Supporting Information

An Oxygen-Economical Nano-Photosensitizer with High Photodynamic Therapeutic Outcome via Simultaneously Reducing Cellular Respiration and Oxygen Depletion of PDT

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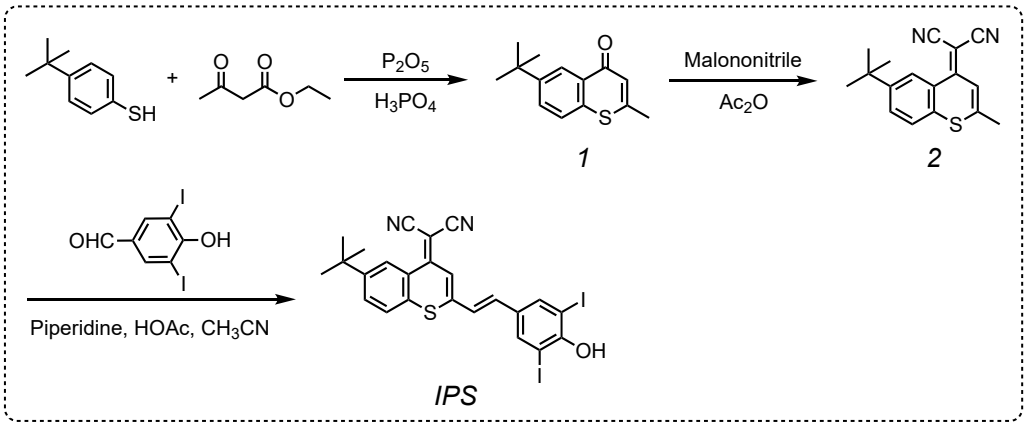
1. Materials

Unless otherwise noted, reagents were used as received from commercial sources. Solvent preparations were carried according to described procedures.[1] Poly (ethylene glycol) methyl ether (PEG-OH; average Mn 5,000), ε-caprolactone (ε-CL), and tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) were purchased from TCI (Shanghai, China). Fetal bovine serum (FBS), Trypsin-EDTA (0.25%), Dulbecco's Modified Essential Medium (DMEM), and 1 × phosphate buffer saline (PBS; pH 7.4) were purchased from Gibco (Life Technologies, AG, Switzerland). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Grand Island Biological Company (USA). Viability/Cytotoxicity Assay kit for animal live dead cells was purchased from US everbright Inc. DNA from calf thymus (DNA-ct), 2′,7′-dichlorfluorescein-diacetate (DCF-DA), 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), dihydroethidium (DHE), atovaquone (ATO), and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were purchased from Sigma-Aldrich. Water was deionized with a Milli-Q SP reagent water system (Millipore) to a specific resistivity of 18.2 MΩcm.

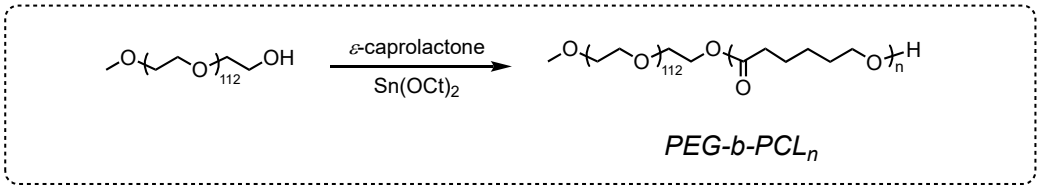
2. Instruments

^1H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker AV400 NMR spectrometer, operating at 400 and 100 MHz, respectively, where chemical shifts (δ in ppm) were determined using partially

or non-deuterated solvent residues as internal references. DMSO-d_6 and $CDCl_3$ were used as the solvents. HPLC analysis were performed with a Shimadzu HPLC system, equipped with a LC-20AT binary pump, an SPD-20A UV-vis detector, and a Symmetry C18 column. UV-vis absorption spectra were recorded on a UH5300 doublebeam UV-vis spectrophotometer (Hitachi). Fluorescence spectra were recorded on an F-4600 (Hitachi) spectrofluorometer. Dynamic light scattering (DLS) study was performed using a Zetasizer NanoZS (Malvern). Morphological analysis was conducted on transmission electron microscopy (TEM; Talos F200C, FEI). MTT assay were monitored by the microplate reader (SpectraMax i3x, MD). Confocal laser scanning microscopy (CLSM) images were acquired using a Leica TCS SP8 microscop8. Fluorescence images of cells were acquired using an inverted fluorescence microscope (ZOE Fluorescent cell Imager, BIO-RAD). Cell flow experiments were detected with flow cytometry (BD LSRFortessa, BD). *In vivo* and *ex vivo* fluorescence imaging of tumor-bearing mice was conducted on NightOWL II LB983 In Vivo Imaging System (Berthold Technologies).



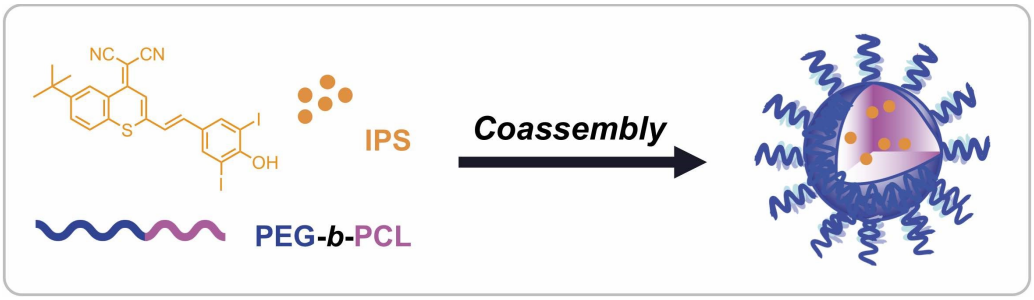
Scheme S1. Synthetic routes employed for the synthesis of IPS.



Scheme S2. Synthetic route employed for the preparation of PEG-*b*-PCL_n diblock copolymer.

3. Preparation of IPS@NPs

The preparation of IPS@NPs was similar to that of ATO-IPS@NPs. Typically, as illustrated in Scheme S3, a THF solution (0.5 mL) of PEG-*b*-PCL_{18} (10 mg) and IPS (0.1 mg) was quickly added into 10 mL deionized water under vigorous stirring at 900 rpm at room temperature. The resulting solution was stirred overnight under this condition. After removing THF by ultrafiltration (10 kD, Amicon), the concentrated aqueous solution of IPS@NPs (or ATO@NPs) was diluted in deionized water to give a stock solution and stored in 4 ℃.



Scheme S3. Schematic illustration of the preparation of IPS@NPs via encapsulating IPS photosensitizers into a polymeric micelle of PEG-*b*-PCL_{18}.

4. Calculation of Drug Encapsulation Efficiency and Loading Efficiency

The calculation formula for drug encapsulation efficiency (EE) and drug loading efficiency (LE) are as follows:

$$EE(\%) = \frac{M_d}{M_{d0}} \times 100\% \qquad \text{S1}$$

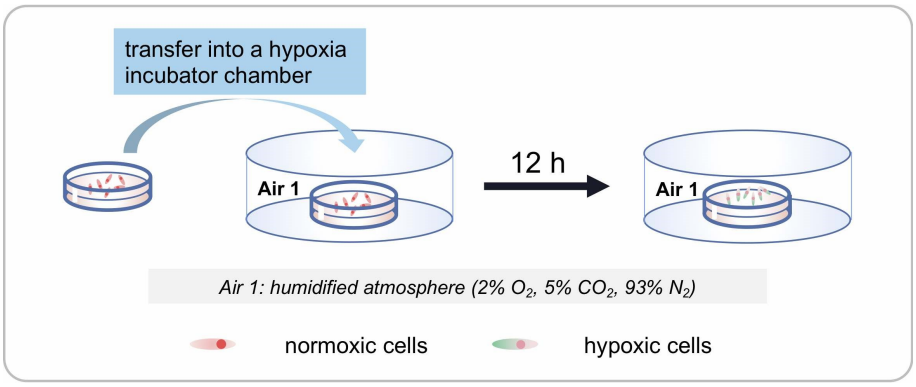
$$LE(\%) = \frac{M_d}{M_{polymer} + M_d} \times 100\% \qquad \text{S2}$$

where M_d represents the weight of the drug molecule encapsulated in the nanomedicine, M_{d0} represents the input weight of the drug molecule when preparing the nanomedicine, and $M_{polymer}$ represents the weight of polymer component of the prepared nanomedicine.

5. Cell Uptake of IPS@NPs

Typically, HeLa cells were plated onto 35 mm confocal dishes and incubated at 37 °C for 24 h. Freshly prepared IPS@NPs (1 μM IPS) in DMEM was added, and the cells were incubated for different time intervals (0.5 and 4 h). Then, the cells were washed with PBS for three times and

imaged immediately by CLSM (Leica TCS SP8 microscope). Ex/Em: 594/680-800 nm.



Scheme S4. Mimic of a severe hypoxic environment (2% O_2) with a hypoxia incubator chamber.

6. Identification of the Photogenerated $O_2^{\bullet-}$ via EPR Spectrometry

Electron paramagnetic resonance (EPR) spectrum was recorded on a Bruker EMXplus 6/1 EPR spectrometer. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was used as a special radical trapping agent for $O_2^{\bullet-}$. Typically, 100 mM DMPO was mixed with 100 μM IPS in DMSO, followed by light irradiation for 5 min. EPR was then performed at a microwave frequency of 9.8 GHz and power of 20 mW with a modulation amplitude and frequency of 1.0 G and 100 kHz, respectively, time constant 0.01 ms, scan time 30 s, receiver gain 30 dB, and center field setting 3512 G.

7. Flow Cytometry Analysis of Intracellular ROS Level.

After incubation with ATO-IPS@NPs (1.0 μM IPS) under normoxia (25% O_2) or hypoxia (2% O_2) environment for 4 h, HeLa cells were washed with PBS (10 mM, pH 7.4) thrice. Freshly prepared DCF-DA solution (20 μM) in DMEM was added and the cells were incubated for 20 min. Then the cells were washed with PBS (10 mM, pH 7.4), followed by light irradiation (580-660 nm, 10 mW/cm^2) or incubated in dark for 3 min and collected by trypsin. After washed with PBS thrice, cells were resuspended in PBS to form a single-cell suspension. The intracellular ROS level was analyzed using flow cytometry (BD LSRFortessa, BD). Ex/Em: 488/500-560 nm. Notably, for experiments in hypoxia, cell culture and treatments were conducted in a hypoxia incubator chamber, and all the DMEM used were bubbled with mixed gas (2% O_2, 5% CO_2, and 93% N_2) for 5 min in advance.

8. Flow Cytometry Analysis of Cell Apoptosis

HeLa cells were incubated with ATO-IPS@NPs (1.0 μM IPS) under normoxia (21% O_2) or hypoxia (2% O_2) for 4 h, washed with PBS (10 mM, pH 7.4) thrice, then subjected to light irradiation (580-660 nm, 10 mW/cm^2) or incubated in dark for 10 min and incubated for additional 2 h. The cells were collected by trypsin without EDTA and washed with cold PBS, then resuspended in binding buffer according to the manufacturer's protocols. The cells were incubated with annexin-V-FITC and PI for 15 min at room temperature in dark. After staining, the induction of apoptosis was determined by analyzing 10000 ungated cells using flow cytometry (BD LSRFortessa, BD). Notably, for experiments in hypoxia, cell culture and treatments were conducted in a hypoxia incubator chamber, and all the DMEM used were bubbled with mixed gas (2% O_2, 5% CO_2, and 93% N_2) for 5 min in advance.

9. *In Vivo* ROS Detection in Tumor with DHE.

4T1 tumor-bearing BALB/c mice (n = 3) were intravenously injected with ATO-IPS@NPs (200 μL, 5 mg/kg IPS). After 8 h, DHE in PBS (25 μL, 50 μM) was injected intratumorally. After 20 min in dark, the mice were imaged on NightOWL II LB983 *In Vivo* Imaging System (Berthold Technologies). Then, the tumor sites of the mice were subjected to light irradiation (633 nm, 0.1 W/cm^2) for 10 min and imaged immediately on the same *in vivo* imaging system. Ex/filter: 530/600 nm.

10. *In Vivo* Biodistribution Analysis.

4T1 tumor-bearing BALB/c mice (n = 3 mice) were intravenously injected with ATO-IPS@NPs (200 μL, 5 mg/kg IPS). The mice were sacrificed at varying time points post-injection (4 h, 8h, 12 h, 24 h, and 48 h) and dissected to collect the major organs (including heart, liver, spleen, lung, and kidney) and tumor tissues for *ex vivo* fluorescence imaging on NightOWL II LB983 *In Vivo* Imaging System (Berthold Technologies). Ex/filter: 630/700 nm. The *in vivo* biodistribution of ATO-IPS@NPs were analyzed based on the mean fluorescence intensity (MFI) of each *ex vivo* tissue.

11. Supplementary Figures

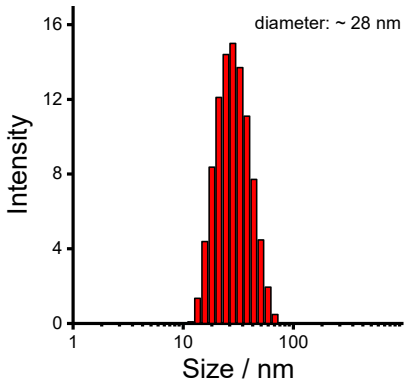


Figure S1. DLS size distribution of IPS@NPs in PBS (pH 7.4).

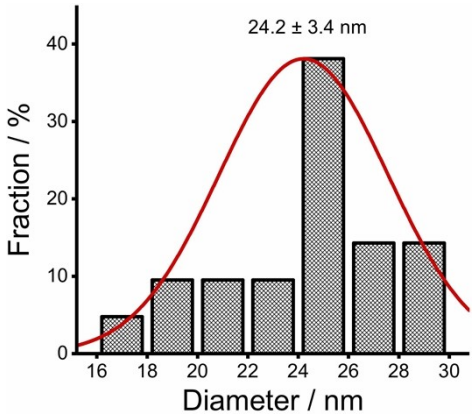


Figure S2. Particle-size distribution of ATO-IPS@NPs s by analyzing the TEM image with ImageJ software.

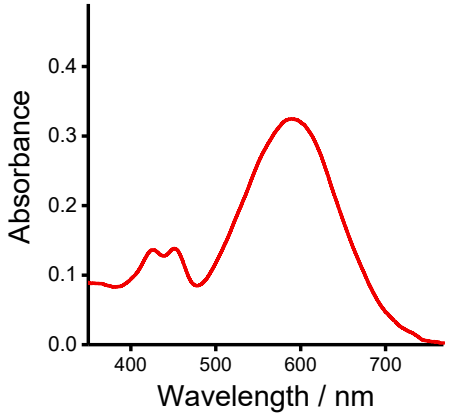


Figure S3. UV-vis spectrum of IPS@NPs in PBS (pH 7.4).

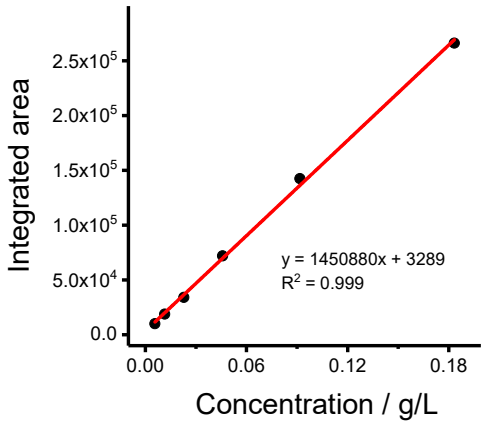


Figure S4. Calibration curve of HPLC peak integration (retention time: 5.8 min) versus concentration recorded for ATO. Detection wavelength is 254 nm.

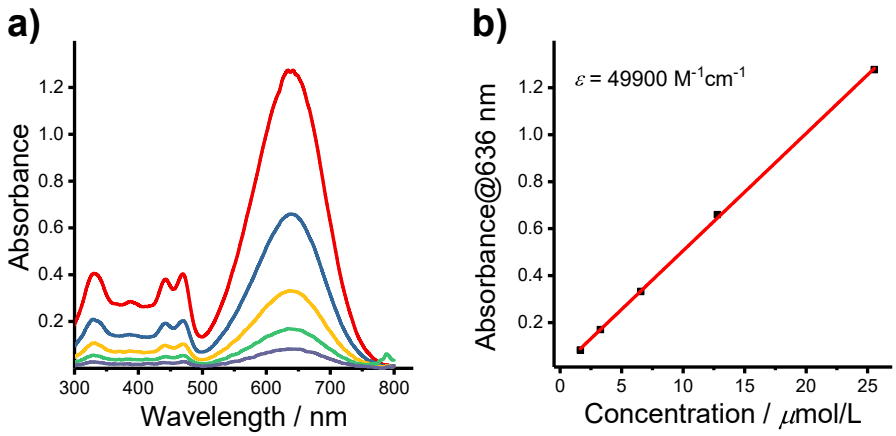


Figure S5. (a) UV-vis absorption spectra recorded for IPS in DMF/water (v/v 9/1) mixture different concentrations. (b) Calibration curve of absorbance at 636 nm versus concentration for IPS. ε represents molar absorption coefficient.

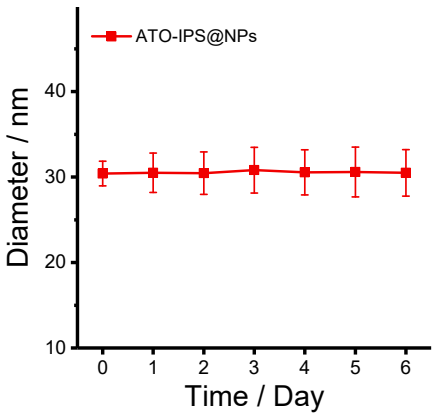


Figure S6. Daily changes in the size of ATO-IPS@NPs over a period of 6 days.

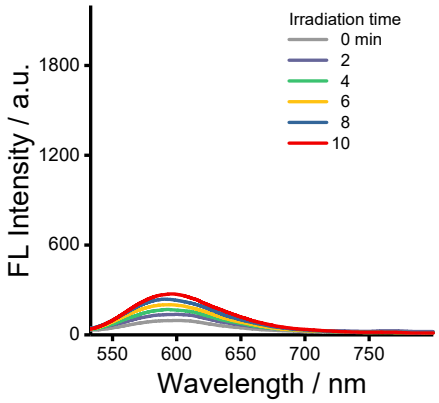


Figure S7. Fluorescence spectra of DHE in PBS upon light irradiation for varying time intervals (Ex: 510 nm; Ex/Em slit: 5/5 nm). Light source: Xe lamp (490-700 nm, 5 mW/cm^2).

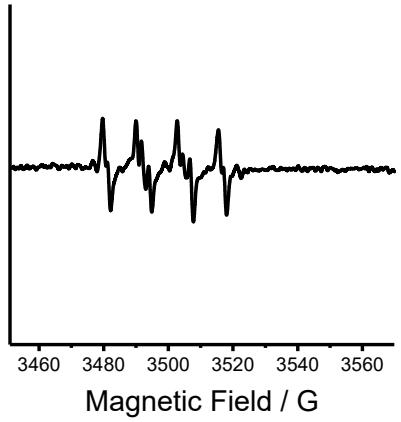


Figure S8. EPR spectrum of IPS in the presence of DMPO upon light irradiation for 5 min.

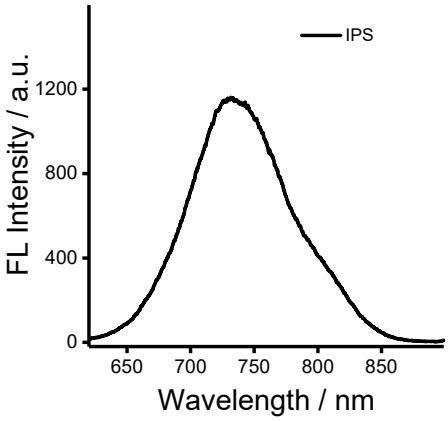


Figure S9. Fluorescence emission spectrum of ATO-IPS@NPs. Ex: 594 nm; Ex/Em slit: 5/5 nm.

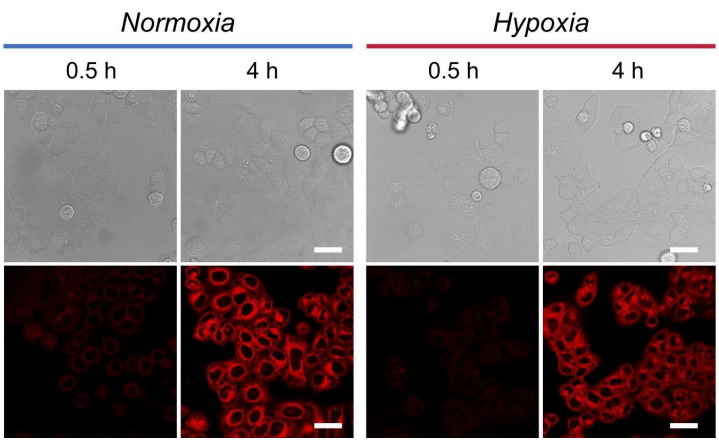


Figure S10. Cell uptake of IPS@NPs by HeLa cells in normoxia (21% O_2) and hypoxia (2% O_2) environment. Scale bar: 50 μm.

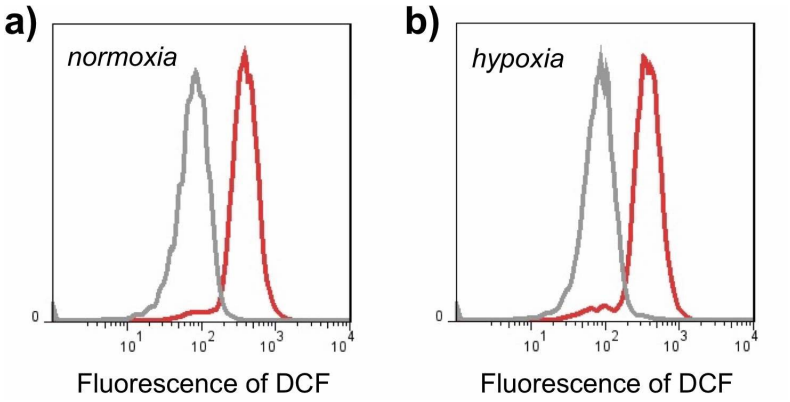


Figure S11. Flow cytometry analysis of the ROS level in **ATO-IPS@NPs**-incubated HeLa cells with (red) or without (grey) light irradiation under (a) normoxic or (b) hypoxic condition using DCF-DA as ROS fluorescence probe.

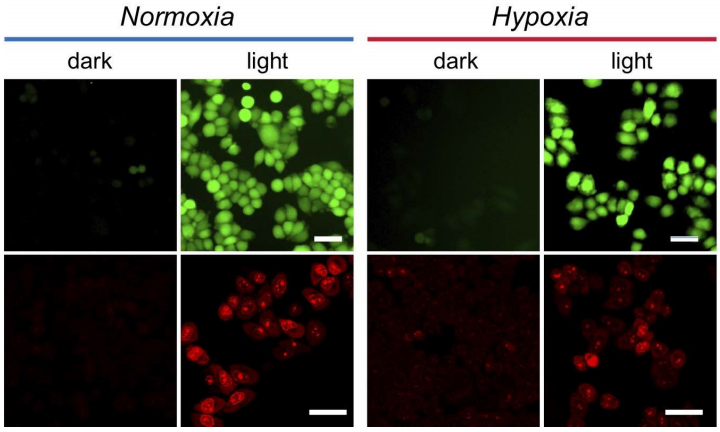


Figure S12. Fluorescence imaging of photogenerated ROS from IPS@NPs in HeLa cells by using DCF-DA and DHE as fluorescence indicators of general ROS and $O_2^{\bullet-}$, respectively. Scale bar: 50 μm.

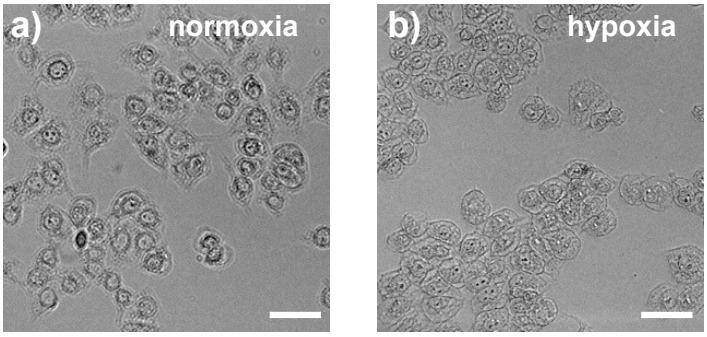


Figure S13. Differential interference contrast (DIC) images of HeLa cells incubated with ATO-IPS@NPs (1 μM IPS) upon light irradiation (led lamp, 580-660 nm, 10 mW/cm^2) for 10 min under (a) normoxia (21% O_2) and (b) hypoxia (2% O_2). Scale bar: 50 μm.

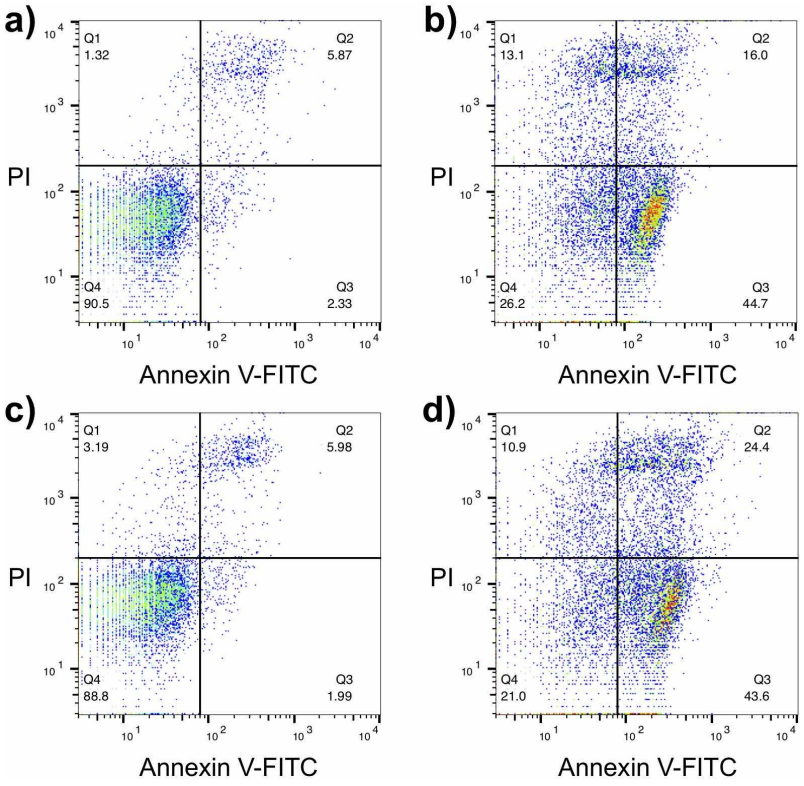


Figure S14. Flow cytometry analysis of cell apoptosis induced by PDT under (a,b) normoxic and (c,d) hypoxic conditions. HeLa cells were treated with ATO-IPS@NPs (containing 1.0 μM IPS) for 4 h followed by (b,d) subjected to light irradiation (580-660 nm, 10 mW/cm^2, 10 min) or (a,c) incubated in dark. After treatment, the cells were incubated for 2 h and then stained with Annexin V-FITC/PI for 15 min.

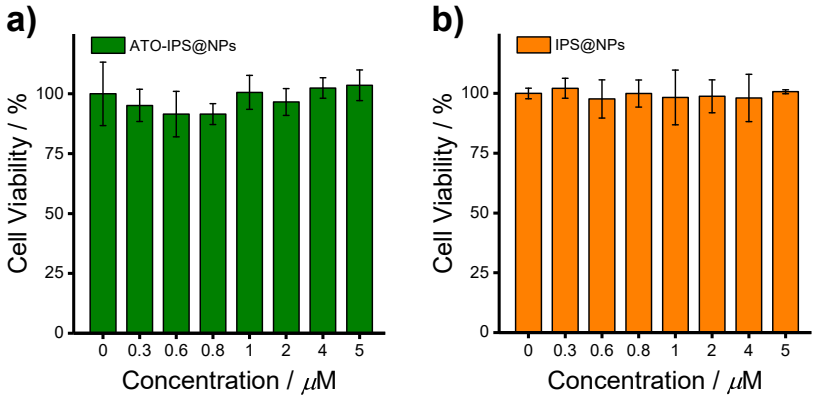


Figure S15. Cell viability of HeLa cells incubated with (a) ATO-IPS@NPs or (b) IPS@NPs of indicated concentrations of IPS component in dark under normoxia (21% O_2) determined by MTT assay (n = 4).

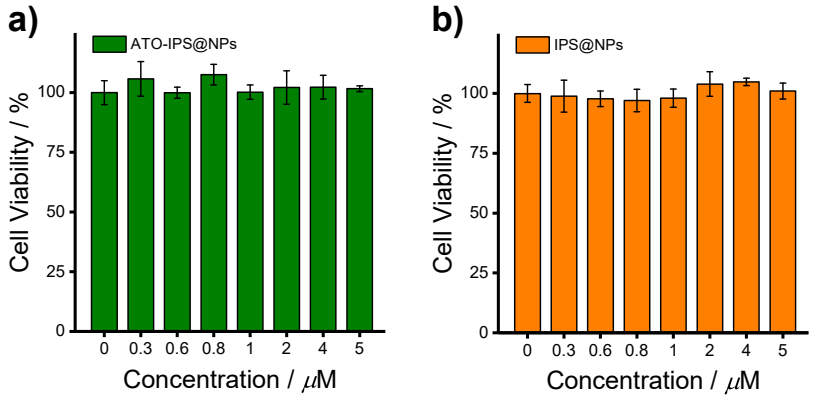


Figure S16. Cell viability of HeLa cells incubated with ATO-IPS@NPs (a) or IPS@NPs (b) of indicated concentrations of IPS component in dark under hypoxia (2% O_2) determined by MTT assay (n = 4).

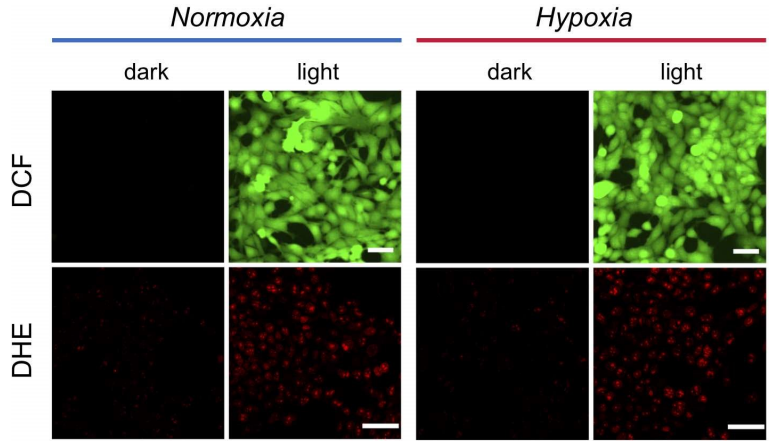


Figure S17. Fluorescence imaging of ROS photogenerated by ATO-IPS@NPs in 4T1 cells by using DCF-DA and DHE as fluorescence indicators of general ROS and $O_2^{\bullet-}$, respectively. Scale bar: 50 μm.

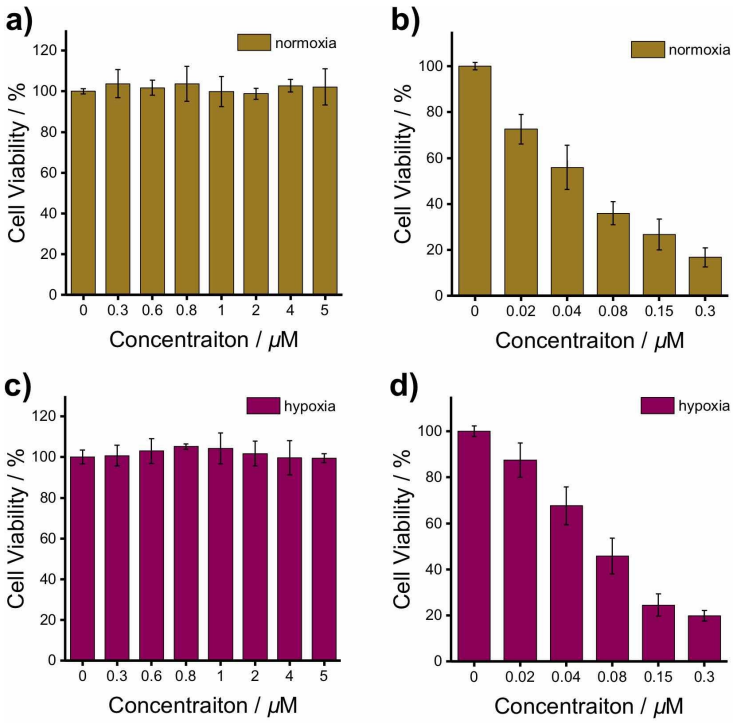


Figure S18. Cell viability of 4T1 cells incubated with ATO-IPS@NPs of indicated concentrations of IPS component (a,c) in dark or (b,d) upon light irradiation (580-660 nm, 10 mW/cm^2) for 20 min under (a,b) normoxia and (c,d) hypoxia determined by MTT assay (n = 4).

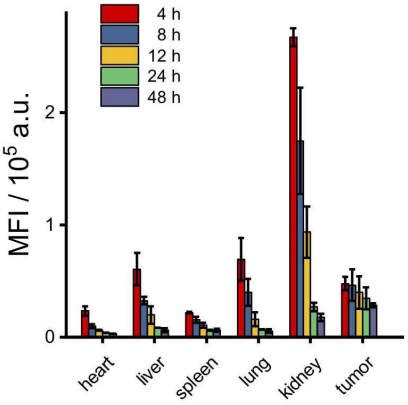


Figure S19. *In vivo* biodistribution of ATO-IPS@NPs via quantitative analysis of IPS fluorescence intensity of the *ex vivo* major organs and tumors at different time intervals post tail vein injection of ATO-IPS@NPs. Data is mean ± SD (n = 3). Ex/Em: 630/700 nm.

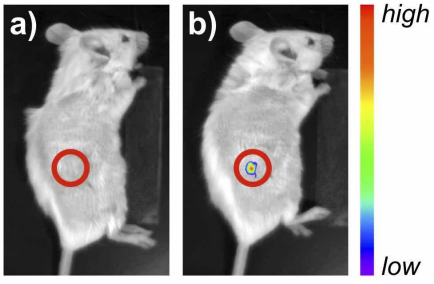


Figure 20. Representative *in vivo* DHE fluorescence images of 4T1 tumor-bearing mice taken (a) before and (b) after light irradiation (633 nm, 0.1 W/cm^2, 10 min) at 8 h post intravenous injection with ATO-IPS@NPs. Ex/filter: 530/600 nm.

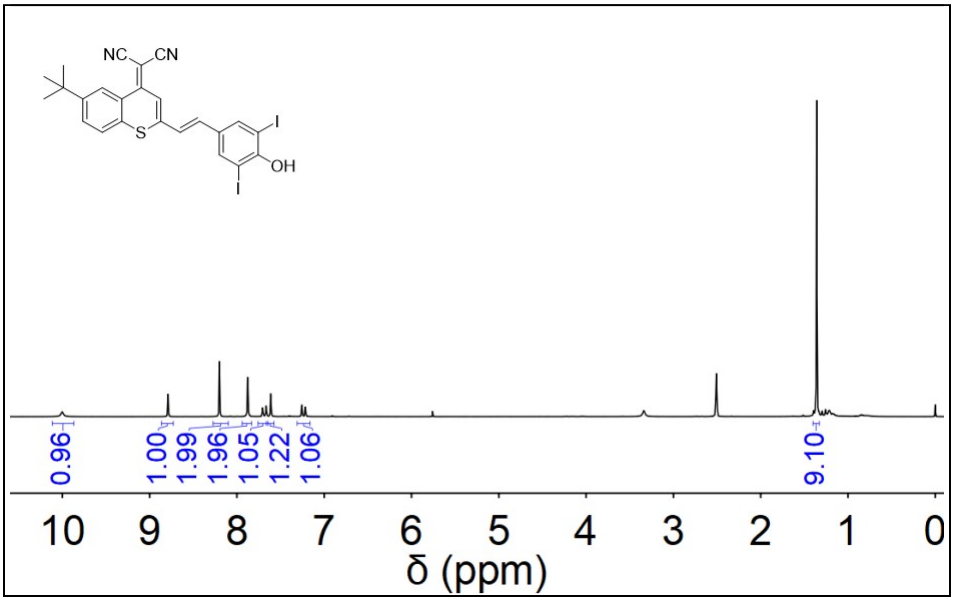


Figure S21. ^{1}H NMR spectrum (400 MHz) of compound IPS in DMSO-d_6 at 25 ºC.

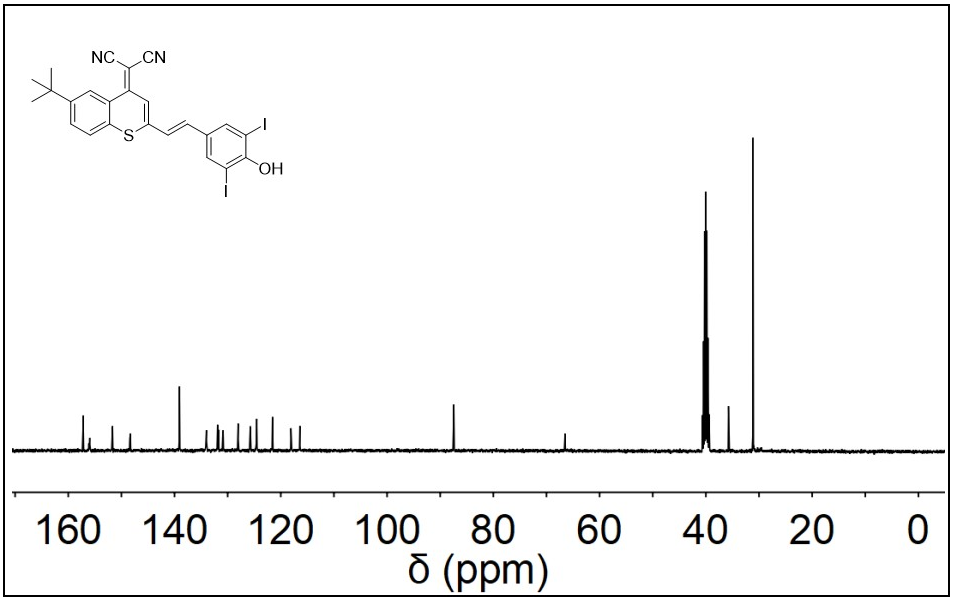


Figure S22. ^{13}C NMR spectrum (100 MHz) of compound IPS in DMSO-d_6 at 25 ºC.

12. References

1. Armarego, W. L. F.; Perrin, D. D. *Purification of Laboratory Chemicals*, Butterworth-Heinemann, **2009**.