Supporting Information

In-Situ H_2 Nanogenerator For Bimodal Imaging–Guided Synergistic Hydrogen/Photothermal Therapies.

Guanglong Yuan, Jieqiong Cen, Jiamin Liao, Yuqin Huang, Liu Jie*.

Experimental Section

Materials: Dopamine hydrochloride, 1, 3, 5-Trimethylbenzene (TMB), Ammonia aqueous solution (NH3·H2O, 30 wt %) and Aminoborane (AB) were purchased from Aladdin Reagent (Shanghai, China). NH2-PEG-NH2 (Mw = 2000) was purchased from Beijing HWRK Chem Co.,LTD. 5,5-dimethyl-1-pyrroline N-Oxide (DMPO) and Pluronic F127 were purchased from Sigma Aldrich Chemical Co. Cell culture medium DMEM/1640 and fetal bovine serum (FBS) were purchased from Gibco (Life Technologies AG, Switzerland). The ROS detection kit, 4', 6-diamidino-2-phenylindole (DAPI) and mitochondrial membrane potential assay kit with JC-1 was purchased from Shanghai Biyuntian Bio-Technology Co., Ltd. The CCK-8 kit was purchased from Dongren Chemical Technology (Shanghai) Co., Ltd. The AV-FITC/PI Apoptosis Detection Kit was purchased from Beijing Sizhengbai Biotechnology Co., Ltd. Calcein-AM/PI live cell/dead cell double staining kit was purchased from Shanghai Bebo Biotechnology Co., Ltd. All reagents and solvents were commercially available and were used without further purification unless otherwise stated, and Ultrapure Milli-Q water (18.2 MΩ cm) was used in all experiments.

Preparation of MPDA NPs: MPDA NPs were synthesized according to reported in literature [1]. In brief,0.60 g dopamine hydrochloride and 0.4 g F127 were added into a mixed solution (40 mL) containing deionized water（20 mL） and ethanol（20 mL） under stirring. Then, 640 μL of TMB was added and sonicated for 2 min in a water bath. Subsequently, 1.4 mL of ammonia solution was dropwise added under stirring. After 2 h reaction, the resultant MPDA nanoparticles were obtained by centrifugation at 13000 rpm/min and washed with ethanol and water several times. The MPDA NPs were dried via vacuum freeze-drying for further use. Pluronic F127 and 1,3,5-trimethylbenzene (TMB) were used as organic templates. Ethanol and ammonia solution were used as catalyst for dopamine polymerization. PDA self-assembled into nanoparticles on TMB template through π – π stacking interaction. Finally, MPDA NPs were obtained after removing the template.
Preparation of AB@MPDA NPs: 0.3 g AB were added into MPDA aqueous solution (10 ml, 30 mg mL^{-1}), and then the mixture was stirred at room temperature for 24 h. AB molecules were trapped into the MPDA

cavities and mesoporous channels by electrostatic adsorption and hydrogen bonding interaction. AB@MPDA nanoparticles were collected by centrifugation and washed by deionized water. The resulting nanoparticles were dried via vacuum freeze-drying for further use. Then, NH_2-PEG-2000 molecules (500 mg) were added into the AB@MPDA aqueous solution（250 mg,10 mL）, and the system was continuously stirring for another 24 h at pH 9.5. PEG molecules were introduced to functionalize MPDA via the Michael addition and/or Schiff base reaction [2]. At last, AB@MPDA-PEG nanoparticles were collected by centrifugation, washed by deionized water, and dried via vacuum freeze-drying.

Characterization: The transmission electron microscopy (TEM) images and element mapping were obtained by a JEOL microscope (JEM-2100F, JEOL, Japan) at an accelerating voltage of 200 kV. Nitrogen sorption isotherms and pore size distribution were measured at 77K with a surface area analyzer (TriStar II 3020, Micromeritcs, USA). Dynamic hydrodynamic diameter (DLS) and zeta potential were analyzed by a nano Zetasizer (Malvern ZEN3600 Zetasizer Nano-ZS, Britain). FT-IR spectra were obtained by NIC0LET iS50+ iN10(USA).

Observation of H_2 bubble growth: AB@MPDA solution(10 μL, 100 μg mL^{-1}) with various pH（5.0, 6.5, 7.4）were taken under the blood cell counting plate to observe the bubble growth at different times points（0, 1, 3, 5, 10 min）.

Measurement of hydrogen release by MB-Pt probe:3 mL mixed solution at 6.5 pH containing AB@MPDA (100 μg mL^{-1}) and MB-Pt probe (3 μg mL^{-1} MB + 0.5 μg mL^{-1} Pt NPs) was prepared. Then, the mixed solution was monitored in real time by the UV measurement.

Evaluation of photothermal effect in vitro: 200 μL (200 μg mL^{-1}) AB@MPDA NPs Were added into 96 well plate. It was irradiated by 808 nm NIR laser (1 W cm^{-2}) for 15 minutes. The temperature of solution was obtained by an infrared imaging camera. The photothermal conversion efficiency (η) of AB@MPDA was determined according to the method reported in the literature [3].

In vitro photothermal imaging performance:

1mL (0-200 μg mL^{-1}) AB@MPDA NPs Were added into eppendorf tubes (1.5 mL). It was irradiated by 808 nm NIR laser (1 W cm^{-2}) for 10 minutes. The solution temperature and photothermal images were obtained by an infrared imaging camera.

In vitro US imaging performance: PBS buffer solution of AB@MPDA (100 μg mL^{-1}) with various pH (5.0,6.5.7.4) were put into eppendorf tubes (5 mL) before imaging and all the droppers were immersed at the same depth in the deionized water. Ultrasound B-mode images of AB@MPDA were acquired using clinical Philips iU elite imaging system with a transducer of L9-3. The center frequency and output energy level were set to 15 MHz and 6%, respectively.

In vitro CT imaging performance: To assess CT contrast efficiency, PBS buffer solution of AB@MPDA (100 μg mL^{-1}) with various pH (5.0,6.5.7.4) were put into eppendorf tubes (1.5 mL). Then all tubes were scanned on a CT (Skyscan 1076) system. CT scanning parameters were as follows: slice thickness, 2.5 mm; the tube voltage of 120 kV, the tube current of 200 mA; field of view, 1024 × 1024. Then, Hounsfield units (HU), which were used to quantify the CT contrast changes, were obtained from a uniform circular region of interest in the resultant CT image for each sample.

Cell culture: The mouse 4T1 breast cancer cells, mouse NIH-3T3 fibroblast cells, human HeLa cervical carcinoma cells, HUVEC Human umbilical vein endothelial cells, HEK-293T emborynic kidney cells, were purchased from China Type Culture Collection (CTCC) obtained from the American Type Culture Collection (ATCC). They were cultured in Dulbecco's modified Eagle's medium (DMEM)(HEK-293T and NIH-3T3 cells) or Roswell Park Memorial Institute-1640 (RPMI-1640)(4T1, HeLa, and HUVEC cells) with 10% (v:v) foetal bovine serum, 100 U mL^{-1}penicillin, and 100 μg mL^{-1}streptomycin in an incubator (Thermo Scientific) at 37 °C under the atmosphere of 5% CO2and 90% relative humidity. To digest cells and subculture, 0.25% (w:v) trypsin was used.

Cell uptake assay: HeLa cells were seeded in glass-bottom culture dishes at a density of 1.5×10^5 cells per dish and incubated for 24 h. Afterwards, the previous medium was removed, and 1 mL of fresh medium containing 100 μg mL^{-1} FITC@MPDA NPs was added. After incubation at various time intervals (1, 2, 4, 8, 12 h), the cells the cells were washed three times with PBS and stained with Lyso-Tracker Red for 30 minutes, and then fixed with paraformaldehyde (4%) for 25 min and stained by DAPI for 5 min. Then, the images of the cells were recorded by a CLSM (Zeiss LSM880 Airyscan, German). For the flow cytometry analysis, the cells were seeded into 6-well plates at 2×10^5 cells per well and treated as mentioned above. At pre-determined time points, the cells in the wells were digested with trypsin, followed by centrifugation (800 rpm, 5 min) and washing with PBS. Finally, the cells were suspended in PBS and then analyzed using a flow cytometry (BD FACSCanto, USA).

Measurement of intracellular hydrogen release: HeLa (or 4T1) cells were seeded into 6-well plates at 2×10^5 cells per well and incubated for 24h. Afterwards, the previous medium was removed, and 1 mL of fresh medium containing 100 μg mL^{-1} MB-Pt probe was added. After incubation for 2 h, the cells were washed with PBS three times and then incubated with 1 mL of fresh medium containing 100 μg mL^{-1} AB@MPDA NPs. After incubation at various time intervals (30 min, 60 min), the cells were digested to be observed and photographed under a microscope.

Cytotoxicity measurement: HeLa (or 4T1) cells were seeded into 96-well plates at 6×10^3 cells per well and incubated for 24h. Then the cells were treated with AB, MPAD, AB@MPAD at various concentrations (12.5–200 μg mL^{-1}, n=5). The control group was treated with PBS only. After 8 h, the medium was replaced with fresh medium, and then the cells were exposed to an 808 nm laser (1 W cm^{-2},10 min). After incubation for another 16 h, the cellular viability was tested by the CCK-8 method.

Live/dead stain of HeLa cells: HeLa cells were seeded into 6-well plates at 2×10^5 cells per well and incubated for 24 h. Then the cells were treated with AB, MPAD, AB@MPAD (200 μg mL^{-1}) respectively. The control group was treated with PBS only. After 8 h, the medium was replaced with fresh medium, and then the cells were exposed to an 808 nm laser (1 W cm^{-2},10 min). After NIR treatment, the cells were washed with PBS, stained with Calcein-AM/PI for 20 min, and imaged by an inverted fluorescence microscope.

Cell apoptosis analysis: HeLa cells were seeded into 6-well plates at 2×10^5 cells per well and incubated for 24 h. Then the cells were treated with AB, MPAD, AB@MPAD (200 μg mL^{-1}) respectively. The control group was treated with PBS only. After 8 h, the medium was replaced with fresh medium, and then the cells were exposed to an 808 nm laser (1 W cm^{-2},10 min). After NIR treatment, the cells were digested with trypsin, stained with AV-FITC/PI Apoptosis Detection Kit, and then analyzed by a flow cytometry (BD FACSCanto, USA).

Intracellular ROS analysis: HeLa/HEK-293T cells were seeded into 6-well plates at 2×10^5 cells per well and incubated for 24 h. Then the cells were treated with PBS, MPAD, AB@MPAD (200 μg mL^{-1}) respectively. After 8 h, the medium was replaced with 1 mL fresh DCFH-DA working fluid for another 30 min incubation, and then the cells were exposed to an 808 nm laser (1 W cm^{-2},10 min). After NIR treatment, the cells were washed with PBS and immediately analyzed by an inverted fluorescence microscope. For the flow cytometry analysis, the cells were treated as mentioned above. After NIR treatment, the cells were suspended in PBS and then analyzed using a flow cytometry.

Mitochondrial membrane potential detection: HeLa/HEK-293T cells were seeded into glass-bottom culture dishes at a density of 1.5×10^5 cells per dish and incubated for 24 h. After incubation with AB@MPDA NPs with different concentrations for 8 h, the HeLa/HEK-293T cells were stained with 1 mL JC-1 dye working solution for 30 min. The red fluorescence (the aggregated JC-1) and green fluorescence (the monomeric JC-1) were observed by using CLSM (Zeiss LSM880 Airyscan, German), and the rate of monomeric JC-1 was quantified by using a flow cytometer (BD FACSCanto, USA) at 37°C.

In vivo therapeutic evaluation: Female BALB/c mice (5 weeks old) were purchased from Beijing Huakang Biotechnology Co., Ltd., China. All animal procedures were performed in accordance with the protocol approved by the Laboratory Animal Science Department of Jinan University. All animal experiments were in accordance with the guidelines of the Animal Care and Use Committee of the Laboratory Animal Science Department of Jinan University. For in vivo therapeutic evaluation, all BALB/c were randomly assigned (n = 3) into 6 groups: (1) PBS; (2) PBS+NIR; (3) MPDA NPs; (4) MPDA NPs+ NIR; (5) AB@MPDA NPs; (6) AB@MPDA NPs+ NIR. They were subcutaneously transplanted with 4T1 mammary cancer cell (1×10^6 cells per site) when they were 6 weeks old. When the tumor volume reached approximately 100 mm^3, mice were injected with different formulations via the tail vein (50 μL, 6 mg kg^{-1}). NIR treatments were conducted after 8 h injection. The first day of treatment was defined as day 0 and the same treatments were repeated on the seventh day. Each mouse's body weight and tumor volume were recorded every two days. The formula for calculating tumor volume was: volume = $W^2 \times L/2$, in which “W" and “L" represented the width and length of the tumor respectively. All mice were sacrificed 24 days later and the tumor was completely stripped and then fixed in PBS containing 4% formaldehyde. Tumor tissues were then stained with TUNEL, H&E, and Ki-67, and pathologically analyzed under a microscope. Other major organs (heart, liver, spleen, lung, and kidney) were also removed for H&E staining to assess acute toxicity.

In Vivo CT Imaging evaluation: On the 14th day of treatment, AB@MPDA NPs (6 mg kg^{-1}) were delivered to mice via intravenous injection through the tail vein. Each mouse was anesthetized by intraperitoneal injection of 1% sodium pentobarbital (4 mL kg^{-1}). CT images and signal intensity were taken from a CT system (Skyscan 1076) at 4, 8, 12 and 24 h postinjection under the same CT scanning parameters as with in vitro measurements.

In Vivo US Imaging evaluation: On the 14th day of treatment, AB@MPDA NPs (6 mg kg^{-1}) were delivered to mice via intravenous injection through the tail vein. Each mouse was anesthetized by intraperitoneal

injection of 1% sodium pentobarbital (4 mL kg^{-1}). US B-mode images and videos of mice were acquired through a clinical Philips iU elite imaging system at 4, 8, 12 and 24 h postinjection under the same US parameters as with in vitro measurements.

In Vivo PTT Effect and imaging evaluation: On the 14th day of treatment, AB@MPDA NPs (6 mg kg^{-1}) were delivered to mice via intravenous injection through the tail vein. Each mouse was anesthetized by intraperitoneal injection of 1% sodium pentobarbital (4 mL kg^{-1}). After 8 h injection, photothermal images and temperature in tumor of mice were acquired through an infrared imaging camera.

Reference

[1] Zhang L, Yang P, Guo R, Sun J, Xie R, Yang W. Multifunctional Mesoporous Polydopamine With Hydrophobic Paclitaxel For Photoacoustic Imaging-Guided Chemo-Photothermal Synergistic Therapy. *Int J Nanomedicine*. 2019; 14:8647-8663.

[2] Park J, Brust TF, Lee HJ, Lee SC, Watts VJ, Yeo Y. Polydopamine-based simple and versatile surface modification of polymeric nano drug carriers. *ACS Nano*. 2014;8(4):3347-3356.

[3] Yang J, Hou M, Sun W, et al. Sequential PDT and PTT Using Dual-Modal Single-Walled Carbon Nanohorns Synergistically Promote Systemic Immune Responses against Tumor Metastasis and Relapse. *Adv Sci (Weinh)*. 2020;7(16):2001088.

Results and Discussion

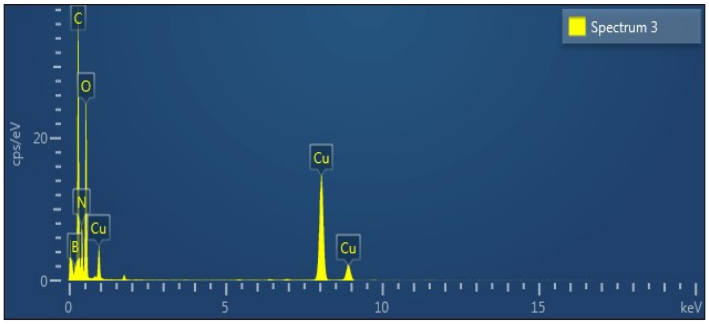

Figure S1. EDS spectra of AB@MPDA.

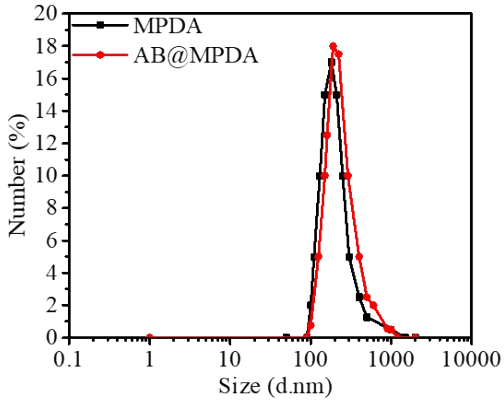

Figure S2. DLS measurements of MPDA and AB@MPDA.

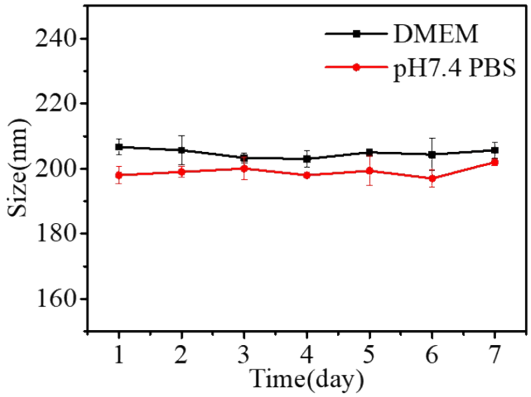

Figure S3. DLS measurements of AB@MPDA in DMEM and PBS within 7day.

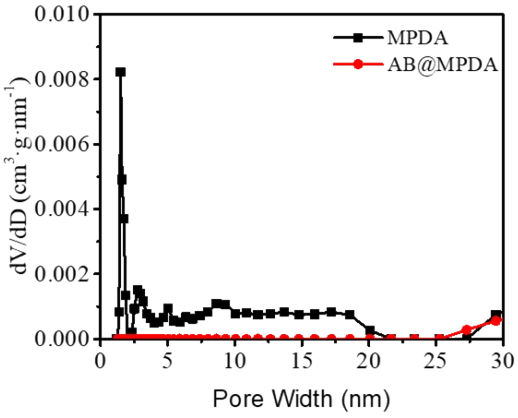

Figure S4. Pore diameter distribution of MPDA and AB@MPDA.

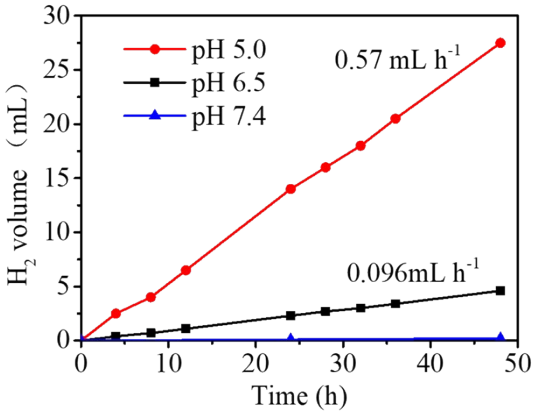

Figure S5. Hydrogen release behavior of free AB.

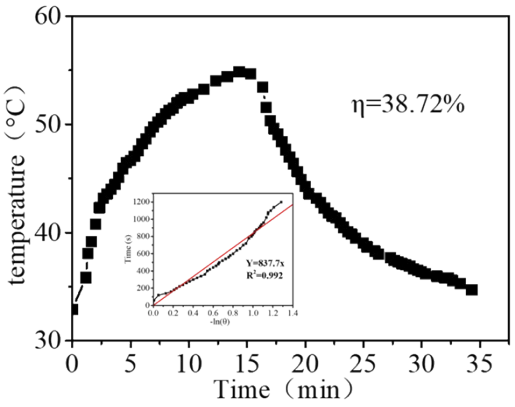

Figure S6. Analysis of photothermal conversion efficiency of AB@MPDA NPs.

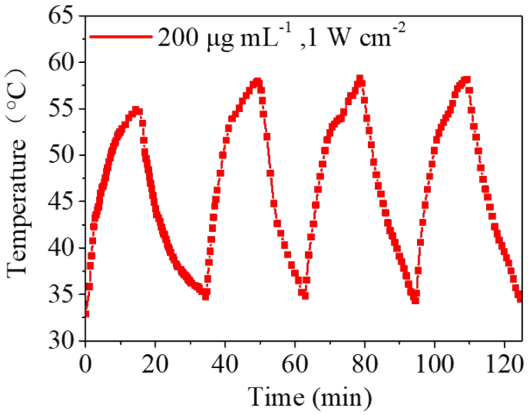

Figure S7. Temperature changes of AB@MPDA NPs (200 μg mL^{-1}) in 4 cycles.

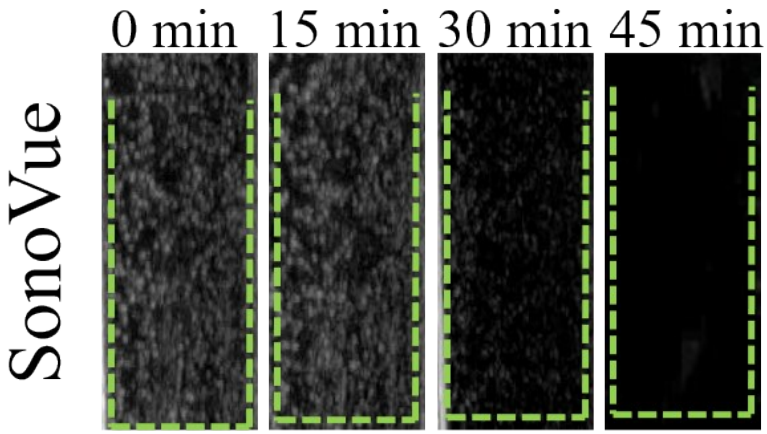

Figure S8. in vitro US B-mode images of SonoVue.

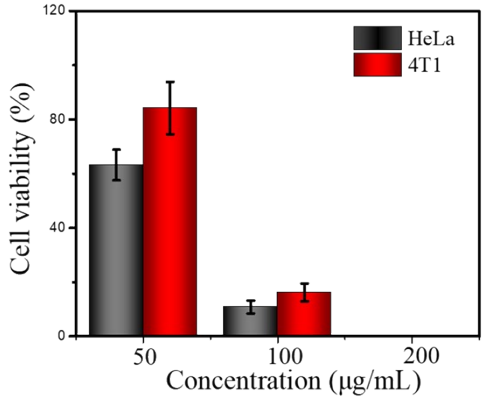

Figure S9. Cell viability of HeLa cells and 4T1 cells treated with AB alone at various concentration.

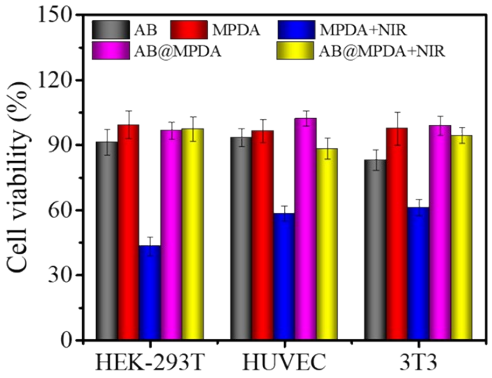

Figure S10. CCK-8 test of normal cells (HEK-293T, HUVEC, NIH-3T3).

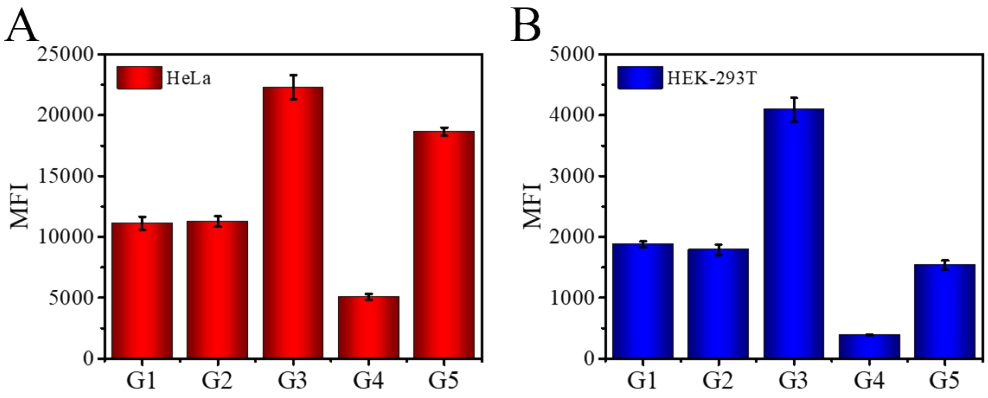

Figure S11. MFI of HEK-293T and HeLa cells with different treatment. (G1, PBS. G2, MPDA. G3, MPDA + NIR. G4, AB@MPDA. G5, AB@MPDA+NIR.).

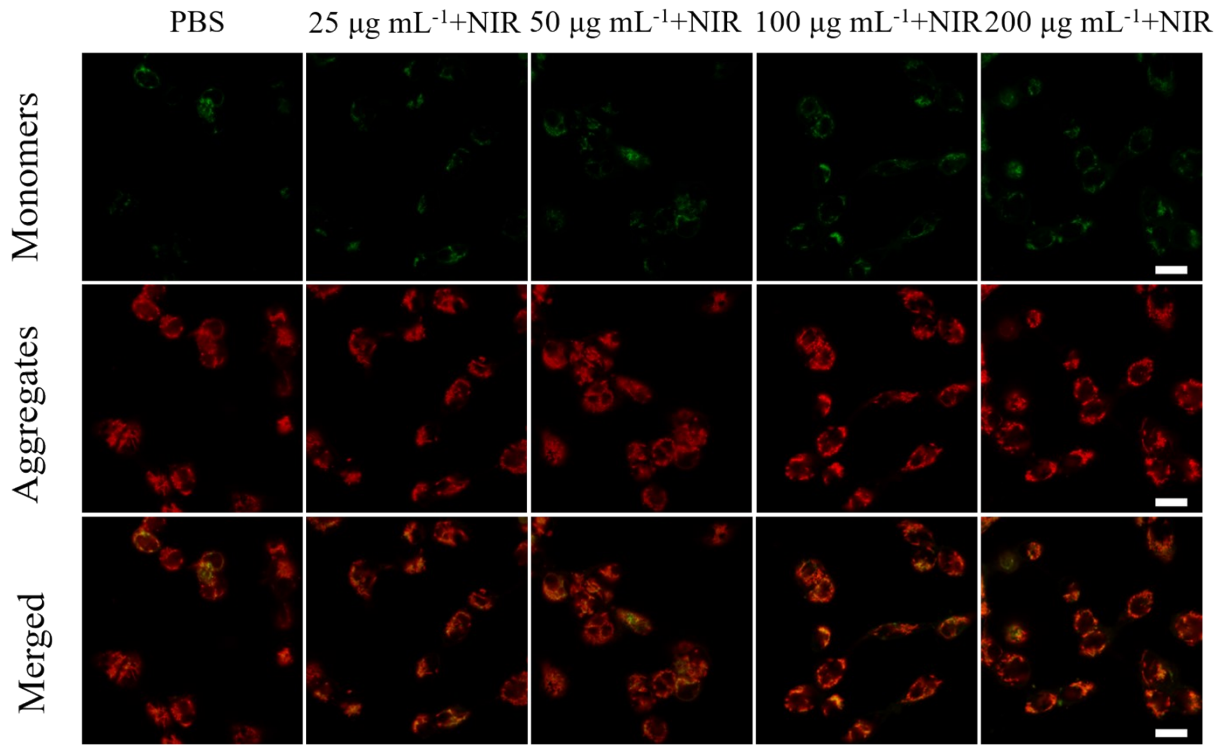

Figure S12. Mitochondrial membrane potential assay of HEK-293T cells. Scale bar: 10 μm.

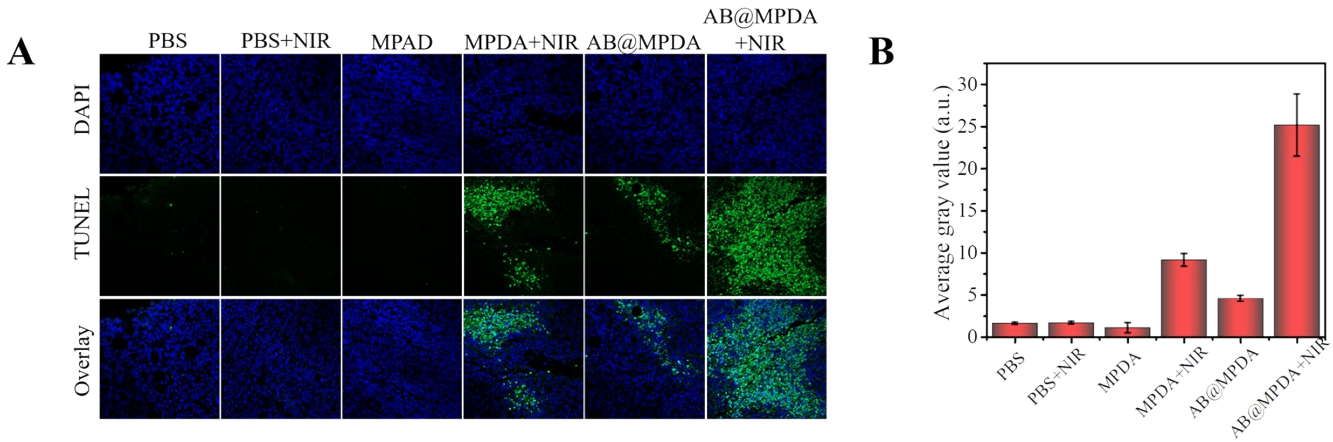

Figure S13. A) TUNEL staining of the sacrificed cancer tissues after various treatments for 24 days and B) corresponding quantitative analysis.

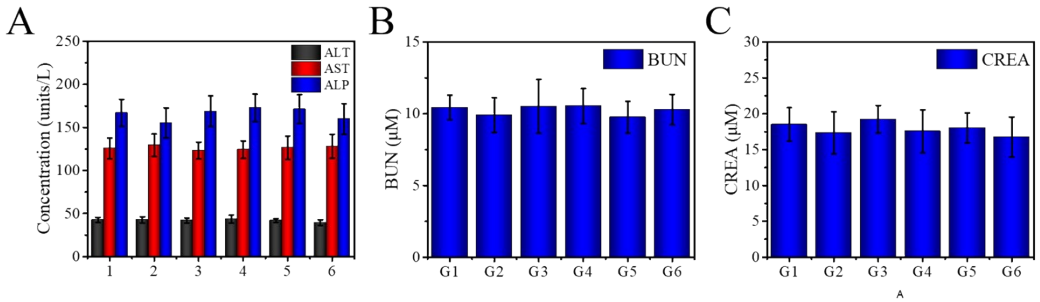

Figure S14. Blood biochemical analyses including liver functions (A) and kidney functions (B, C). ALP, alkaline phosphatase; AL T, alanine transaminase; AST, aspartate transaminase; CREA, creatinine; BUN, blood urea nitrogen (n =4). Mean value and error bar are defined as mean and s.d., respectively. (G1, PBS. G2, PBS+NIR. G3, MPDA. G4, MPDA + NIR. G5, AB@MPDA. G6, AB@MPDA+NIR)

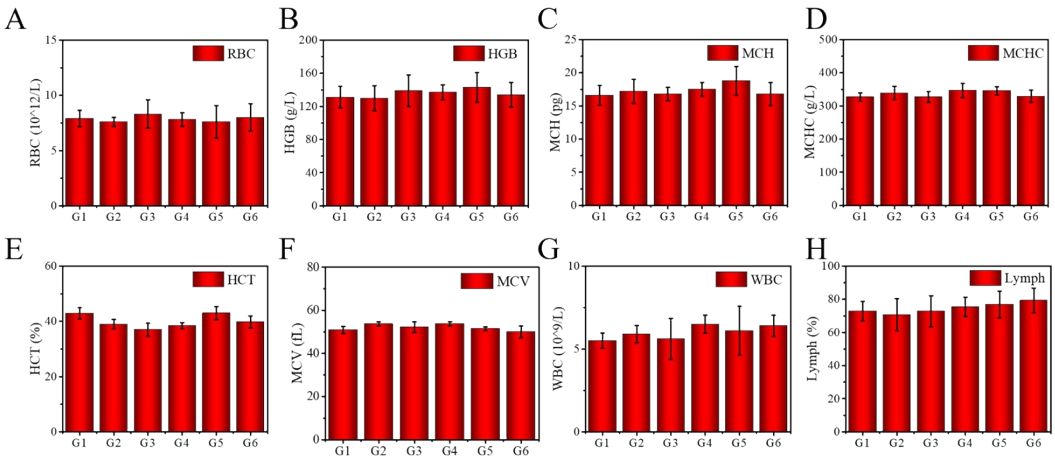

Figure S15. assay of complete blood panel. A) red blood cells, B) hemoglobin, C） mean corpuscular hemoglobin, D) mean corpuscular hemoglobin concentration, E) hematocrit, F） mean corpuscular volume, G)white blood cells and H) lymph. (G1, PBS. G2, PBS+NIR. G3, MPDA. G4, MPDA + NIR. G5, AB@MPDA. G6, AB@MPDA+NIR)

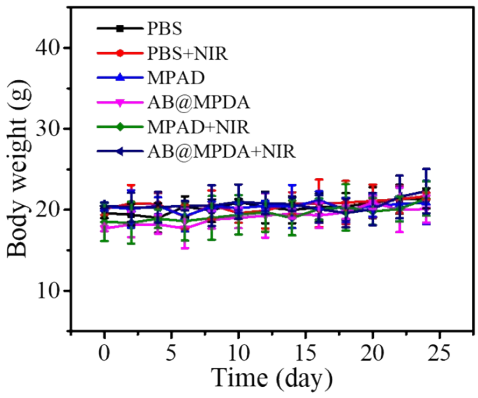

Figure S16. Weight of mice during 24 days of treatment.

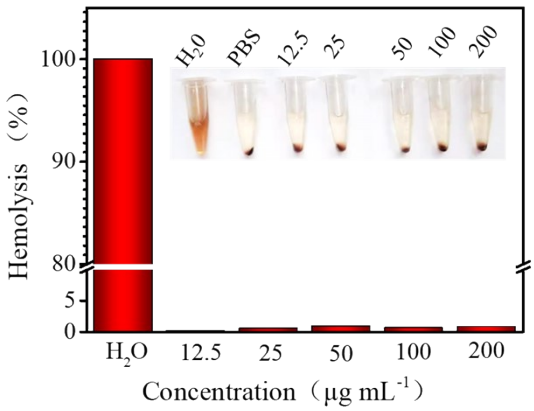

Figure S17. Hemolysis rate test of AB@MPDA with different concentration.

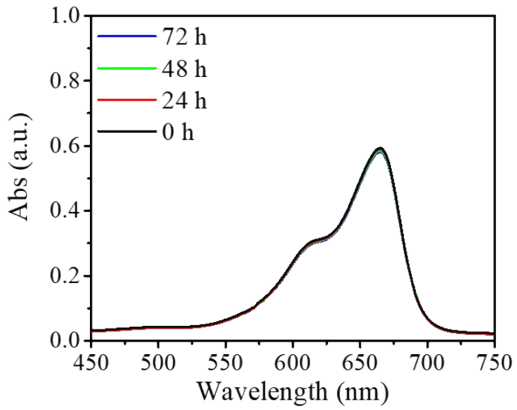

Figure S18. Drug leakage detection by MB-Pt probe at pH 7.4.