Supporting Information

Ultrasound responsive erythrocyte membrane-derived hybrid nanovesicles with controlled drug release for tumor therapy

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Experimental Section

Materials

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), Didodecyldimethylammonium bromide (DDAB) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) were purchased from Xi'an Ruixi Biological Technology Co., Ltd (Xi'an, China). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-PE) were obtained from Avanti Polar Lipids Inc. (Alabaster, USA). Cholesterol was purchased from J&K Scientific Co., Ltd (Beijing, China). Doxorubicin hydrochloride (DOX) was bought from Beijing HuaFeng United Technology Co., Ltd. (Beijing, China). Hematoporphyrin monomethyl ether (HMME) were obtained from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). 1,3-Diphenylisobenzofuran (DPBF) was obtained from Sigma Aldrich (Shanghai, China). RPMI 1640 culture medium, phos- phate-buffered saline (PBS) and fetal bovine serum (FBS) were bought from Gibco (Grand Island, USA). LysoTracker™ Deep Red was purchased from Thermo Fisher Scientific (Waltham, USA). 3, 3'-dioctadecyloxacarbocyanine perchlorate (DiO) and 5-(and-6)-carboxy-2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) were obtained from Shanghai Yisheng Biological Technology Co., Ltd. (Shanghai, China). MTT Cell Proliferation and Cytotoxicity Assay Kit was obtained Beyotime Biotechnology Inc (Shanghai, China). Deionized (D.I.) water was prepared from Millipore (Bedford, USA). All the other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

Preparation of erythrocyte membrane-derived nanovesicles (NEM)

Firstly, whole blood samples were taken from the orbit of male BALB/c mice into a heparin tube and maintained at 4 °C. The plasma and the buffy coat were removed from the whole blood by centrifugation at 1000 g for 5 min at 4 ℃, and washed with pre-cooled PBS for three times. Then the obtained erythrocytes were treated with 0.25× PBS hypotonic solution in an ice bath for 30 min, the supernatant hemoglobin was removed by centrifugation at 20000 g for 10 min at 4°C, and washed five times with 0.25× PBS. Finally, the erythrocyte membrane was sonicated for 5 min in an ice bath and subsequently extruded through 400 nm, 200 nm, and 100 nm polycarbonate porous membrane to obtain NEM.

Preparation of DOX/HMME-loaded cationic liposomes (DOX/HMME@FA-Lipo)

Briefly, DOTAP, DOPE, cholesterol, FA-PEG2000-DSPE, and HMME were dissolved in 10 mL chloroform and methanol (2:1, v/v) solution with a fixed molar ratio 75: 100: 80: 1: 20. Then the organic solvent was removed with rotavapor to generate a thin lipid film on the round-bottomed flask at 50 ℃ for 1 h until the solvent was completely evaporated. Subsequently, the dried lipid film was hydrated with 3 mL of ammonium sulfate solution (200 mM), and then the liposomes was obtained via an extrusion process by mini-extruders using 200 nm and 100 nm polycarbonate membranes. The free ammonium sulfate was removed by dialysis method (3500 Da). DOX (drug:lipid = 1:10, w/w) was subsequently mixed with the liposomes suspension, and the free DOX was removed by dialysis, the DOX/HMME@FA-Lipo was obtained ultimately. PEG2000-DSPE without folic acid was used instead of FA-PEG2000-DSPE to prepare folate-free targeting liposomes DOX/HMME@Lipo. In addition, as a control group,

the saturated cationic liposomes DOX/HMME@FA-dLipo was prepared via the same method using DABD and DSPE instead of DOTAP and DOPE.

Fabrication of erythrocyte membrane-derived hybrid nanovesicles (DOX/HMME@FA-NL)

In order to verify the membrane fusion process between NEM and the cationic liposomes, the Förster resonance energy transfer (FRET) study was conducted. Firstly, NBD-PE (Ex/Em = 460/535 nm) / Rhod-PE (Ex/Em = 560/583 nm) doped liposomes (NR-Lipo) was prepared flow the same method as above, the molar ratio of DOTAP, DOPE, cholesterol, FA-PEG2000-DSPE, NBD-PE and Rhod-PE was 75: 100: 80: 1: 2: 2. Then NR-Lipo was added to the NEM supernatant solution at the phospholipid molar ratios (NR-Lipo: NEM) of 1:0.5, 1:1, 1:2 and 1:4, and incubated at 45 ℃ for 30 min. In addition, NR-Lipo and NEM were incubated at 45 ℃ for 0, 5, 15 and 30 min at the phospholipid molar ratio of 1:1. The fluorescence spectrum of each sample was detected between 500 and 650 nm using an excitation wavelength of 460 nm on a FluoroMax-4 spectrofluorometer (HORIBA, USA).

To fabricate DOX/HMME@FA-NL, NEM and DOX/HMME@FA-Lipo were incubated at 45°C for 30 minutes at a phospholipid ratio of 1:1, and then the obtained DOX/HMME@FA-NL was separated and purified by size exclusion chromatography. According to the same fusion strategy, the control groups HMME@FA-dNL, DOX/HMME@NL and DOX/HMME@FA-dNL can be prepared respectively.

Fluorescence colocalization analysis

In order to further confirm the membrane fusion process, NEM were incubated in the presence of green fluorescent dyes DiO (1.0×10^{-5} M) (Ex/Em = 488/520 nm) for 30 min at 37 °C, then centrifuged at 20,000 g for 60 min to remove free dye, and obtained DiO-labeled NEM. Next,

the Rhod-PE labeled cationic liposomes (R-Lipo) was prepared flow the same method as above. Finally, DiO-labeled NEM were incubated with R-Lipo at 45°C for 30 minutes, the fluorescence co-localization was observed using a FV1000-IX81 CLSM (OLYMPUS, Japan).

SDS-PAGE protein analysis

For SDS-PAGE protein analysis, NEM, DOX/HMME@FA-Lipo and DOX/HMME@FA-NL mixed with SDS-PAGE sample loading buffer were heated at 100 °C for 10 min. Then the samples were loaded into a 12% Bis-Tris gel and run at 110 V by a DYY-7C electrophoresis system (Liuyi Instrument, China). SDS-PAGE ruler prestained protein ladder (Thermo Fisher, USA) was used to track protein migration. The resultant gel was stained in Coomassie Blue 2 h at room temperature and washed overnight for subsequent imaging.

Detection of reactive oxygen species (ROS)

DPBF was used to detect the generation of ROS under ultrasound. The solutions of DOX/HMME@FA-NL and DOX/HMME@FA-dNL at the same HMME concentration (10 μg mL^{-1}) were mixed separately with 30 μL DPBF solution (2 mg mL^{-1}) and then exposed to an 1MHz ultrasound at a power density of 1.5 W cm^{-2} for different times. The UV-vis absorption spectra were detected between 350 and 550 nm using a TU-1901 UV-vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China)

Ultrasound responsive controlled drug release study

For studying the effect of ultrasound on DOX releasing, the DOX/HMME@FA-NL with a HMME concentration of 20 μg mL^{-1} divided into four groups were placed into dialysis bags (3500 Da). Then, the packaged dialysis bags were soaked in 40 mL of PBS with gentle shaking at 37 ℃. After 2 h, the solution in the dialysis bags were exposed to ultrasound irradiation with

different power respectively (0, 0.5, 1.0 and 1.5 W cm^{-2}) for 5 min. The DOX content outside the dialysis bags were detected using fluorescence spectrophotometer. In addition, the DOX release behavior from DOX/HMME@FA-NL and DOX/HMME@FA-dNL with or without ultrasound irradiation (1.5 W cm^{-2}) for 5 min at 0 h, 2 h, and 4 h were also investigated as the above methods.

Cell Culture

Murine hepatocellular carcinoma cell line H22 and human umbilical vein endothelial cells (HUVEC) were obtained from China Center for Type Culture Collection (CCTCC). Cells were cultured in RPMI 1640 culture medium supplemented with 10% FBS at 37 °C in the presence of 5% CO_2.

Cellular Uptake Assay

H22 cells and HUVEC cells were seeded in 6-well plates at a density of 1.5×10^5 cells per well overnight. Then the culture medium was replaced with DOX/HMME@FA-NL solution at a DOX dose of 1.0 μg mL^{-1}, and incubated for 1, 6 and 12 h. Subsequently, the cells were fixed with 4% paraformaldehyde for 20 min and stained with DAPI (5 μg mL^{-1}) before imaging using FV1000-IX81 CLSM. For quantification, the cells were collected and resuspended in the flow tube with 350 μL of PBS. The quantification of intracellular fluorescence intensity of DOX was measured on CytoFLEX flow cytometer (Beckman, USA).

Intracellular ROS detection

The intracellular ROS level was detected via CLSM and flow cytometry, and DCFH-DA was employed as a ROS probe. Cells were incubated with HMME@FA-NL and DOX/HMME@FA-NL at the same HMME dose of 5.0 μg mL^{-1} for 6 h, respectively. Then the

cells were washed with PBS and incubated with fresh medium containing 10 μM DCFH-DA for further incubation 30 min. Subsequently, the cells were washed with PBS for 3 times and then irradiated by ultrasound with the power of 1.5 W cm^{-2} for 1 min. Finally, after incubating for 30 min, the cells were fixed with 4% paraformaldehyde and observed by CLSM, or collected for flow cytometry analysis.

In vitro cytotoxicity

H22 cells were seeded in 96-well plates and then treated with DOX/HMME@FA-NL at HMME concentrations from 1.25 to 20.0 μg mL^{-1} or DOX concentrations from 1.00 to 16.0 μg mL^{-1} for 24 h without ultrasound irradiation. Another treated 96-well plates with the same protocols intended to evaluate the sonodynamic toxicity were incubated for 6 h firstly, then irradiated by ultrasound with the power of 1.5 W cm^{-2} for 1 min and incubated for another 18 h. Cell viability was measured by the MTT method according to the manufacturer's protocol. To confirm the effect of ultrasound-responsive drug release on cytotoxicity, the cytotoxicity of HMME@FA-NL, DOX/HMME@FA-dNL, DOX/HMME@NL and DOX/HMME@FA-NL at the same HMME dose of 10.0 μg mL^{-1} were also evaluated as the above methods.

Tumor mouse model

Male BALB/c mice were purchased from the Center for Disease Control and Prevention in Hubei Province, China. And all animal studies were performed according to the guidelines approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology (HUST, Wuhan, China). The H22 tumor-bearing BALB/c mice were obtained by subcutaneous injection of 2×10^6 H22 cells into the backside of male BALB/c mice. Tumors were allowed to grow for a week, reaching about 100

mm^3, before further studies were performed.

Hemolysis assay

The blood compatibility of DOX/HMME@FA-NL was evaluated via hemolysis assay. Fresh blood samples were taken from the orbit of male BALB/c mice into a heparin tube at 4 °C. Then the plasma was removed from erythrocyte by centrifugation at 800 g for 10 min. The obtained erythrocyte was incubated with DOX/HMME@FA-NL at different HMME concentrations (3.125, 6.25, 12.5, 25, 50, and 100 μg mL^{-1}) for 2 h. Finally, the mixtures were centrifuged at 800 g for 10 min, and the absorbance of the supernatants at 575 nm were measured by using UV-vis absorption spectrum. Erythrocyte incubated with deionized water and saline was used as positive control (+) and a negative control (-), respectively.

Tumor targeting study

Firstly, erythrocyte membrane-derived hybrid nanovesicles with or without FA targeting group were labeled by the near-infrared fluorescent dye DiR respectively as the above methods. Then H22 tumour-bearing BALB/c mice were randomly divided into two groups and intravenously injected with DiR@FA-NL and DiR@NL. The accumulation of DiR@FA-NL and DiR@NL in tumour tissues were observed on an IVIS Lumina XR System (Caliper Life Sciences, USA) The excitation and emission wavelengths of fluorescence were 730 and 790 nm.

Anti-tumour assay *in vivo*

H22 tumour-bearing BALB/c male mice were randomly divided into eight groups: PBS group, US group, DOX/HMME@NL group, DOX/HMME@NL + US group, DOX/HMME@FA-dNL group, DOX/HMME@FA-dNL + US group, DOX/HMME@FA-NL group and DOX/HMME@FA-NL + US group. The mice were administered via the tail vein at a dose of

5 mg kg^{-1} of HMME or 4 mg kg^{-1} of DOX. After 48 h post-injection, the tumors were irradiated with or without ultrasound irradiation (1.5 W cm^{-2}, 5 min). Mice weight and tumor volume of each group were measured every day. The tumor volume (V, mm^3) was calculated to be V = ab2/2, where a and b represent the length and width of tumors, respectively. All mice were sacrificed at the end of the treatment, and their tumors were resected and imaged.

Statistical Analysis

Data were analyzed by Student's t-test, *p-values of <0.05 were considered significant, and **p-values of <0.01 were considered highly significant.

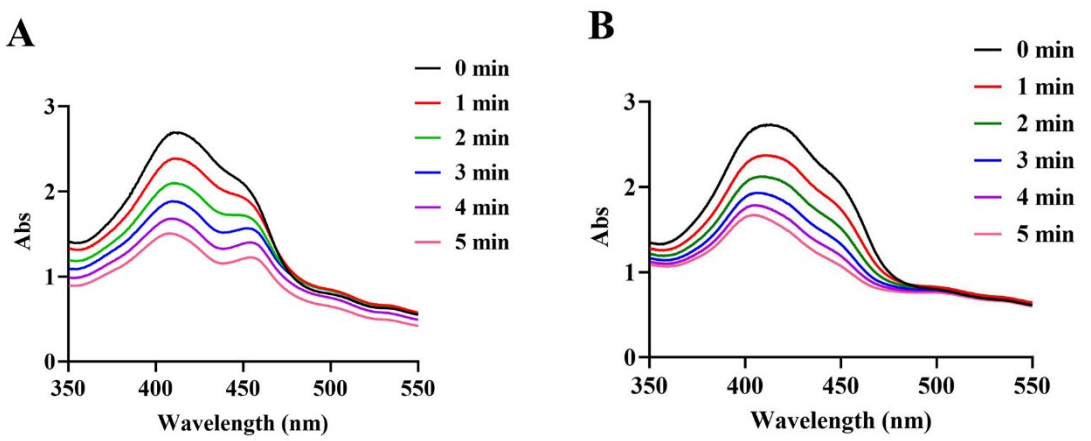

Fig. S1 Time-dependent DPBF absorption spectra of DOX/HMME@FA-dNL (A) and DOX/HMME@FA-NL (B) under US irradiation (1MHz, 1.5 W cm-2) for varied durations.

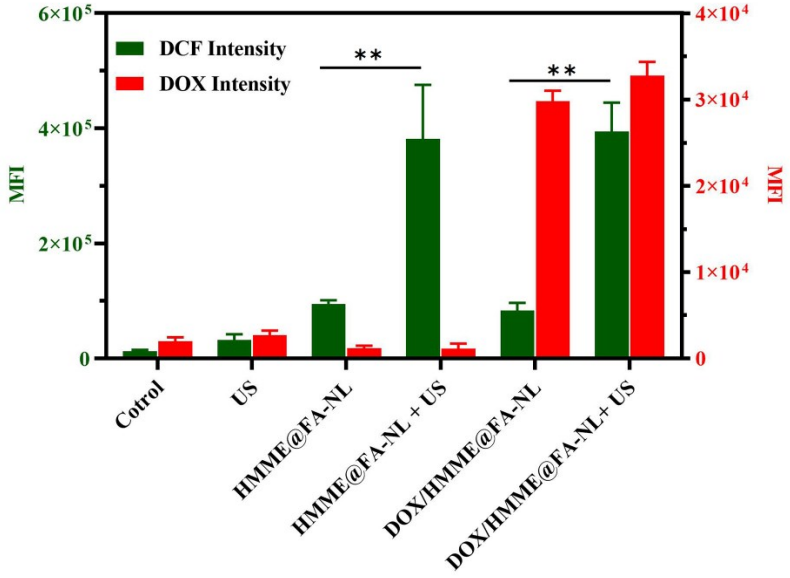

Fig. S2 Mean fluorescence intensity of DCF and DOX fluorescence for H22 cells after treatment with Control, US, HMME@FA-NL, HMME@FA-NL+US, DOX/HMME@FA-NL and DOX/HMME@FA-NL+US. *$P < 0.05$, **$P < 0.01$

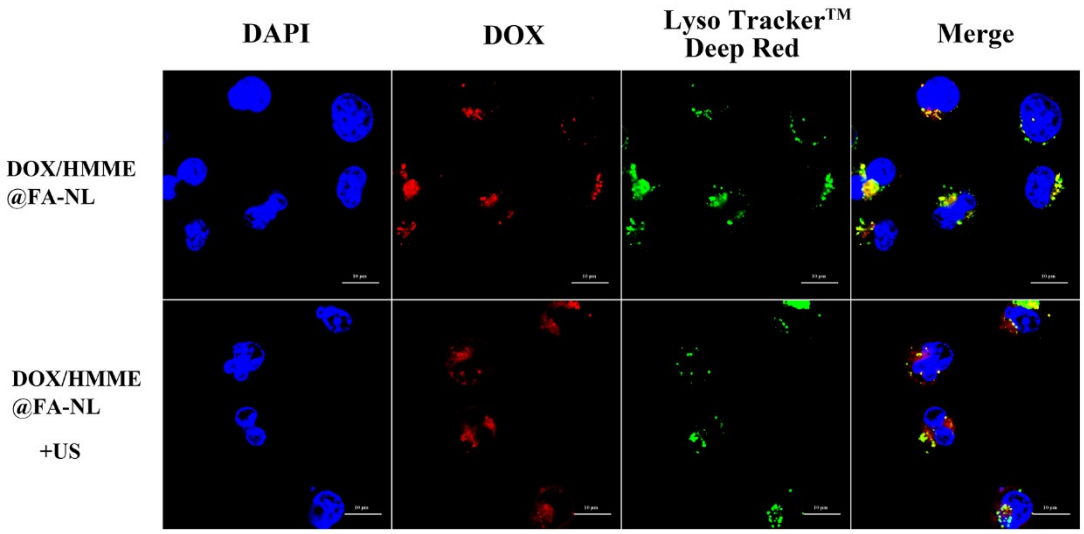

Fig. S3 CLSM images of H22 cells incubated with DOX/HMME@FA-NL for 6 h with or without US irradiation (1.5 W cm^{-2}, 1 min). Cell nucleus were labeled with DAPI (blue). Lysosomes were labeled with LysoTracker™ Deep Red (green)

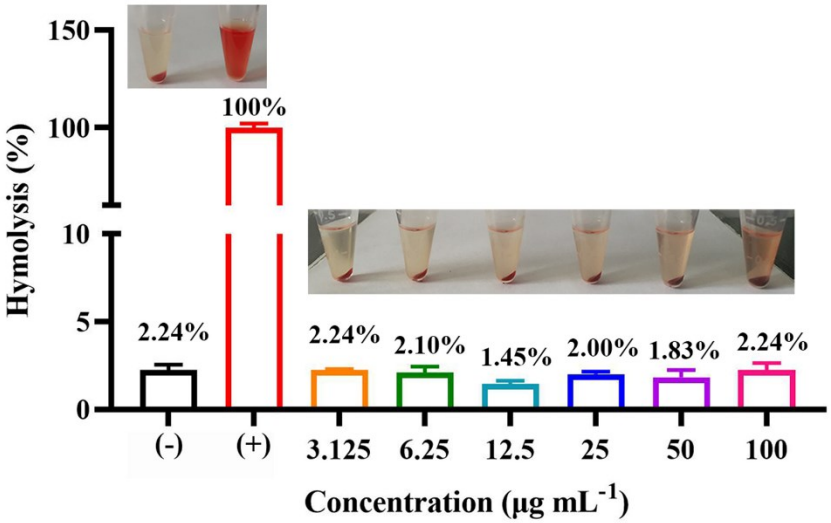

Fig. S4 Hemolysis percentages and photographs of DOX/HMME@FA-NL in physiological saline at concentrations of 3.125, 6.25, 12.5, 50 and 100 μg mL^{-1}. Erythrocyte cells respectively incubated with deionized water and physiological saline were used as the positive (+) and negative (-) control

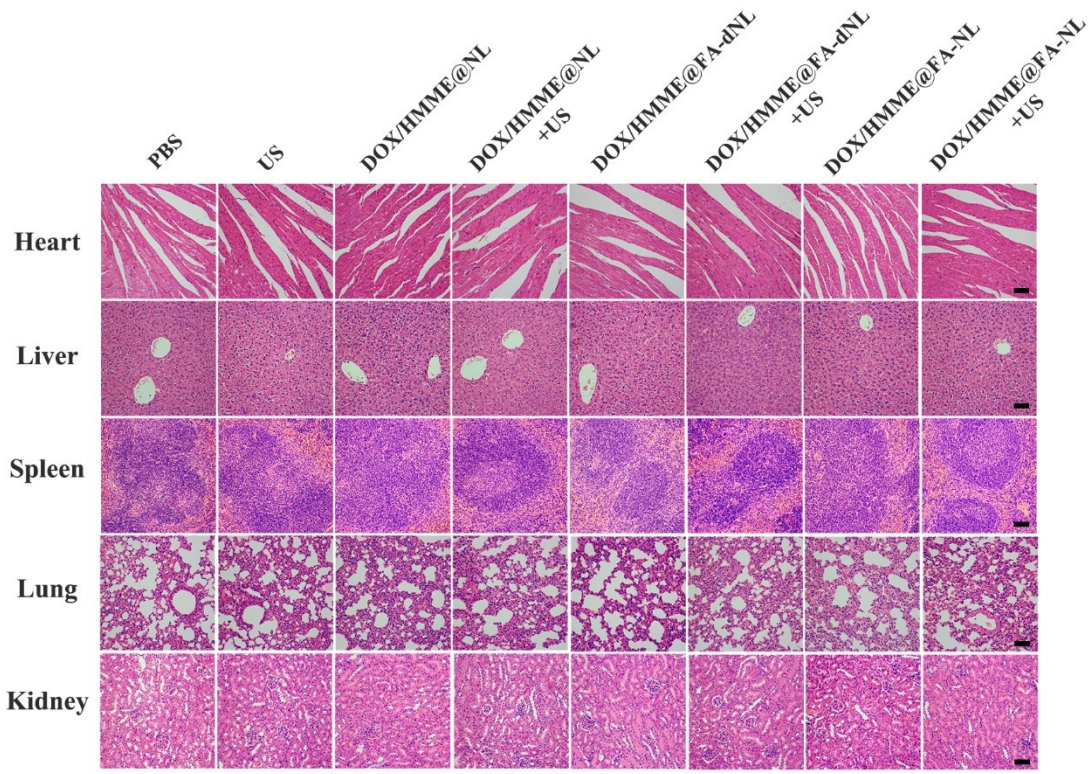

Fig. S5 H&E staining images of heart, liver, spleen, lung, and kidney after the indicated treatments. Scale bars = 20 μm.