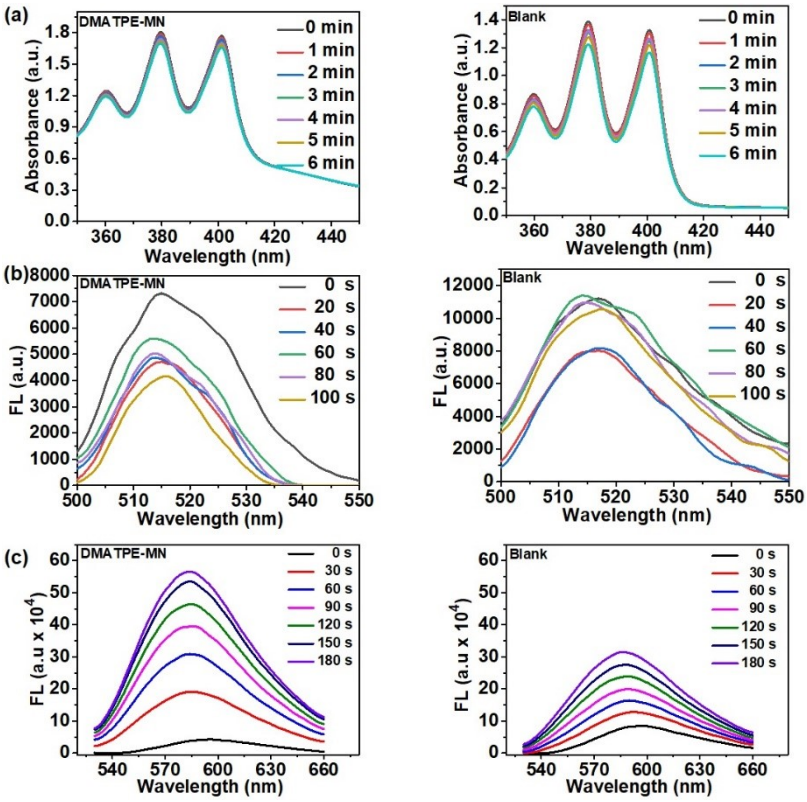


Fig. S6 (a) The absorption spectra of ABDA in the presence or absence of DMATPE-MN after light irradiation. (b) The fluorescence spectra of APF in the presence or absence of DMATPE-MN after light irradiation. (c) The fluorescence spectra of DHE in the presence or absence of DMATPE-MN after light irradiation.

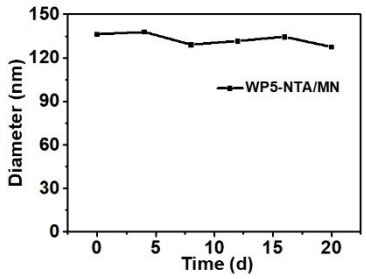


Fig. S7 The sizes changes of WP5-NTA/MN during 20 day.

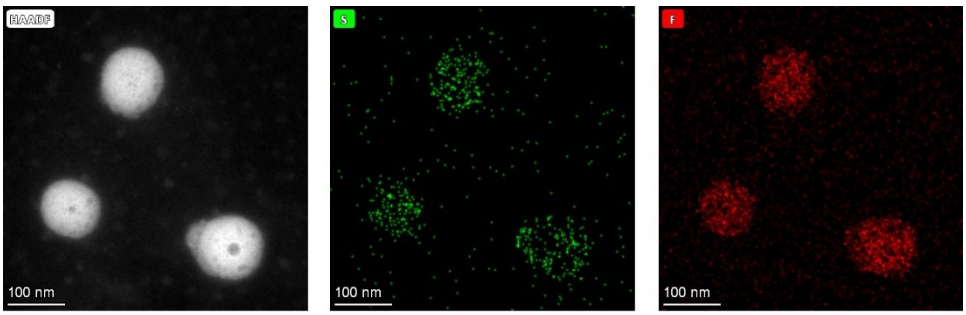


Fig. S8 The two-dimensional element mappings for the detected elements S and F of WP5-NTA/MN.

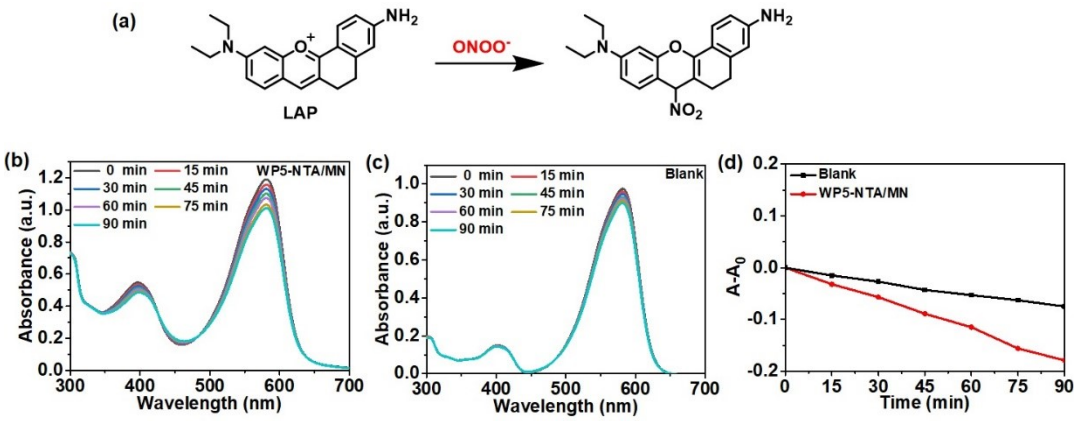


Fig. S9 (a) The $ONOO^-$ detection mechanism of LAP. (b) The absorption spectra of LAP with WP5-NTA/MN aqueous solution (200 μg/mL) after light irradiation. (c) The absorption spectra of LAP without after light irradiation. (d) The changes of the absorbance of LAP at 590 nm in the presence or absence of WP5-NTA/MN after light irradiation.

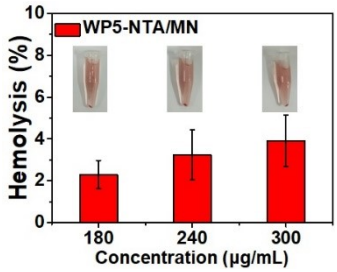


Fig. S10 Percentage of hemolysis of the red blood cells incubated with WP5-NTA/MN of different concentrations. Data are presented as the average ± standard deviation (n = 6) (Inserted graph: red blood cells incubated with WP5-NTA/MN of the conrresponding concentrations).

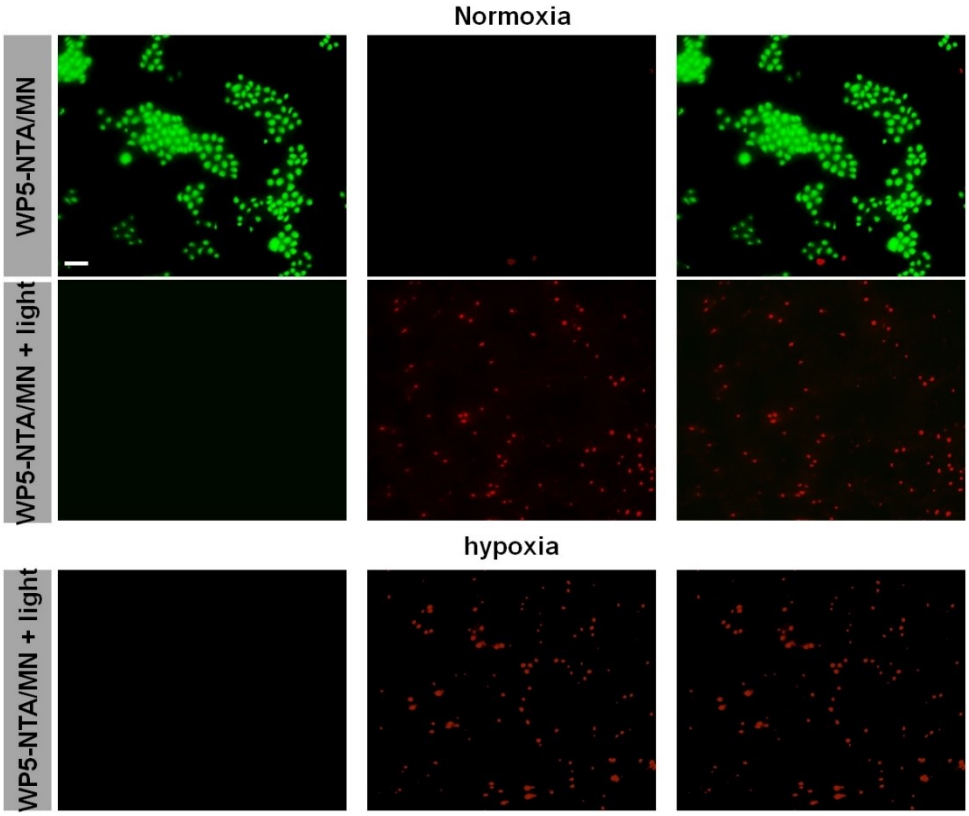


Fig. S11 The Calcein-AM and PI staining imaging of HeLa cells after received different treatments (Live cell: green; dead cell: red. Scale bar: 50 μm).

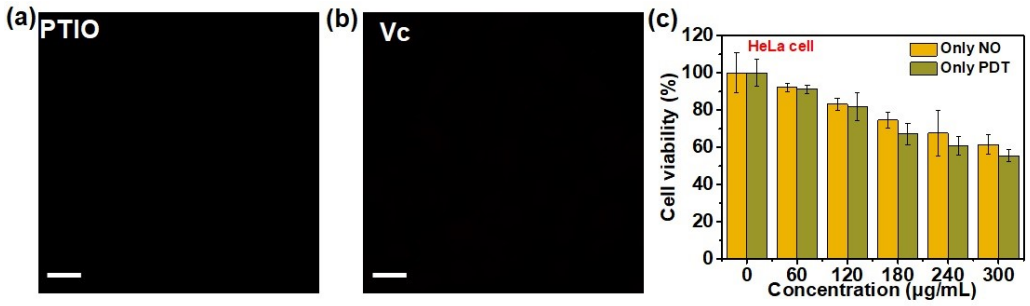


Fig. S12 (a) The detection of NO in HeLa cells after incubated with PTIO and WP5-NTA/MN under light irradiation. (b) The detection of NO in HeLa cells after incubated with Vc and WP5-NTA/MN under light irradiation. Scale bar: 50 μm. (c) The viability data of HeLa cell after received NO therapy alone or PDT alone.

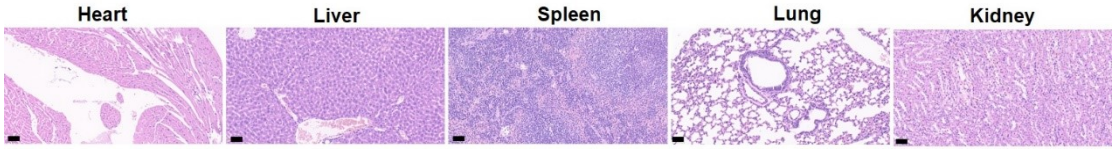


Fig. S13 H&E staining images of major organ slices from mice after treatments (Scale bars: 50 μm).

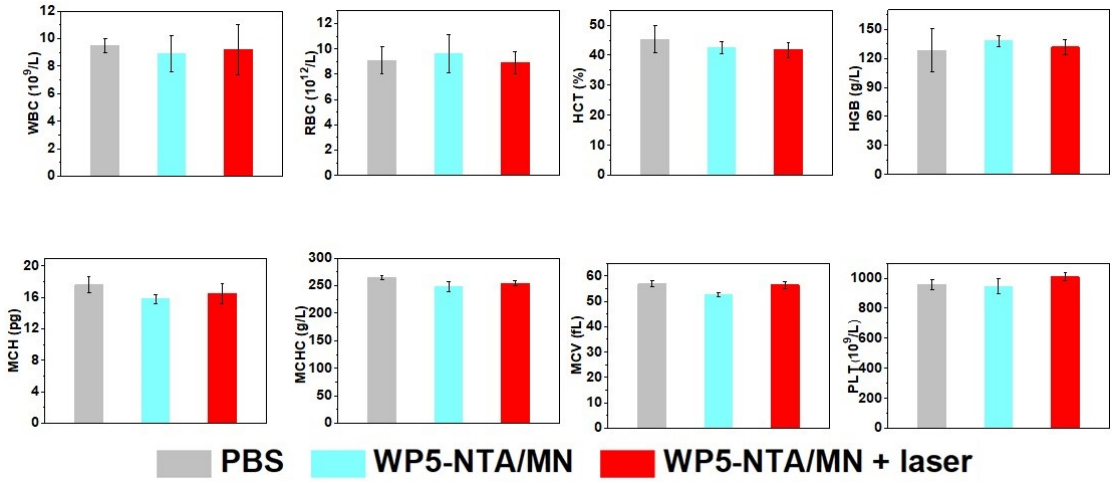


Fig. S14 Parameter values including white blood cell (WBC), red blood cell (RBC) hematocrit (HCT), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) and platelets (PLT) of routine examination for all the mice.

S1 B. Guo, X. Cai, S. Xu, S. M. A. Fateminia, J. Liu, J. Liang, G. Feng, W. Wu and B. Liu, Decoration of Porphyrin with Tetraphenylethene: Converting Fluorophore with Aggregation-Caused Quenching to Aggregation Induced Emission Enhancement. *J. Mater. Chem. B*, 2016, **4**, 4690−4695.

S2 B. Lu, Y. Huang, J. Xia and Y. Yao, Active targeted drug delivery system constructed from functionalized pillararenes for chemo/photodynamic synergistic therapy. *Front. Chem. Sci. Eng.*, 2024, **18**, 138.