Electronic Supplementary Information

Light-enhanced Hypoxia-responsive Nanoparticles for Deep Tumor Penetration and Combined Chemo-Photodynamic Therapy

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Materials and Instruments

Pluronic P123 (P123, MW=5800 Da), branched PEI (MW=600 Da), PEG2000, N-ethyl-N′-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDCI), 1-hydroxy-7-azabenzotriazole (HOAT), p-nitrobenzoic acid, Pluronic F127 (MW = 12600 Da), Chlorine e6 (Ce6) and doxorubicin hydrochloride (DOX·HCl) , 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,3-diphenylisobenzofuran(DPBF) were obtained from Sigma (St. Louis, MO, USA). Trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco-BRL(Burlington, Canada). The RPMI DMEM medium and penicillin-streptomycin were purchased from Bristol-Myers Squibb Trading Co. Ltd. (Shanghai,China). 4′,6-diamidino-2-phenylindole (DAPI) was purchased from the Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). ROS-ID® Hypoxia/Oxidative Stress Detection Kit was obtained from Enzolifesciences. Rat liver microsomes were prepared according to the literature.[1] All chemicals were of reagent grade and used without further purification.

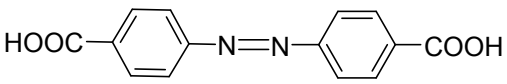
^{1}H NMR (400 MHz) were recorded on a Bruker DRX-400 spectrometer (Bruker, Ettlingen, Germany). FT-IR spectra was recorded on a Varian Excalibur™ FT-IR spectrometer. UV spectra was recorded on a Techcomp UV2310 UV/vis spectrophotometer. The transmission electron microscopy image determined by a HT-

7700 TEM. The average hydrodynamic diameter and ζ-potential were determined at 25 °C by DLS using a 90 Zeta Plus particle size analyzer (Brookhaven Instruments Corp), with a laser light wavelength of 635 nm at a scattering angle of 90°. Fluorescence spectrophotometer was recorded on a RF-5301PC spectrometer (Shimadzu, Japan). Cells were analyzed by CLSM (Radiance2100, Bio-Rad) and inverted fluorescence microscope (Leica DMLB&DMIL). Spectrophotometrical was measured at an ELISA reader (model 680, Bio-Rad).

Cells and Animals.

Michigan Cancer Foundation-7 (MCF-7) cells were purchased from American Type Culture Collection (Rockville, MD). The MCF-7 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin and streptomycin. The cells were cultured at 37 °C in a humidifed atmosphere containing 5% CO_2. Normal female nude mice and MMTV-PyMT mice (4–6 weeks old, 16–18 g) were purchased from the Zhejiang Chinese Medical University and maintained in a pathogen-free environment under controlled humidity and temperature. The animal experiments were performed in accordance with the China Animal Protection Law (CAPN), and the protocols were approved by the Zhejiang University Animal Care and Use Committee.

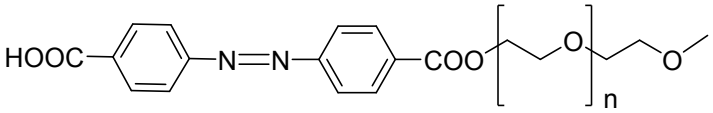
Synthesis of AZO.



Briefly, *p*-nitrobenzoic acid (6.50 g, 38.9 mmol), NaOH (25.0 g, 625 mmol) were dissolved in water (120 mL) at 50 °C, followed by addition of the solution of glucose (10 g, 227 mmol) in water (75 mL) in 30 min. The reaction mixture was stirred vigorously at 50~60 °C for 8 h in N_2 atmosphere. After cooling to room temperature, the mixture was adjusted to pH=6 with glacial acetic, after which a mud-like precipitate was collected by filtration. The solid was recrystallized from hot K_2CO_3 solution for twice, and 3.94 g (75.2% yield) bright orange solid

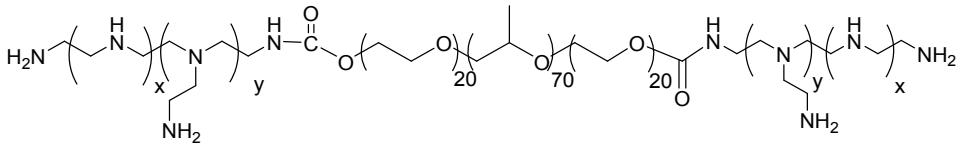
of **AZO** was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ(TMS, ppm) 7.64-7.62 (2 H, t), 6.75-6.77 (2 H, t).

Synthesis of AZOPEG.



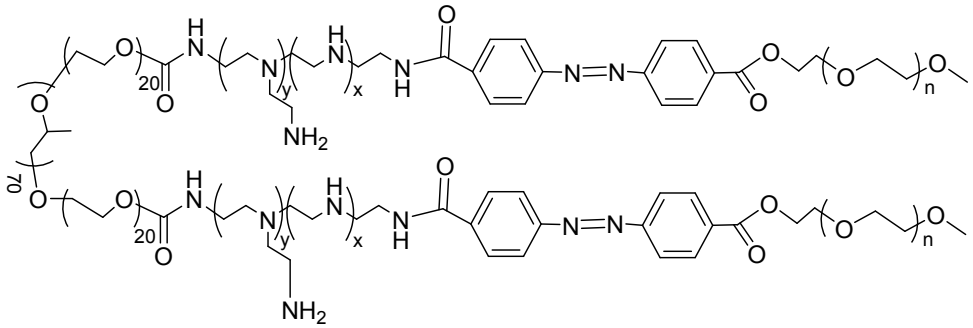
The compound AZO (405 mg, 1.5 mmol), EDCI (286 mg, 1.5 mmol), and HOAT (204 mg, 1.5 mmol) were dissolved in 30 mL of anhydrous dichloromethane, followed by addition of 0.2 mL of Et_3N. 400 mg mPEG (2000 Da, 0.2 mmol) in 20 mL of anhydrous dichloromethane was added drop-by-drop under nitrogen atmosphere at 0 °C. The reaction mixture was stirred overnight and dialyzed in water using a dialysis tube (MWCO = 2000) for 24 h. Then mixture was filtered to remove the precipitate and freeze-dried to yield compound AZOPEG as white powders (254 mg, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ(TMS, ppm) 7.62-7.60 (2 H, t), 6.74-6.76 (2 H, t), 3.58-3.53 (576 H, m), 3.23 (9 H, s).

Synthesis of PP6.



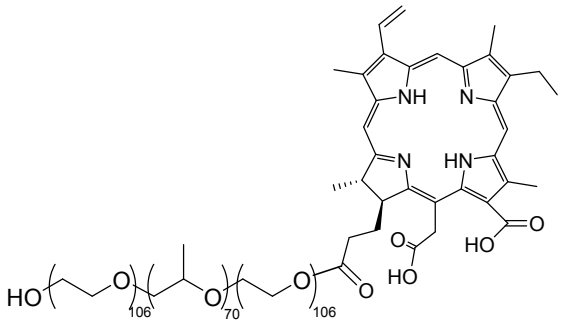
For the synthesis of PP6, Pluronic P123 (0.58 g, 0.1 mmol), CDI (0.17 g, 1.0 mmol), were dissolved in 30 mL DMSO, then 0.2 mL triethylamine was added. The solution was stirred at room temperature for 6 h under nitrogen, then the mixture was added dropwise to PEI (0.60 g, 1.0 mmol) solution in 10 mL DMSO. After stirring for 12 h, the mixture was dialyzed in water and freeze-dried to yield the desired product PP6 (yield: 65.8%). ^{1}H NMR (400 MHz, $CDCl_3$) δ(TMS, ppm) 3.71-3.30 (370 H, m), 2.81-2.43 (112 H, m), 1.03 (210 H, s).

Synthesis of PAP.



The compound AZOPEG (700 mg, 0.3 mmol), EDCI (95 mg, 0.5 mmol), and HOAT (68 mg, 0.5 mmol) were dissolved in 30 mL of anhydrous dichloromethane, followed by addition of 0.1 mL of Et_3N. 700 mg PP6 (0.1 mmol) in 20 mL of anhydrous dichloromethane was added drop-by-drop under nitrogen atmosphere at 0 °C. The reaction mixture was stirred overnight and dialyzed in water using a dialysis tube (MWCO = 7000) for 24 h. Then mixture was filtered to remove the precipitate and freeze-dried to yield compound PAP as white powders (437 mg, 37.7% yield).

Synthesis of FC.



F127 (MW=12220, 1222 mg, 0.1 mmol), EDCI (95 mg, 0.5 mmol), and HOAT (68 mg, 0.5 mmol) were dissolved in 30 mL of anhydrous dichloromethane, followed by addition of 0.1 mL of Et_3N. 40 mg Ce6 (0.07 mmol) in 20 mL of anhydrous dichloromethane was added drop-by-drop under nitrogen atmosphere at 0 °C. The reaction mixture was stirred overnight and dialyzed in water using a dialysis tube (MWCO = 10000) for 24 h. Then mixture was filtered to remove the precipitate and freeze-dried to yield compound FC as dark green powders (594 mg, 46.2% yield).

Fourier transform-infrared (FT-IR) spectra.

Fourier transform-infrared (FT-IR) spectra of PP6PEG, PAP and AZOPEG was

recorded on a Varian Excalibur™ FT-IR spectrometer .

Preparation of PAP-FC Nanomicelles.

The dichloromethane solution of PAP (10 mg/mL, 1 mL) and various concentration of FC (10 mg or 5 mg or 3 mg or 2 mg in 1 mL dichloromethane) were added into 8 mL DI water under continuous sonication with a microtip-equipped probe sonicator (DS2060) for 10 min to form different mass ratios of PAPFC mixture. Then the mixed solution was stirred overnight to volatilize dichloromethane thoroughly and filtrated through a 0.22 μm filter membrane to remove the insoluble polymer. Finally, the micelles were collected by lyophilization.

Preparation of DOX-loaded PAP-FC Nanomicelles .

The dichloromethane solution of DOX (3 mg/mL, 1 mL), PAP (10 mg/mL, 1 mL) and FC (5 mg/mL, 1 mL) were added into 7 mL DI water under continuous sonication with a microtip-equipped probe sonicator (DS2060) for 10 min. Then the mixed solution was stirred overnight to volatilize dichloromethane thoroughly and filtrated through a 0.22 μm filter membrane to remove the insoluble polymer. Finally, the PAPFC/DOX micelles were collected by lyophilization.

To determine drug encapsulation efficiency (EE) and drug loading content (LC), lyophilized PAPFC/DOX nanomicelle powders were dissolved in acetone and centrifuged. The amount of encapsulated DOX in the supernate was detected by fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan). LC and EE were then calculated according to the following equations:

$$EE(\%) = \frac{weight\ of\ DOX\ in\ micelles}{weight\ of\ DOX\ in\ feed} \times 100\%$$

$$LC(\%) = \frac{weight\ of\ DOX\ in\ micelles}{weight\ of\ DOX\text{-}loaded\ micells} \times 100\%$$

Determination of particle size and ζ-potential.

Size distribution of PAPFC nanomicelles with the ratio of "m(FC)/m(PAP)=5:10 " in water determined by a Zetasizer 3000 dynamic light scattering (DLS). The inset is the transmission electron microscopy image determined by a HT-7700 TEM.

The ζ-potential of FC, PAP and different ratios of PAPFC nanomicelles were determined at 25 °C by DLS using a 90 Zeta Plus particle size analyzer (Brookhaven Instruments Corp.).

Determination of CMC.

The CMC of FC, PAP and different ratios of PAPFC was determined by a fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan) using pyrene as a fluorescence probe. The pyrene fluorescence excitation wavelength was set to 334 nm with an emission wavelength of 393 or 373 nm. The intensity ratios of I_{393}/I_{373} from the excitation spectra were plotted against the micelle concentration to determine the CMC.

In *Vitro* DOX Release.

The hypoxic condition in *vitro* was prepared by bubbling nitrogen gas into the reaction solution (0.1 M potassium phosphate buffer (pH=7.4)) for 30 minutes. Then rat liver microsomes (226 g/3 mL) pre-incubated at 37 °C for 5 min and 50 μM NADPH as a cofactor for reductases was added at 5 min.

The in vitro release profles of PAPFC/DOX nanomicelles with the ratio of "m_{FC}/m_{PAP}=5:10" (the following PAPFC ratio is the same) were studied by dialyzing the DOX-loaded micelle suspension in PBS (pH 7.4) with horizontal shaking (100 rpm) in hypoxia and normoxia condition at 37 °C. The drug-loaded suspension (2.0 mL) was dialyzed against a 20.0 mL solution (MWCO = 1000 Da). At predetermined time intervals, the medium outside of the dialysis tube was collected and displaced with fresh solution. The samples were analyzed by fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan).

Gel penetration chromatogram (GPC).

Degradation of PAP nanomicelle in hypoxia condition was analyzed by GPC. PAP nanomicelle in normoxia condition and PEG was set as the control groups. The mobile phase is dimethylformamide with a flow rate of 1.0 mL/min at 60 °C.

Cellular Uptake Study.

MCF-7 cells were purchased from American Type Culture Collection (Rockville, MD) and cultured in RPMI DMEM culture medium supplemented with 10% fetal bovine serum. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. For microscopic observation, 5×10^4/well MCF-7 cells were seeded onto 24-well plates and incubated at 37 °C for 18 h. After being co-incubated with 50 μg/mL of PAP nanomicelles loaded with DOX in fresh culture medium, cells were incubated at 37 °C for 4 h in normoxia or hypoxia condition. Then cells were washed twice with ice-cold PBS and counterstained with DAPI to label nuclei. Coverslips were mounted on slides and analyzed by CLSM (Radiance2100, Bio-Rad). And finally the cells were visualized at interval time with CLSM.

Determination of ROS in vitro.

1,3-Diphenylisobenzofuran (DPBF) was used as the ROS probe. In brief, DPBF which dissolved in the ethanol (10 μL, 1.35 mg/mL) was added into the solution of PAPFC loaded with Ce6 (3 mL, 100 μg/mL). The mixture was then irradiated with 660 nm light for designated time of 8 mW/cm^2. After that, the absorption spectra of mixture were determined on Techcomp UV2310 UV/*vis* spectrophotometer at fixed time, respectively. The ROS quantum yields of PAPFC loaded with Ce6 in solution were measured according to the absorption intensity at 410 nm.

In *Vitro* Cytotoxicity Study.

The cytotoxicity of PAPFC nanomicelles was assessed by MTT assay under normoxia and hypoxia condition. The MCF-7 cells were seeded in 96-well plates at a density of 8000 cells/well for 18 h. The culture medium was then replaced by a serum-free medium containing various concentrations of free DOX, PAPFC with light and PAPFC/DOX with or without light. After 24 h incubation, the medium was replaced with the MTT solution (0.5 mg/mL in serum free DMEM medium) for 4 h. After removal of MTT medium, the formazan crystals were dissolved in 100 μL of DMSO and the microplates were agitated for 10 s at a medium rate prior to the

spectrophotometrical measurement at a wavelength of 570 nm on an ELISA reader (model 680, Bio-Rad). The untreated cells served as the 100% cell viability control, and the completely died cells served as the blank. The relative cell viability (%) related to control cells was calculated by the formula below:

$$V\% = \frac{[A]_{experimental} - [A]_{blank}}{[A]_{control} - [A]_{blank}} \times 100\%$$

where V% is the percent of cell viability, $[A]_{experimental}$ is the absorbance of the wells culturing the treated cells, $[A]_{blank}$ is the absorbance of the blank, and $[A]_{control}$ is the absorbance of the wells culturing untreated cells.

Intracellular oxygen consumption and ROS generation detection assay.

ROS/IDR Hypoxia/Oxidative Stress detection kit (Enzo Life Sciences, US) was used to determine the oxygen consumption and the generation of ROS inside cells. MCF-7 cells were incubated in confocal dish with 70% confluency. The sufficient volume of the hypoxia/oxidative stress detection mixture that contains various ratios of the PAPFC (total concentration is 100 μg/mL). After incubated for desirable time (2 h for PAPFC) in dark, the PAPFC treated MCF-7 cells were exposed to the 660 nm laser irradiation at 8 mW/cm^2 for 6 min. After that, the MCF-7 cells were washed with PBS for three times, and inverted fluorescence microscope (Leica DMLB&DMIL) was used to image. The ROS detection requires a filter set compatible with fluorescein (Ex/Em: 490/525 nm), and the hypoxia detection filter set with Texas Red (Ex/Em: 596/670 nm).

Drug-Loaded Micelle Penetration in Multicellular Spheroids (MCs).

A total of 5 × 10^4 MCF-7 cells per well were seeded in 96-well Corning spheroid microplates (Corning, USA) and incubated at 37 °C. MCs formed spontaneously after 5 d. DOX-loaded micelles were divided into four groups: PP-FC/DOX with light, PAP/DOX with light, PAPFC/DOX without light, PAPFC/DOX with light. The DOX-loaded micelles in fresh culture medium were co-cultivated with the spheroids for 4 h (DOX-equivalent dose: 5 μg/mL) with/without light (8 mW/cm^2, 6 min, 660

nm). Then the medium was removed, and the spheroids were rinsed with PBS. The spheroids were imaged by confocal laser scanning microscopy ((Radiance2100, Bio-Rad)). Representative confocal images were taken for every 1.5 μm section from the top to the middle of the spheroid.

In Vivo Drug-Loaded Micelle Penetration in Solid and MMTV-PyMT Tumor.

The animal experiments were performed in accordance with the CAPN, and the protocols were approved by the Zhejiang University Animal Care and Use Committee. The MCF-7 cells were inoculated subcutaneously in the right abdominal region of BALB/c mice. The genetically modified MMTV-PyMT transgenic mice were constructed according to the literature.[2] The tumors were allowed to grow to 100 mm^3 before the experiment began. The tumor volume (V) was calculated using the following formula:

$$v(mm^3) = \frac{\pi}{6} \times length(mm) \times width(mm)^2;$$

Mice bearing MCF-7 tumors were randomly divided into three groups treated with DOX/light, PAPFC/DOX/dark, PAPFC/DOX/light (DOX-equivalent dose: 5 μg/mL X 200 μL per mice; Drug-loading efficiency is 5%; light condition: 660 nm, 8 mW, 30 min). The tumor-bearing mice were sacrificed 24 h after treatment. The tumors were collected, fixed in 10% formalin and embedded in paraffin blocks to prepare tumor sections at a thickness of 5 μm. After deparaffinization, the tissue sections were stained with DAPI, and the tissue sections were visualized using confocal laser scanning microscopy (Radiance2100, Bio-Rad).

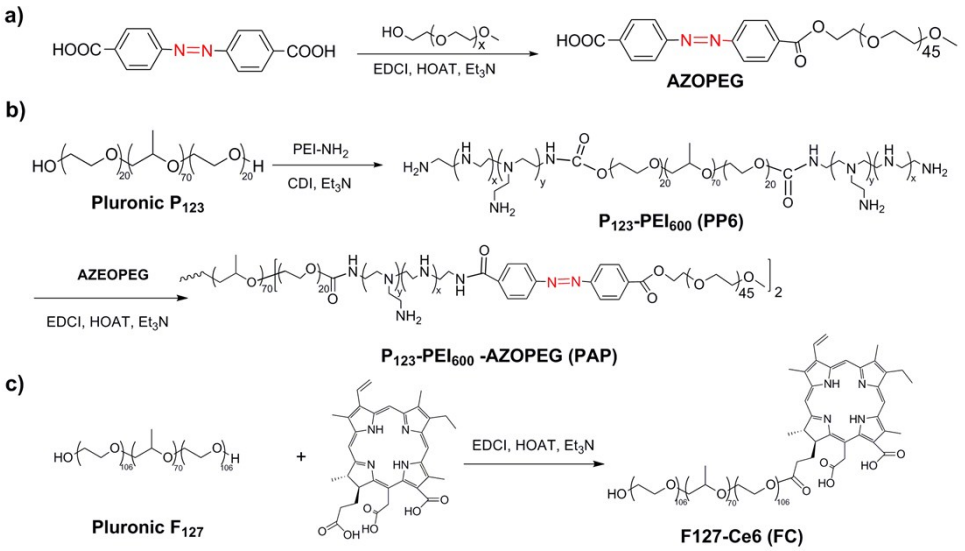


Figure S1. Synthesis route of the PAP and FC.

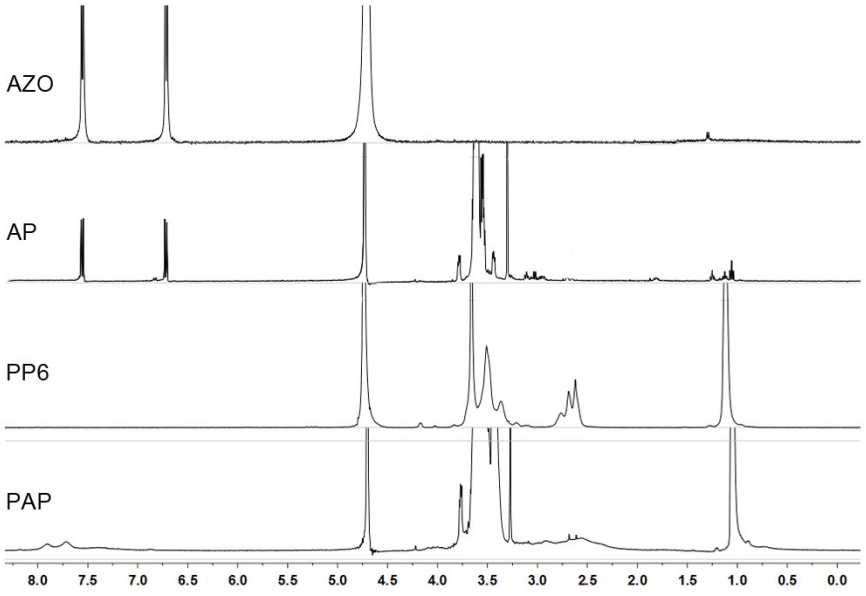


Figure S2 1H NMR spectra of AZO, AP, PP6, PAP in D_2O.

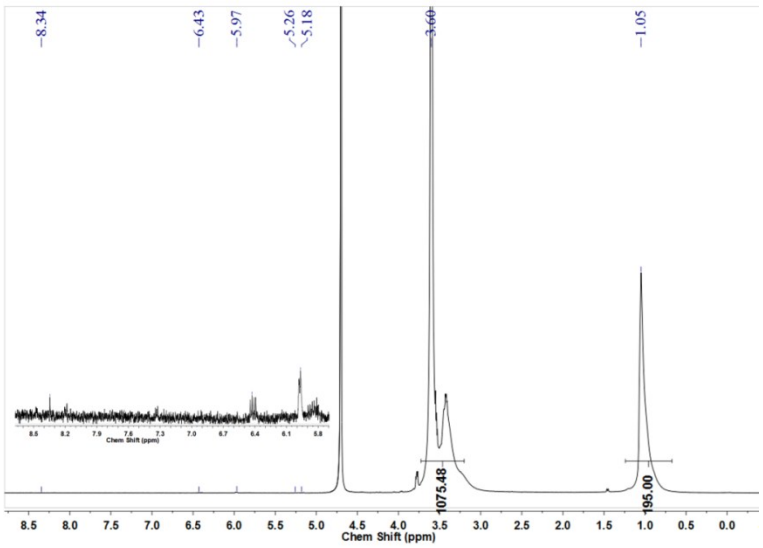


Figure S3. ^{1}H NMR (400 MHz) spectra of FC recorded on a Varian 400 MHz spectrometer.

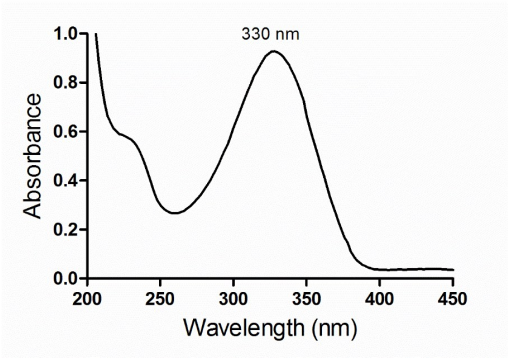


Figure S4. UV/vis absorption spectra of PAP in PBS recorded by Techcomp UV2310 UV/*vis* spectrophotophotometer.

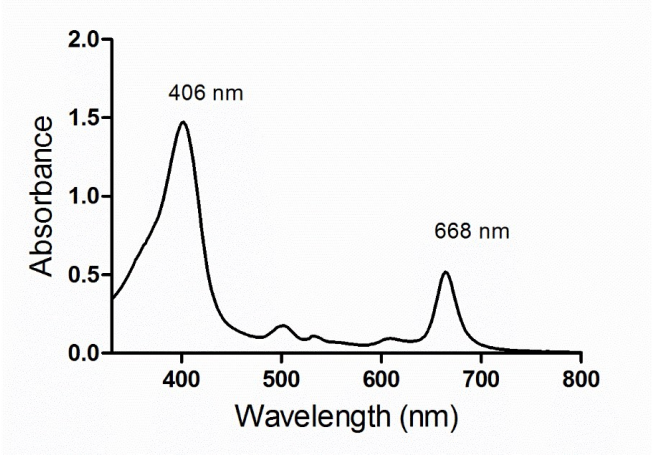


Figure S5. UV/vis absorption spectra of FC was recorded by Techcomp UV2310 UV/*vis* spectrophotometer.

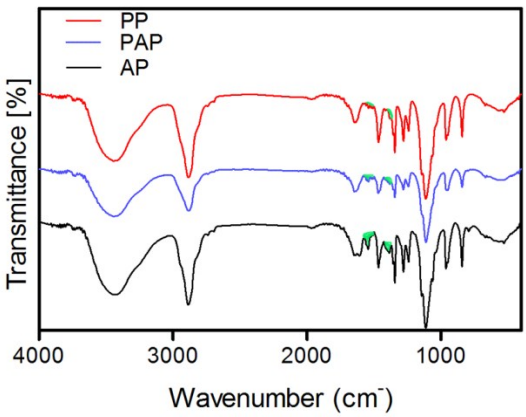


Figure S6. Fourier transform-infrared (FT-IR) spectra of PP, PAP and AP recorded by Varian Excalibur™ FT-IR spectrometer.

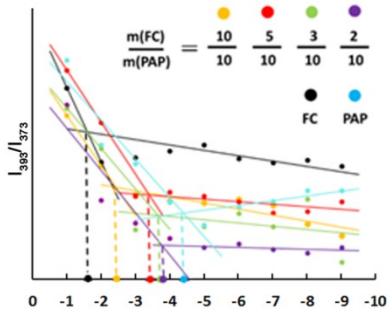


Figure S7. Fluorecence intensity ratios of I_{393} / I_{373} from the excitation spectra of PAPFC nanomicelles with different m_{FC}/m_{PAP} ratio. The critical micelle concentration (CMC) was detected using pyrene as the fluorescence probe.

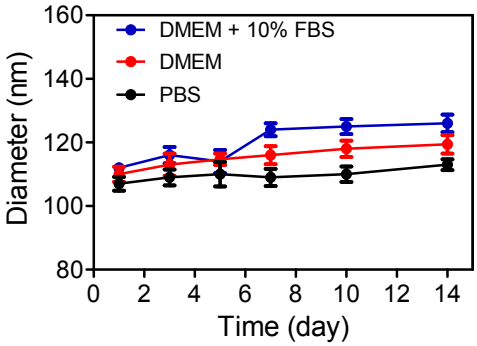


Figure S8. Time-dependent colloidal stability of PAP-FC NPs in PBS, PBS containing DMEM medium and PBS containing DMEM medium with 10% FBS at 37 °C.

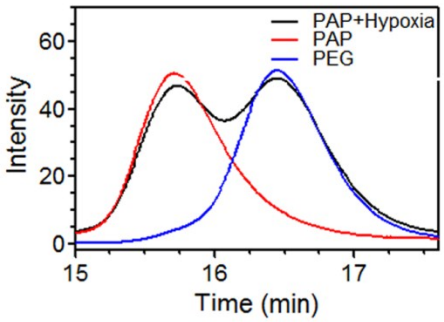


Figure S9 Gel penetration chromatogram (GPC) of PAP NPs treated in hypoxia condition. PAP NPs in normoxia condition and mPEG2000 was set as the control groups.

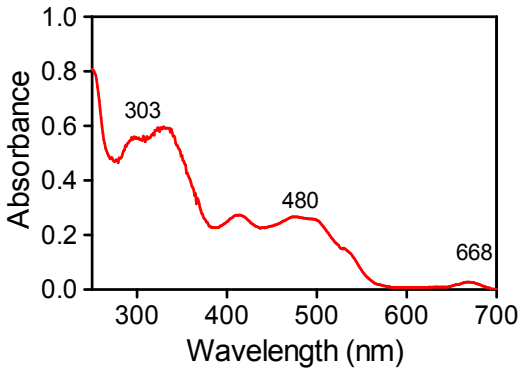


Figure S10. UV/vis absorption spectra of PAP-FC/DOX NPs in PBS. The remarkable absorption peaks at 330, 480, 668 nm was attributed to azobenzene, DOX and Ce6 respectively, which proved the successful co-encapsulation of DOX and Ce6 in the same nanocarrier.

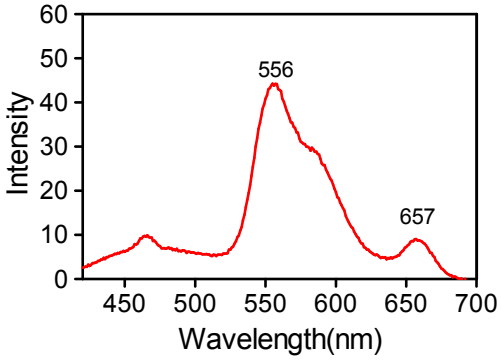


Figure S11. Fluorescence emission spectra of PAP-FC/DOX NPs in PBS. The PL peaks at 556,657 nm was attributed to DOX and Ce6 respectively. The excited wavelength 401 nm.

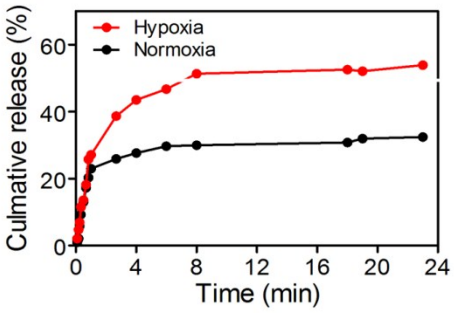


Figure S12 DOX release behaviors from PAP-FC/DOX NPs (mFC/mPAP = 5/10) in hypoxia or normoxia condition.

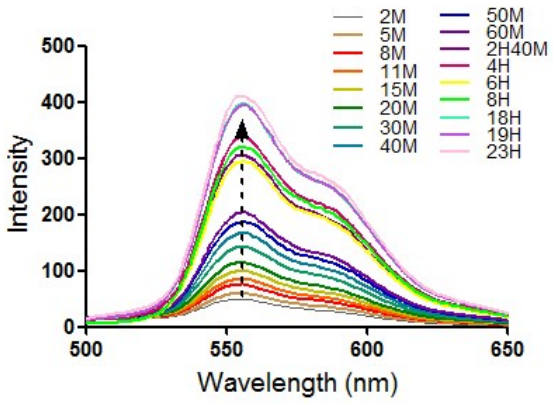


Figure S13. Fluorescence emission spectra of PAP-FC/DOX NPs in hypoxia condition.

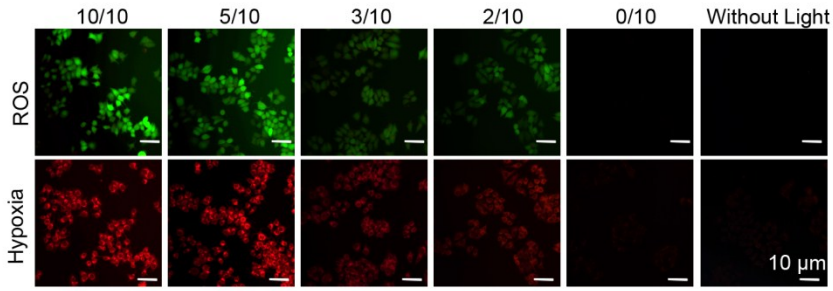


Figure S14. ROS and hypoxia generation detection after incubating with PAP-FC/DOX NPs with different mFC/mPAP ratio. Images of the MCF-7 cells were stained with ROS and hypoxia probes .

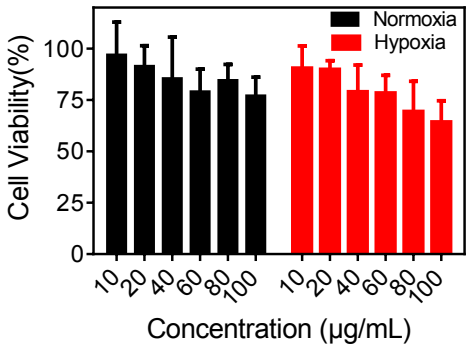


Figure S15. Cell viabilities of normal HEK-293 cells after the incubations with PAP for 24 h both in hypoxia and normoxia condition.

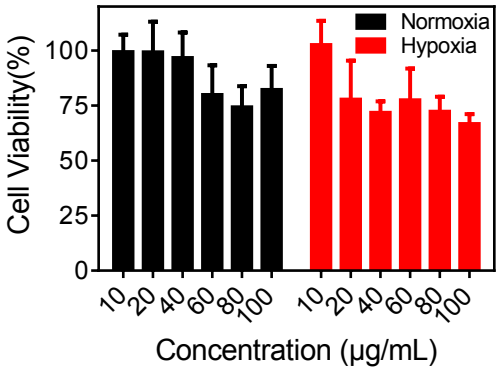


Figure S16. Cell viabilities of MCF-7 cancer cells after the incubations with PAP for 24 h both in hypoxia and normoxia condition.

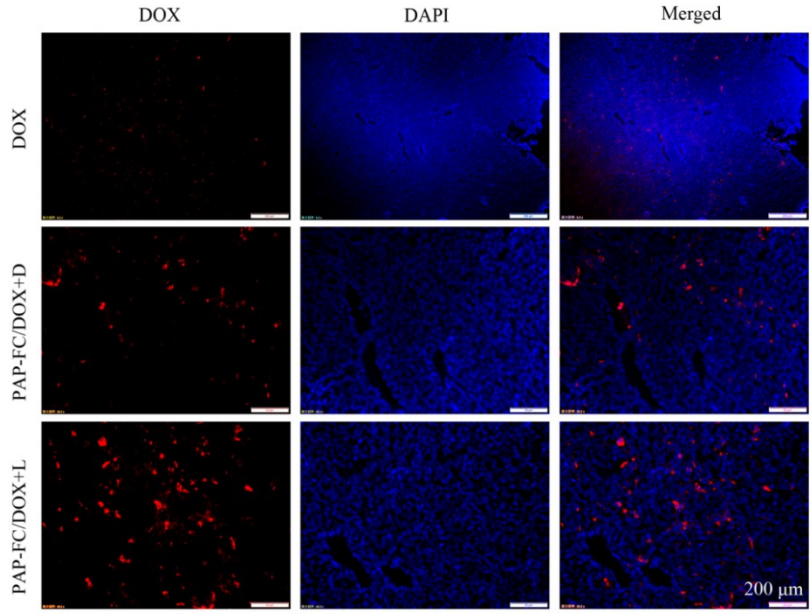


Figure S17. Confocal images of frozen tumor sections showing the improved tumor delivery and penetration of NPs in MMTV-PyMT tumor-bearing mice. DOX was shown in red and the nucleus in blue (DAPI).

Notes and references

[1] Piao W, Tsuda S, Tanaka Y, et al. Development of azo‐based fluorescent probes to detect different levels of hypoxia. Angew. Chem. Int. Edit, 2013, **52**(49): 13028-13032.

[2] Fantozzi, A., & Christofori, G.. Mouse models of breast cancer metastasis. *Breast Cancer Research*, 2006, **8**(4), 212.