Electronic Supplementary Information

for

Carbonic anhydrase IX-targeted nanovesicles potentiated ferroptosis by remodeling the intracellular environment for synergetic cancer therapy

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Materials

Poly(lactide-coglycolide)-poly(ethylene glycol)-NHS (PLGA-PEG-NHS) was purchased from Hunan Hua Teng Pharmaceutical Co., Ltd. 4-(2-aminoethyl) benzene sulfonamide (AEBS) was obtained from Sigma-Aldrich. Chlorin e6 (Ce6) was purchased from Rhawn (Shanghai, China). Hemoglobin (Hb) and hydrogen peroxide (H_2O_2) were purchased from Aladdin Reagent, Ltd (Shanghai, China). N, N-Diisopropylethylamine (DIPEA) was obtained from Macklin Biochemical Co., Ltd (Shanghai, China). 9,10-anthracenediyl- bis(methylene)-dimalonic acid (ABDA) was purchased from Sigma-Aldrich (Shanghai, China). Dichlorofluorescin diacetate (DCFH-DA) was purchased from Yeasen Biotech Co., Ltd. Annexin V-FITC/Propidium Iodide (PI) and apoptosis Detection Kit were purchased from Bestbio. 3,3',5,5'-tetramethylbenzidine (TMB), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymet (BCECF-AM) were obtained from Beyotime Biotechnology. C11-BODIPY$^{581/591}$ was purchased from Good Laboratory Practice Bioscience (Montclair, USA). Liproxstatin-1 was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). GPX4 rabbit polyclonal antibody was purchased from Beyotime Biotechnology. Diagnostic kits for blood urea nitrogen (BUN), uric acid (UA), creatinine (Cr), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Synthesis of PLGA-PEG-AEBS

In a dry 50 mL round bottom flask, DMF (25 mL) and PLGA-PEG-NHS (500 mg, 1 mmol) were successively introduced at room temperature. Then AEBS (201 mg, 1 mmol) and DIPEA (210 μL, 1.2 mmol) were added to the mixed solution and stirred overnight. The obtained products were purified by dialysis with a 2 kDa cut-off membrane, and were then dried through lyophilization.

Preparation of PAHC NV

PLGA-PEG-AEBS (5 mg) and 1.5 mg Ce6 were successively introduced in 1 mL of DMSO solution and stirred for 30 min. Then the mixed solution was added into Hb solution (0.5 mg/mL, 10 mL) and the obtained mixture was stirred for another 6 h. The desired PAHC was collected by centrifugation (10000 rpm, 10 min) and washed three times with deionized water. The PHC was prepared using the same protocol, except that PLGA-PEG-NHS was utilized instead of PLGA-PEG-AEBS.

Quantification of Ce6 and Hb content in PAHC NV

For the PAHC NV, the loading capacity of Ce6 and Hb was determined using a UV-vis

spectrometer according to previous literature [1, 2]. To estimate the amount of Ce6 and Hb loaded by PAHC NV, the PAHC NV were collected by centrifugation, and the free Ce6 and Hb in the supernatant was quantified by using UV calibration curve. The loading rate was calculated by the following formula: drug loading content (wt%) = (the total mass of the drug - mass of the drug in supernatant)/the total mass of the nanoparticles × 100%.

Characterizations of PAHC NV

The chemical structure of PLGA-PEG-AEBS was detected by ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR, Bruker AV600, Switzerland). The 1H data was obtained on a 600 MHz NMR spectrometer and DMSO-d6 as solvent unless otherwise stated. Hydrodynamic diameters and zeta potentials of PAHC was measured by dynamic light scattering (DLS, Zetasizer Nano ZS-90, Malvern, UK). The morphology of the as-synthesized samples was observed using a transmission electron microscopy (TEM, JEM-2100, JEOL, Japan). The UV–vis absorption spectrum was measured using a spectrometer (UV-2550, Shimadzu, Japan). The fluorescence spectrum was implemented on a fluorescence spectrophotometer (Hitachi F-2500, Japan).

SDS-PAGE

All samples were mixed with an equal volume of sample buffer (Bio-Rad) containing 5% (v/v) beta-mercaptoethanol, and then boiled for 5 min. A 4% stacking gel with a 10% resolving gel was assembled on a minivertical gel apparatus and each lane loaded with 20 μg of protein. The gel was run at 120 V for approximately 1 h. After electrophoresis, the gel was stained with Coomassie blue R250 for 30 min and then the gel was destained using destaining buffer (10% acetic acid and 20% methanol).

Fenton-like reaction

We used TMB as indicator to inspect the •OH generation of PAHC, which could decompose H2O2 to •OH via the Fenton reaction. PAHC (150 μg/mL, 200 μL), H2O2 (1 mM, 100 μL), and TMB (500 μg/mL, 0.5 mL) were added into 2 mL of PBS (pH = 5.5). After that, the UV–vis absorption spectrum of the samples was scanned at different time points.

ESR measurements of •OH

5,5-Dimethyl-1-pyrroline-Noxide (DMPO) was used as the trapping reagent to identify the •OH radicals by the ESR spin-trap technique (Bruker A300, Germany). PAHC (100 μg/mL), H_2O_2 (5 mmol/L), and DMPO (25 mM) was added into 2 mL of PBS. ESR spectra were recorded after reacting for 10 min.

Oxygen release capability of PAH

The oxygen release behavior of free Hb and PAH (Hb: 0.2 mg/mL) was inspected with $Ru(dpp)_3Cl_2$ as a fluorescence quenching O_2 indicator. Hb (or PAH) and $Ru(dpp)_3Cl_2$ were mixed in deoxygenated aqueous solution, and then the fluorescence intensity was recorded at indicated time intervals.

Photodynamic effect study

ABDA was used to inspect the production of singlet oxygen. In brief, 100 μL ABDA (20 μg/mL) was mixed with various samples (1 mL) in which the content of Ce6 in PAHC solution was same with free Ce6 solution (4 μg) in 2mL of deoxygenated water. The fluorescence spectrum of ABDA at the predetermined time interval was measured (λ_{ex}=360 nm) to monitor ABDA consumption.

ESR measurements of $^1O_2$

2,2,6,6-Tetramethyl-4-piperidone monohydrate (TEMP) was used as the trapping reagent to identify the $^1O_2$ radicals by the ESR spin-trap technique. PAHC (100 μg/mL), and TEMP (50 mM) was added into 2 mL of deoxygenated water. All samples were exposed to 638 nm laser (0.5 W/cm^2) for 3 min and ESR spectra were then recorded.

Colloidal stability of PAHC

Phosphate-buffered saline (PBS, Gibco Life Technologies (Switzerland) buffer was used as biological media to assess the colloidal stability of PAHC, which is crucial to their biological utility. Therefore, the PAHC was incubated in PBS buffer and stored at 4 ℃ for 7 days. At the same time, the changes of particle size and polymer dispersity index (PDI) were monitored at different incubation time points by DLS.

Cell culture

Murine breast cancer cells (4T1) were purchased from American Type Culture Collection (ATCC, MD, USA). Cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37 ℃ under 5% CO_2.

In vitro cellular uptake

4T1 cells were seeded into 6-well plates at a density of 1 × 10^5 cells/well and cultured for 24 h, and the medium was discarded. Then, the cells were incubated with free Ce6 and PAHC (2 μg/mL, Ce6 equivalent) for 6 h. For the pro-blocking group, 4T1 cells were pre-treated with AEBS for 2 h. After that, the cellular uptake was stopped, 4T1 cells were rinsed with PBS three times and fixed with 4% of paraformaldehyde. Last, DAPI was applied to stain the cell nuclei and the CLSM (Zeiss LSM 510 Meta, Germany) was utilized to visualize the samples.

Meanwhile, to quantitatively assessed the *in vitro* cellular uptake of PAHC, flow

cytometry (ATTUNE 22 NXT, Thermo Fisher Scientific, USA) was applied to measure the fluorescence intensity of Ce6 in cells. 4T1 cells were seeded into 6-well plates at a density of 1×10^5 cells/well and cultured for 24 h, and the medium was discarded. Then, the cells were incubated with free Ce6 and PAHC (2 μg/mL, Ce6 equivalent) for 6 h. For the pro-blocking group, 4T1 cells were pre-treated with AEBS (2 μg/mL) for 2 h. After that, the cells were scrapped off and intracellular fluorescence intensity was detected by flow cytometry.

Intracellular pH assessment

4T1 cells were seeded in 6-well plates at 1.0×10^5 cells per well and cultured in a normoxic environment (21% oxygen) for 24 h. Subsequently, the cells were treated with culture medium (without serum) containing PBS, Hb, Ce6, PHC, PAHC, and PAHC(AEBS pretreated) in a normoxic environment (21% oxygen) for another 12 h. At the end of incubation, the BCECF-AM fluorescent dye (5 μM) was added and co-incubated for 40 min to assess the intracellular pH changes.

To quantitatively assessed the intracellular pH change of PAHC-treated cells, flow cytometry was applied to measure the fluorescence intensity of BCECF-AM in cells. The experimental methods were according to previous literature with slight modifications [3]. After 4T1 cells were co-incubated with BCECF-AM dye (5 μM) and washed by PBS three times, the PBS (pH 6.6, 6.8, 7.0, 7.2, or 7.4) containing nigericin (15 μg/mL) was added and co-incubated for 10 min. At the end of incubation, the intracellular fluorescence intensity was detected by flow cytometry.

Intracellular ROS detection

4T1 cells were seeded in 6-well plates at 1.0×10^5 cells per well and cultured in a normoxic environment (21% oxygen) for 24 h. Subsequently, the cells were treated with culture medium (without serum) containing PBS, Hb, Ce6, PHC, PAHC, and PAHC(AEBS pretreated) in a normoxic environment (21% oxygen) or hypoxic environment (1% oxygen) for another 6 h. Next, the cells in laser irradiation groups were exposed to 638 nm laser (0.5 W/cm^2) for 3 min. At the end of incubation, the DCFH-DA fluorescent dye was added and co-incubated for 30 min to assess the intracellular ROS levels.

In vitro cytotoxicity analysis

The *in vitro* cytotoxicity of the PAHC against 4T1 cells was investigated by the MTT assay. In brief, 4T1 cells were plated in 96-well plates (1×10^4 cells per well) and cultured for 24 h. Then, the cells were incubated with the different formulations at various concentrations in 100 μL of complete DMEM in a normoxic environment (21% oxygen) or hypoxic environment (1% oxygen) for 12 h. Subsequently, the culture

medium was replaced by fresh DMEM (100 μL) to remove the residual medicine. The cells were exposed to 638 nm laser irradiation (0.5 W/cm^2, 3 min). After that, the cells in all groups were cultured for another 12 h and the cell viability was measured by the MTT assay based on the absorbance at the wavelength of 570 nm.

To evaluate the cytotoxicity of treatments more intuitively, the cells treated with PBS, Ce6, Hb, PHC, and PAHC at a Ce6-equivalent dose of 1 μg/mL in a normoxic environment (21% oxygen) or hypoxic environment (1% oxygen) for 12 h. Subsequently, the culture medium was replaced by fresh DMEM (100 μL) to remove the residual medicine. The cells were exposed to 638 nm laser irradiation (0.5 W/cm^2, 3 min). After that, the cells in all groups were cultured for another 12 h and stained with calcein-AM/PI and observed with fluorescence microscopy.

Apoptosis assessment in vitro

To measure cellular apoptosis with flow cytometry, the cells treated with PBS, Ce6, Hb, PHC, and PAHC at a Ce6-equivalent dose of 1 μg/mL in a normoxic environment (21% oxygen) or hypoxic environment (1% oxygen) for 12 h. Then, the culture medium was replaced by fresh DMEM to remove the residual medicine. The cells were exposed to 638 nm laser irradiation (0.5 W/cm^2, 3 min) and then cultured for another 12 h. Prior to analysis with flow cytometer, the cells were harvested and stained with annexin V-FITC (5 μL) and PI (10 μL).

CLSM of intracellular LPO assay

4T1 cells were seeded into 6-well plates at a density of 1 × 10^5 cells/well and cultured for 24 h, and the medium was discarded. Then, the cells were incubated with PBS, Hb, Ce6, PHC, PAHC, and PAHC+Lip-1 (0.8 μg/mL, Ce6 equivalent) for 12 h. Subsequently, the culture medium was replaced by fresh DMEM to remove the residual medicine. The cells were exposed to 638 nm laser irradiation (0.5 W/cm^2, 2 min) and incubated for another 6 hours. After that, the cells were incubated with C11-BODIPY$^{581/591}$ fluorescent probe (10 μM) for 30 min and the CLSM (Zeiss LSM 780, Germany) was utilized to visualize the samples.

Western Blot Analysis

4T1 cells were seeded into 6-well plates at a density of 1 × 10^5 cells/well and cultured for 24 h, and the medium was discarded. Then, the cells were incubated with PBS, Hb, Ce6, PHC, PAHC (1 μg/mL, Ce6 equivalent) for 12 h. Subsequently, the culture medium was replaced by fresh DMEM to remove the residual medicine. The cells were exposed to 638 nm laser irradiation (0.5 W/cm^2, 2 min) and incubated for another 6 hours. The cells lysates were collected and analyzed by electrophoresis run on 12% denaturing polyacrylamide gels.

Intracellular GSH, GPX4, and MDA assay

4T1 cells were seeded in 6-well plates at 1.0 × 10^5 cells per well and cultured with different formulations at a Ce6-equivalent dose of 1 μg/mL for 12 h. Then, the culture medium was replaced by fresh DMEM to remove the residual medicine. The cells were exposed to 638 nm laser irradiation (0.5 W/cm^2, 3 min) and then cultured for another 12 h. The GSH amount was evaluated using GSH assay kit; the GPX4 activity was measured using GSH peroxidase assay kit, and MDA amount was monitored using MDA assay kit.

Animal model establishment

Female BALB/c mice (4-6 weeks, 15 - 18 g) were provided from Xiamen University Laboratory Animal Center. All animal study protocols were in consistent with the principles and procedures, which defined in the Guide for the Care and Use of Laboratory Animals, approved by Institutional Animal Care and Use Committee of Xiamen University (NO. XMULAC20190001). To establish the 4T1 tumor-bearing mice model, the cells (about 5×10^6) were inoculated subcutaneously into the right thigh of the mice. When the tumor volumes of 4T1 tumor-bearing mice reached about ~100 mm^3, the mice were utilized for subsequent experiments. The tumor volume was estimated by the formula: V = L × D^2/2, in which L and D was the longest and the shortest diameter of the tumor, respectively.

Fluorescence imaging

To evaluate *in vivo* distribution of PAHC, 4T1 tumor-bearing mice were randomly assigned to three groups: (1) Free ICG; (2) ICG labelled PHC; (3) ICG labelled PAHC. After intravenous injection of corresponding drugs at a Ce6-equivalent dose of 0.8 mg/kg, the *in vivo* fluorescence distribution was observed using an *in vivo* imaging system (Living Image) (λ ex= 740 nm, λ em= 820 nm). At 48 h after injection, the mice were sacrificed, and their tumor tissues and main organs (heart, liver, spleen, lung, and kidneys) were excised for FL imaging *ex vivo*.

In vivo antitumor efficiency evaluation

4T1 subcutaneous tumor-bearing mice were randomly divided into 5 groups (n = 5): (1) PBS; (2) Hb; (3) Ce6; (4) PHC; (5) PAHC. The formulations were intravenously injected into 4T1 tumor-bearing mice at a Ce6-equivalent dose of 0.8 mg/kg every three days. 24h after injection, the corresponding mice were subjected to 638 nm laser irradiation (1 W/cm^2, 3 min). The tumor volumes and body weights of mice were monitored every three days over a period of 15 days. At the end of treatment, all mice were sacrificed and the tumors as well as the major organs were collected,

photographed, weighed, and then fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining. The tumor growth inhibition rate (TGIR) was calculated according to the following equation: TGIR (%) = $(W_C - W_T)/W_C \times 100\%$, where W_C was the tumor weight of the PBS group and W_T represented the tumor weight of mice after the treatments. For survival rate assay, the 4T1-bearing mice in each group (n = 5) were observed until death to determine survival percentage and complete curve rate. Notably, the mice were euthanized for humane reasons when the tumor volume reached 2000 mm3, which was considered as the end point of survival data. Survival was assessed using Kaplan–Meier analysis (using OriginPro 8.5 software). All the mice in the PAHC-treated group were kept alive on the 36th day, while all untreated tumor-bearing mice of the control group died at this time.

Hemolysis assay

Fresh rabbit blood sample (2.0 mL) was stabilized by heparin and then washed with 8.0 mL of PBS. Then, the sample was centrifugated (4000 rpm, 5 min) and the supernatant was removed to furnish the fresh red blood cells (RBCs). The RBCs were washed by PBS three times. After that, the PAHC with various concentrations was incubated with 2% (V_0/V_1) of RBC dispersions at 37°C for 12 h. All samples were centrifugated (4000 rpm, 5 min) and the supernatants were collected to monitor the hemoglobin contents. Finally, the released hemoglobin was determined with UV-vis spectrometer at the wavelength of 540 nm. The hemolysis was calculated as follows: hemolysis (%) = $(A_{sample} - A_{PBS})/(A_{water} - A_{PBS}) \times 100\%$, where A_{sample}, A_{PBS}, and A_{water} are the absorbance values of the sample group, the normal saline group, and the distilled water group, respectively.

Animal care statement

GPX4 rabbit polyclonal antibody was purchased from Beyotime Biotechnology. Rabbit red blood cells used in the hemolysis study were collected and provided by Xiamen University Laboratory Animal Center. Female BALB/c mice (4-6 weeks, 15-18 g) were provided from Xiamen University Laboratory Animal Center. All animal study protocols were in consistent with the principles and procedures of Guide for the Care and Use of Laboratory Animals and the Principles for the Utilization and Care of Vertebrate Animals, Xiamen University. All animal studies were approved by the Institutional Animal Care and Use Committee of Xiamen University (NO. XMULAC20190001).

Statistical analysis.

Quantitative data are presented as mean ± s.d. Statistical comparisons were indicated as *P < 0.05, **P < 0.01, and ***P < 0.001, which was considered significant,

moderately significant, and highly significant, respectively.

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[3] S.-C. Chao, G.-J. Wu, S.-F. Huang, N.-T. Dai, H.-K. Huang, M.-F. Chou, Y.-T. Tsai, S.-P. Lee, S.-H. Loh, Functional and molecular mechanism of intracellular pH regulation in human inducible pluripotent stem cells, World Journal of Stem Cells 10(12) (2018) 196-211.

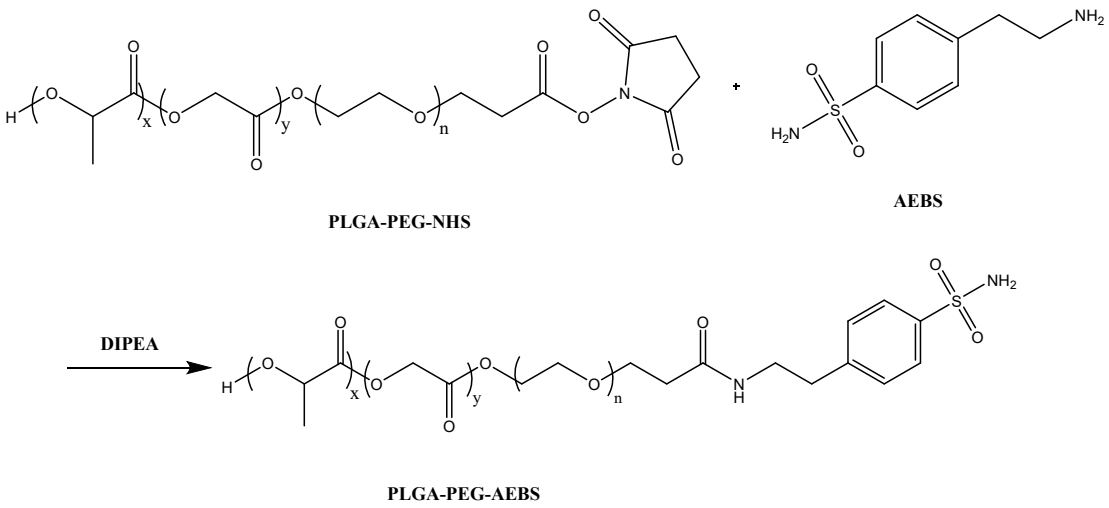

Figure S1. Chemical structures and synthetic routes of PLGA-PEG-AEBS.

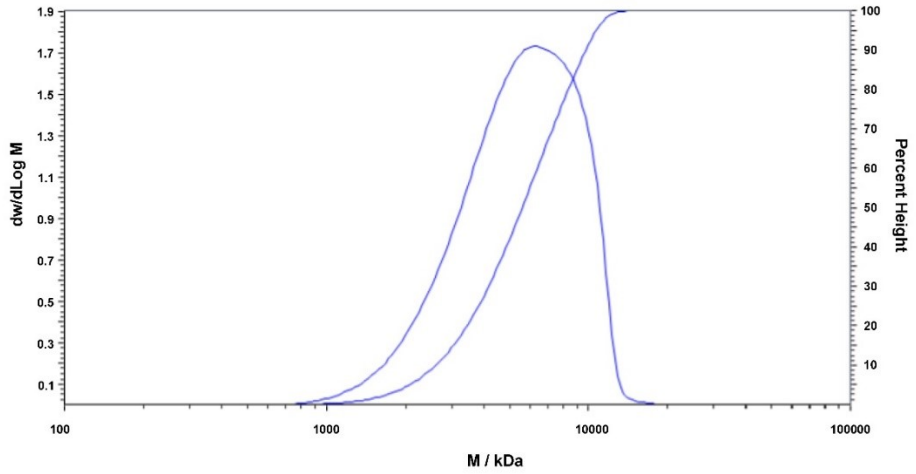

Figure S2. The GPC chromatogram of PLGA-PEG-AEBS in THF.

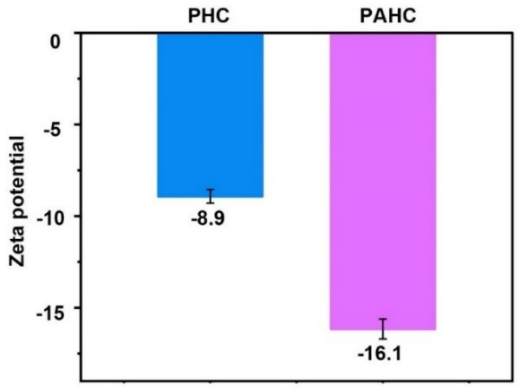

Figure S3. ζ-potential of PHC and PAHC.

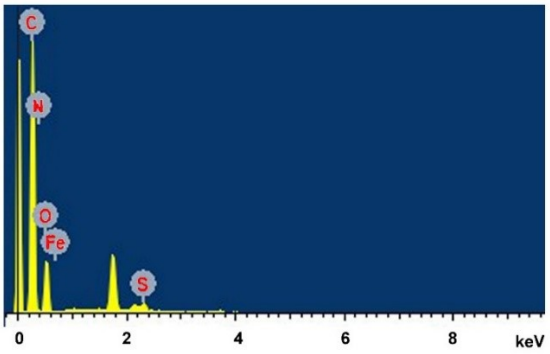

Figure S4. EDS analysis of PAHC NV.

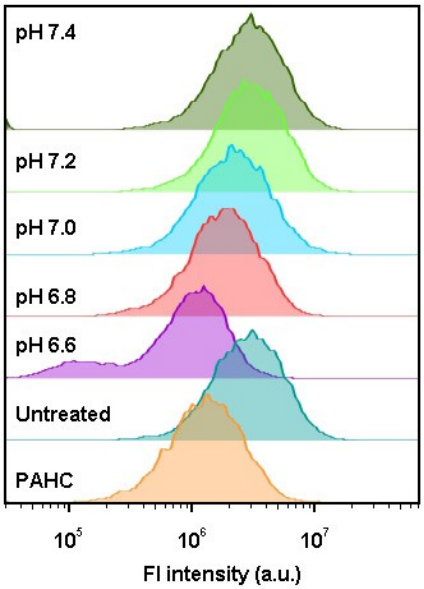

Figure S5. Flow cytometric analysis of intracellular pH change

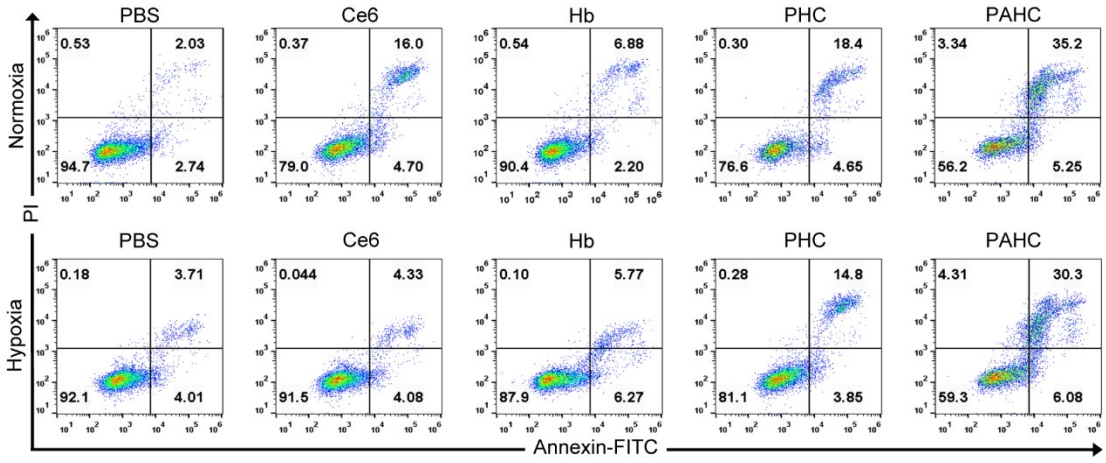

Figure S6. Apoptosis evaluation of 4T1 cells after incubation with PBS, Ce6, Hb, PHC, and PAHC.

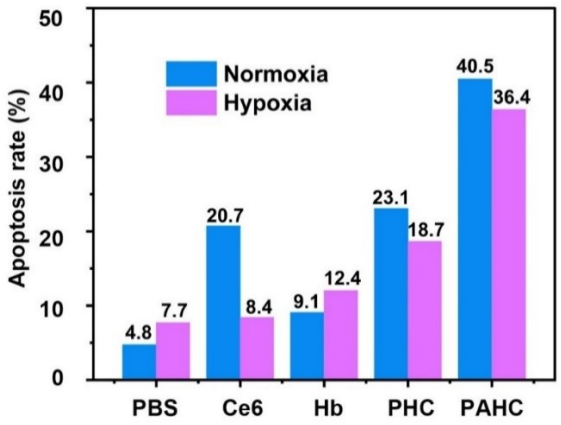

Figure S7. Apoptosis rate of 4T1 cells after various treatments.

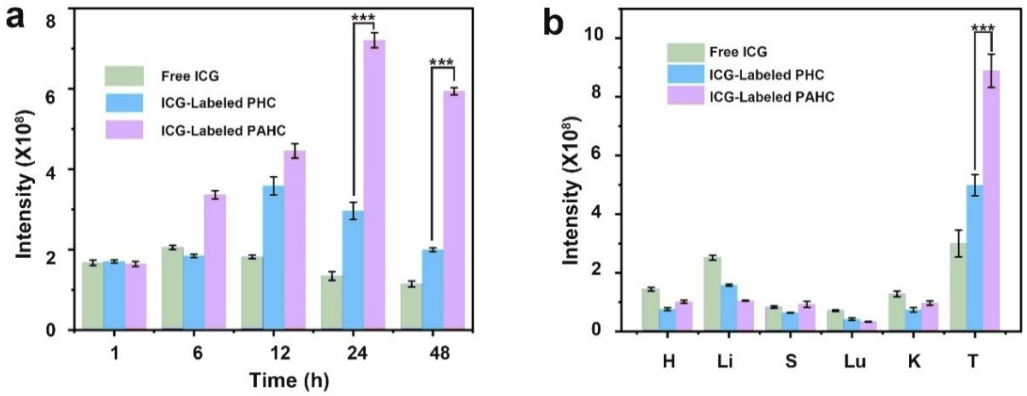

Figure S8. (a)Time-dependent averaged fluorescence intensity of the tumor site at 2, 5, 12, 24, and 48 h post i.v. injection and (b) averaged fluorescence intensity of excised main organs and tumors at 48 h post-injection.

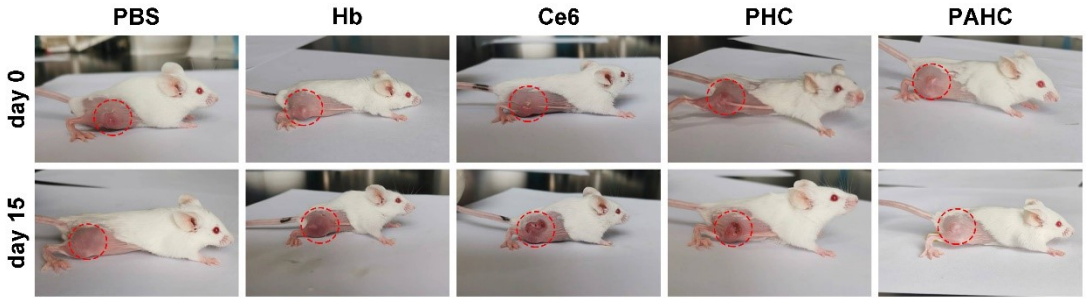

Figure S9. Tumor growth photographs of 4T1 subcutaneous tumor-bearing mice during different therapies.

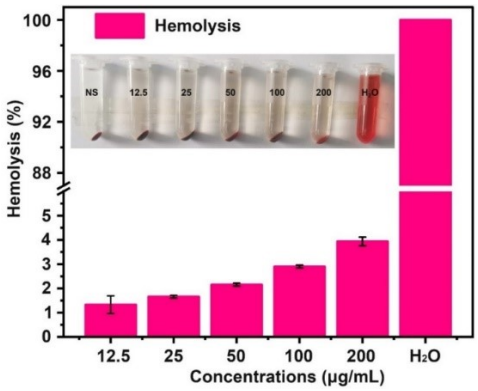

Figure S10. *In vitro* hemolysis percentage of PAHC incubated with rabbit red blood cells for 12 h. The inset image showed RBCs treated with PAHC at various concentrations for 12 h.

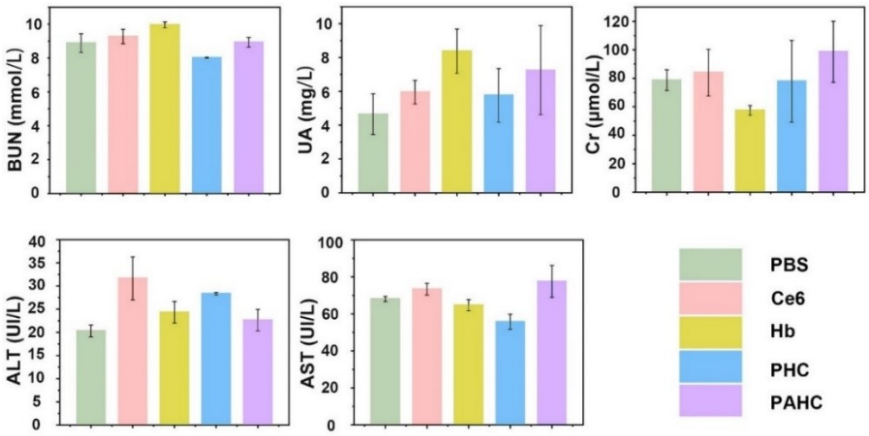

Figure S11. Serum biochemical indexes analysis after different treatments at 15 days. BUN, blood urea nitrogen; UA, uric acid; Cr, creatinine; ALT, alanine aminotransferase; and AST, aspartate aminotransferase.

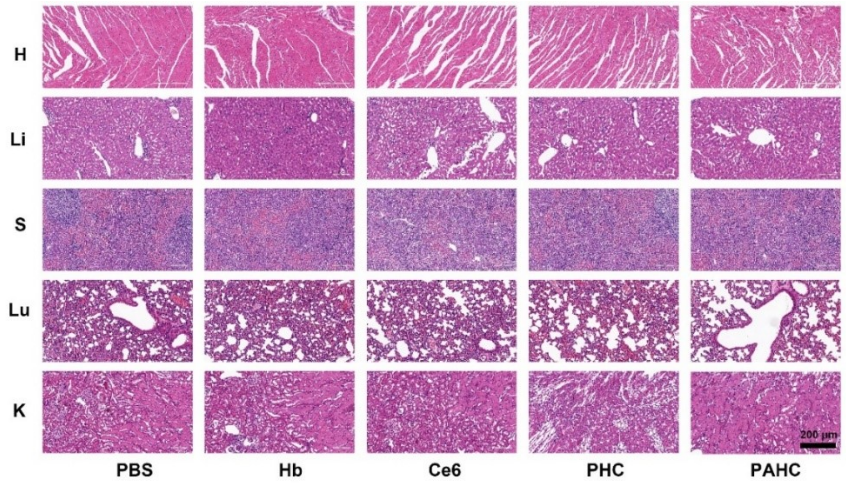

Figure S12. H&E staining of main organs obtained from the mice treated with different formulations. (scale bar: 200 μm).