Supporting Information

Artificial chimeric exosomes for anti-phagocytosis and targeted cancer therapy

Kai-Long Zhang,[a] Ying-Jie Wang,[a] Jin Sun,[b] Jie Zhou,[a] Chao Xing,[a] Guoming Huang,[b] Juan Li*[ac] and Huang-Hao Yang*[a]

[a] MOE Key Laboratory for Analytical Science of Food Safety and Biology, Fujian Provincial Key Laboratory of Analysis and Detection Technology for Food Safety, State Key Laboratory of Photocatalysis on Energy and Environment, College of Chemistry, Fuzhou University, Fuzhou 350002, P. R. China

[b] College of Biological Science and Engineering, Fuzhou University, Fuzhou 350116, P. R. China

[c] Institute of Molecular Medicine, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Jiao Tong University, Shanghai, 200240, P. R. China

*Correspondence to [email: lijuan@fzu.edu.cn], [email: hhyang@fzu.edu.cn]

Experiment Sections

Chemicals. Indocyanine green (ICG) and doxorubicin (DOX) were purchased from Sigma-Aldrich (USA). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and cholesterol were purchased from Avanti Polar Lipids Inc. (USA). BCA protein assay kit was obtained from Beyotime (Shanghai, China). All other organic reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). All solutions were prepared using ultrapure water (18.2 MΩ resistivity) as the solvent.

Cell culture and animals. Human breast carcinoma MCF-7 cells, Human epithelial carcinoma HeLa cells and mouse macrophage RAW264.7 cells were purchased from ATCC (American Type Culture Collection). MCF-7 cells were cultured in RPMI-1640 medium (Gibco) with 10% FBS (Gibco) at 37 °C in a humidified atmosphere with 5% CO_2. HeLa cells and RAW264.7 cells were grown in DMEM containing 10% FBS, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate at 37 °C in a humidified incubator with 5% CO_2. Animals received care in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Institute of Materia Medica, CAS. Female BALB/c nude mice and female ICR mice were purchased from Shanghai Experimental Animal Center (Shanghai).

Extraction of membrane proteins from MCF-7 cancer cells and red blood cells. MCF-7 cancer cell membranes and red blood cell membranes were achieved by hypotonic lysis, mechanical membrane disruption and then differential centrifugation,

according to a previously reported extrusion approach. To obtain membrane proteins, the membrane material was added to membrane protein extraction reagents and incubated at 4 °C for 1 h. The hybrid membrane proteins contain MCF-7 cancer cell membrane proteins and red blood cell membrane proteins at 1:1 mass ratio.

Preparation of biomimetic artificial chimeric exosomes (ACEs). ACEs were prepared by the thin layer evaporation (TLE) method and extrusion process. Briefly, 10.8 mg DPPC, 2.4 mg DSPC, 7.2 mg DOPC, 1.2 mg cholesterol (Avanti Polar Lipids) and 0.25 mg ICG were dissolved in 2 mL organic mixture containing 1.5 mL chloroform and 0.5 mL methanol. Then the solvent was evaporated through an IKA Rotary Evaporator RV10 (IKA, Germany) to form a thin film. Films were hydrated with 70 μg of hybrid membrane proteins, MCF-7 cancer cell membrane proteins, or red blood cell membrane proteins dispersed in PBS to assemble ACEs, AMEs or AREs, respectively, by heating and vortexing at 45 °C for 20 min. Conventional liposomes were prepared as described above with PBS alone. Lipid suspension was then extruded 20 times through cellulose acetate membranes (100 nm pore-size) at 45 °C, using a mini-extruder (Avanti Polar Lipids Co. Ltd., USA). The encapsulation of DOX was achieved by the $(NH_4)_2SO_4$ gradient method. The obtained vesicles were then dialyzed overnight through a 1000 kDa dialysis bag to eliminate raw material and unincorporated proteins.

Characterization of biomimetic artificial chimeric exosomes (ACEs). Vesicle size and zeta potential of liposomes, AREs, AMEs and ACEs were measured using a Zetasizer Nano ZS instrument (Malvern, England). The morphology of liposomes,

AREs, AMEs and ACEs was determined using an FEI Tecnai G20 microscope (FEI NanoPorts, USA) after staining with 3% (w/v) phosphotungstic acid. Encapsulation efficiency was determined using a Shimadzu LC-20AT HPLC system (Japan) with a reverse phase C-18 Ultra sphere ODS column (250 × 4.6 mm, 5 mm, Beckman, USA).

Atomic Force Microscopy (AFM) analysis. In order to stabilize the vesicles, samples, as reported in the main text, were prepared on a mica surface in a 0.1% APTES coating to avoid their collapse, followed by performing AFM analysis with the Bruker Mulitmode 8 microscope.

Analysis of membrane protein composition. The membrane protein composition of red blood cell membrane, AREs, MCF-7 cell membrane, AMEs and ACEs was characterized by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method and Western blotting assay. Briefly, the membrane proteins from different samples were quantified by the BCA assay kit (Beyotime, China), and samples with equal protein amounts were added to 10% SDS-polyacrylamide gel, using SDS-PAGE electrophoresis buffer as running buffer in the Mini-PROTEAN Tetra System (Bio-Rad, CA, USA) based on the manufacturer's instructions to separate different molecular weights of proteins. Subsequently, protein was stained by Coomassie brilliant blue and imaged. For Western blotting assay, proteins on the gel were then transferred to PVDF membrane using the Mini-PROTEAN Tetra System (Bio-Rad) at 100 V for 1 h. Then the membrane was blocked and incubated with the primary antibodies of anti-CD47 (Abcam, USA), anti-EpCAM (Abcam, USA), anti-N-cadherin (Abcam, USA), and anti-galectin-3 (Abcam, USA), followed with horseradish

peroxidase-conjugated anti-rabbit IgG. Protein signals were measured by a gel imaging system (ChemiDocTM Touch, Bio-Rad, America).

The simultaneous presence of RBCs membrane proteins and MCF-7 cell membrane proteins on each individual ACE was examined by high-sensitivity flow cytometer (HSFCM). After extraction, RBCs membrane proteins and MCF-7 cell membrane proteins were respectively labelled with TAMRA-SE dyes and FITC Dyes by using Labeling Kit (G-Biosciences, USA). The fluorescent labelled ACEs was prepared with the hybrid fluorescent labelled membrane proteins and then evaluated by a high-sensitivity flow cytometer. In addition, ACEs were incubated with transferrin-conjugated gold (5 nm) at 37 °C for 1h, and ACE morphology was determined by TEM after staining with 3% (w/v) phosphotungstic acid.

Stability test of ACEs in PBS and FBS. Liposomes, AREs, AMEs and ACEs solution (10 mg/mL) was respectively prepared and mixed with PBS and 20% FBS. The mixture was incubated at 37 °C, and vesicle size was determined at intervals using DLS.

***In vitro* drug release.** Liposomes, AREs, AMEs and ACEs fabricated as described above were dissolved in PBS, sealed in dialysis tubes (MWCO = 3.5 kDa), and then dialyzed in 57 mL of PBS with 2% Tween 80. At predetermined time points, a 100 μL aliquot of the solution outside the dialysis tube was withdrawn, and DOX concentration was determined using HPLC analysis.

***In vitro* targeting of MCF-7 cells.** MCF-7 cells and HeLa cells (200 μL of medium, 2 × 10^4 cells/well) were seeded in eight-well chambered cover glasses and incubated under the condition of 5% CO_2 at 37 °C for 24 h. The old medium was replaced with

medium containing liposomes, AREs, AMEs and ACEs at a DOX dose of 10 μM. After 2 h of incubation, cells were washed three times with PBS, fixed with 4% paraformaldehyde solution for 20 min, stained with Hoechst 33258 for 10 min, and then rinsed three times with PBS. Finally, cellular uptake was observed by a CLSM microscope (Nikon A1, Japan).

***In vitro* cellular uptake of macrophage RAW264.7 cells.** To evaluate the cellular uptake of mouse macrophage RAW264.7 cells, the cells were incubated with liposomes, AREs, AMEs and ACEs at a DOX dose of 10 μM for 2 h. Subsequently, the cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min before imaging using a CLSM microscope (Nikon A1, Japan).

***In vitro* cytotoxicity assay.** In a typical experiment, MCF-7 cells were seeded in 96-well plates and then incubated with 100 μL of varying concentrations of liposome, AREs, AMEs and ACEs without DOX for 24 h at 37 ^{o}C in a humidified 5% CO_2 atmosphere. Cell viability was determined by the CCK-8 method according to the manufacturer's protocol. The *in vitro* cytotoxicity assay was performed using a similar procedure, except that MCF-7 cells were incubated with liposomes, AREs, AMEs and ACEs, and the final concentration of DOX in all formulations was in the range of 10 to 1×10^{-4} μg/mL.

***In vivo* imaging.** The MCF-7 xenograft tumor model was established by subcutaneous injection of 1×10^7 MCF-7 cells into the back of the hind leg of female nude mice. When the volumes of MCF-7 tumors reached 100-200 mm^3, the nude mice were randomly divided into four groups and injected with liposomes, AREs, AMEs and

ACEs (200 μL, 200 μg/mL ICG) via the tail vein, respectively. The fluorescence signals of ICG were obtained by an SI Imaging Amix small animal imaging system (Spectral Instruments Imaging Co., USA) (ex: 745 nm; filter: 830 nm).

***In vivo* biodistribution.** In the drug biodistribution experiments, female BALB/c nude mice were subcutaneously injected with 1×10^7 MCF-7 cells on the back of the hind leg. When the tumor volume reached 200 mm^3, the mice were randomly divided into four groups (n = 3) and intravenously injected with liposomes, AREs, AMEs and ACEs at a DOX dose of 6 mg/kg. Twenty-four h after treatment, the mice were sacrificed, and the major organs, including heart, liver, spleen, lung, kidneys, and tumor, were collected for DOX analyses. The organs or tissues were washed with 0.9% saline before being weighed and then cut into small pieces and homogenized. Two hundred μL of ice-cold acetonitrile containing 0.5% acetic acid were used to extract DOX, and then the mixture was centrifuged at 13,000 rpm for 10 min. Finally, the supernatant was concentrated and subjected to HPLC to determine DOX levels, and the corresponding DOX tissue concentrations were calculated accordingly.

***In vivo* pharmacokinetics.** In the plasma pharmacokinetic study, female ICR mice were randomly assigned to 4 groups (n = 3) and intravenously injected with liposomes, AREs, AMEs and ACEs, respectively, at a DOX dose of 6 mg/kg. Blood samples were collected into heparinized tubes at 15 and 30 min and 1, 2, 4, 8, 12 and 24 h after administration and centrifuged at 5,000 rpm and 4 °C for 10 min to harvest plasma samples. Acetonitrile was added to the samples to precipitate all proteins and extract

the content of the drug. After centrifugation, the organic layer was collected and concentrated, followed by examination of the DOX concentration using HPLC analysis.

***In vivo* therapy.** To develop the human tumor xenograft, MCF-7 cells were harvested and implanted into the back of the hind leg of nude mice (1×10^7 cells). When the tumors reached a mean volume of approximately 80 mm^3, mice were randomly divided into five treatment groups (n = 5): liposomes, AREs, AMEs, ACEs (DOX dose of 5 mg/kg) and the control group (PBS). The treatment was implemented by i.v. injection every 3 days for 5 times. Animal weight and tumor volume were measured, and tumor volume was calculated as tumor volume = $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. Tumor growth curves were plotted using the average tumor volume versus days after the first treatment. All mice were sacrificed at the end of the experiment, and their tumors were resected and imaged.

H&E Histology. At the end of the experiment, tumor-bearing mice were sacrificed, and the tumor, heart, liver, spleen, lung, and kidney were collected for H&E assays to evaluate the histopathologic toxicity of the tissues.

Statistical Analyses. Data were presented as mean ± standard deviation (SD). Statistical comparisons were performed using unpaired Student's *t* test for two group comparisons. Differences were considered statistically significant when $p < 0.05$.

Supporting Tables:

Table S1. DOX and ICG encapsulation efficiency (EE) and membrane protein incorporation efficiency (IE) of liposomes, AREs, AMEs and ACEs. (Means ± SD, n = 3)

Types of samples	DOX EE (%)	ICG EE (%)	IE (%)
Liposomes	93.6±1.2	90.2±0.8	—
AREs	91.2±2.0	83.4±1.3	45.6±1.4
AMEs	91.4±2.3	84.1±0.9	44.9±2.0
ACEs	90.7±1.8	83.9±1.1	45.3±2.2

Table S2. Pharmacokinetic parameters of DOX following intravenous administration of liposomes, AREs, AMEs and ACEs at the dose of 6 mg/kg (n =3 for each administration).

Parameters	Liposomes	AREs	AMEs	ACEs
$t_{1/2\alpha}$ (h)	0.152	0.514	0.161	0.393
$t_{1/2\beta}$ (h)	0.186	30.507	1.547	17.716
V1 (L/kg)	0.059	0.124	0.059	0.12
CL1 (L/h/kg)	0.062	0.012	0.062	0.012
$AUC_{(0-t)}$ (mg/L*h)	118.736	354.398	167.272	318.517
$AUC_{(0-\infty)}$ (mg/L*h)	145.486	554.861	207.658	467.871
K_{10} (1/h)	1.052	0.095	1.052	0.1
K_{12} (1/h)	0.005	0.872	1.302	1.015
K_{21} (1/h)	3.749	0.4	0.602	0.687

Supporting Figures:

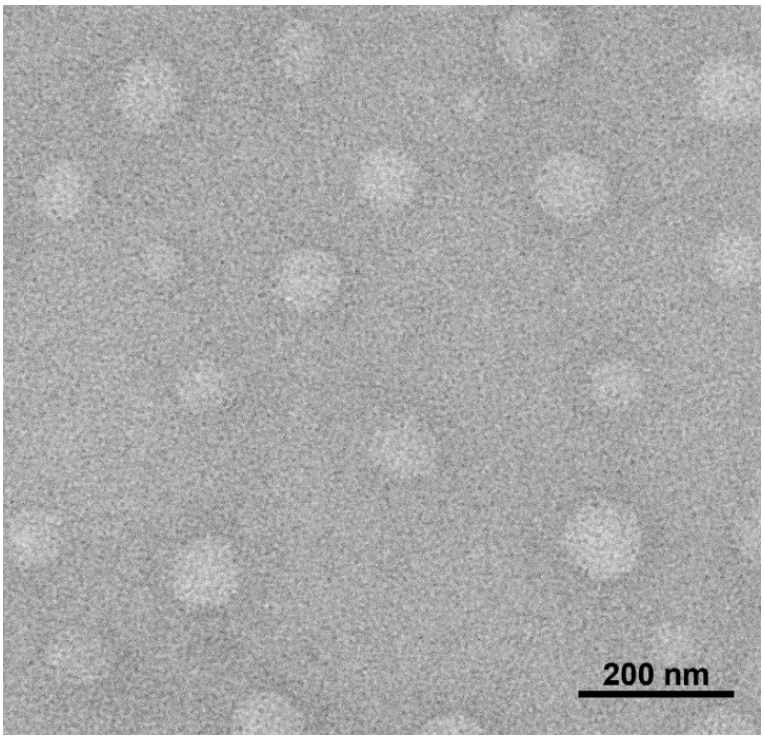

Fig. S1 TEM image of liposomes negatively stained with 3% phosphotungstic acid.

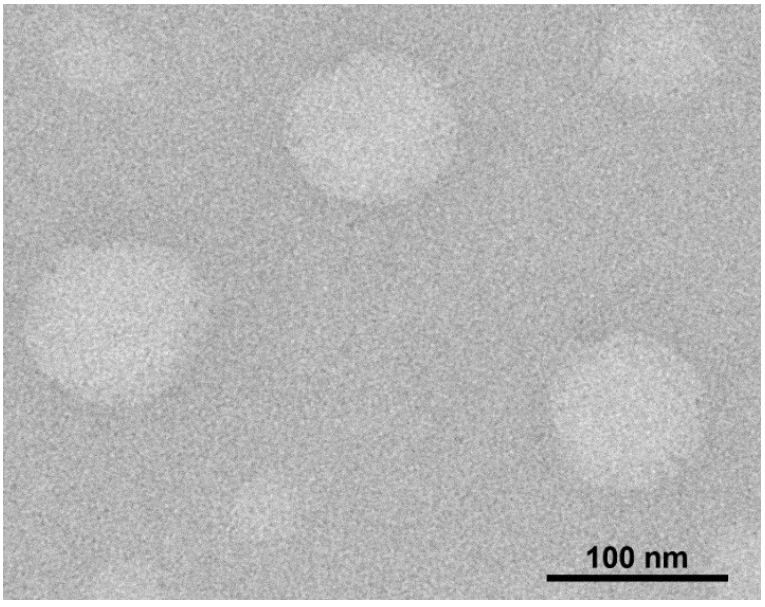

Fig. S2 TEM image of AREs negatively stained with 3% phosphotungstic acid.

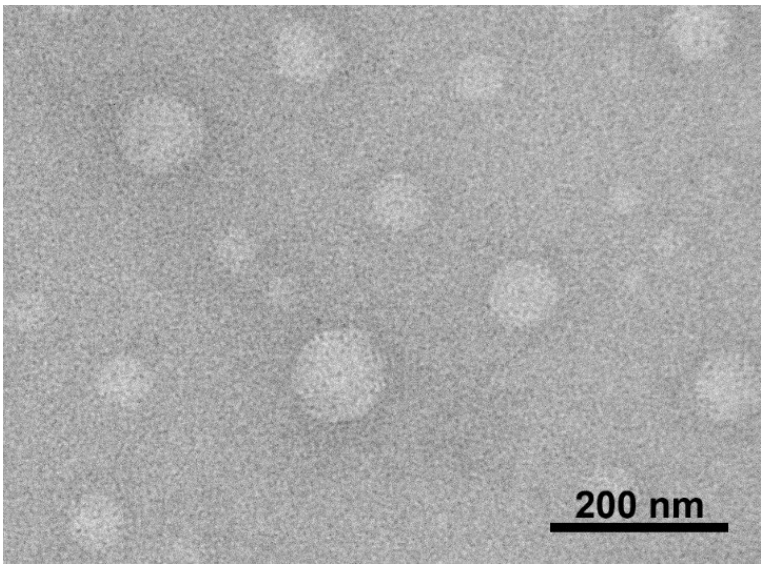

Fig. S3 TEM image of AMEs negatively stained with 3% phosphotungstic acid.

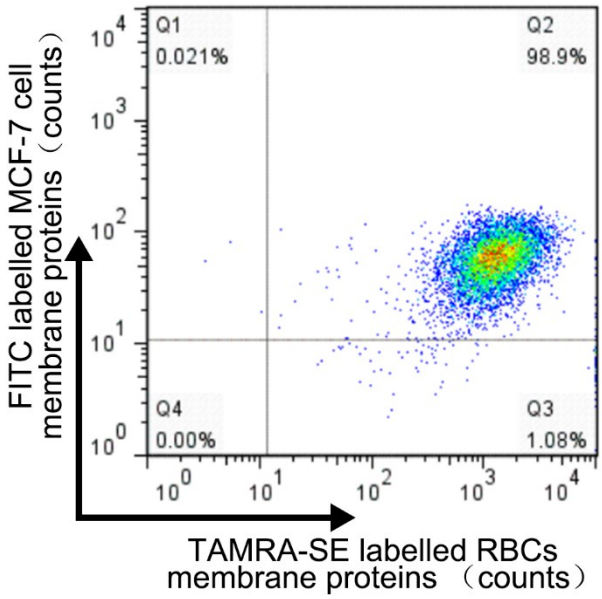

Fig. S4 HSFCM analysis of TAMRA-SE labelled RBCs membrane proteins and FITC labelled MCF-7 cell membrane proteins on each individual ACE.

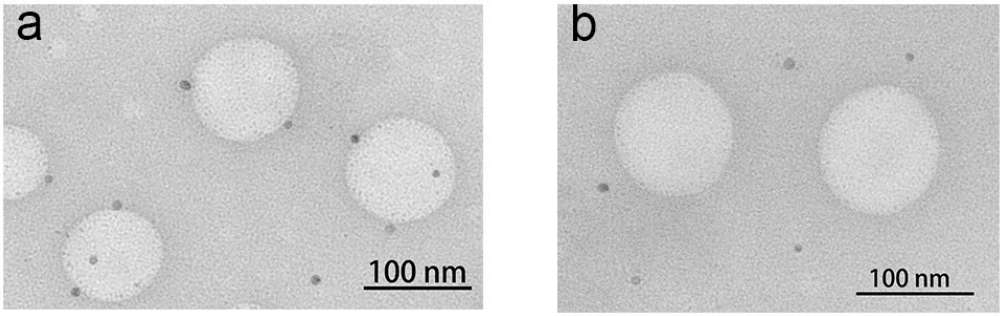

Fig. S5 TEM images of ACEs (a) and liposomes (b) incubated with transferrin-conjugated gold NPs (5 nm) negatively stained with 3% phosphotungstic acid.

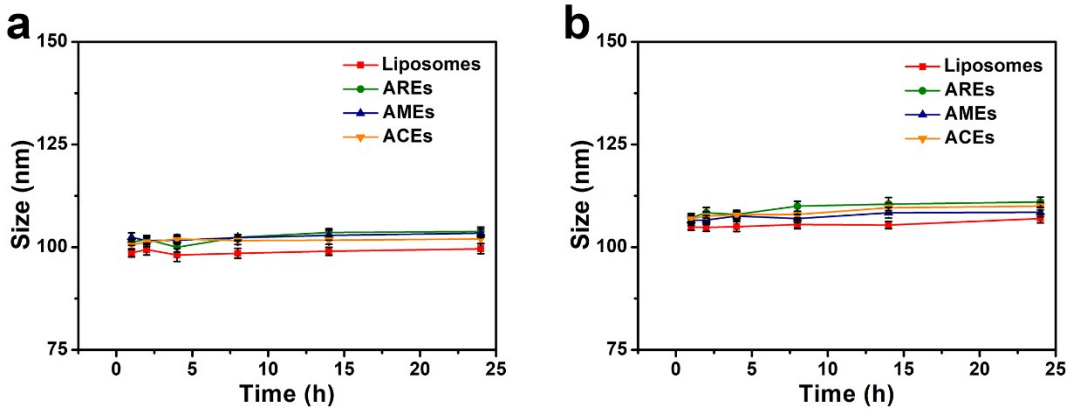

Fig. S6 Hydrodynamic diameter distributions of liposomes, AREs, AMEs and ACEs in PBS (pH 7.4) (a) and 20% fetal bovine serum (FBS) (b) over 24 h. (Means ± SD, n = 3)

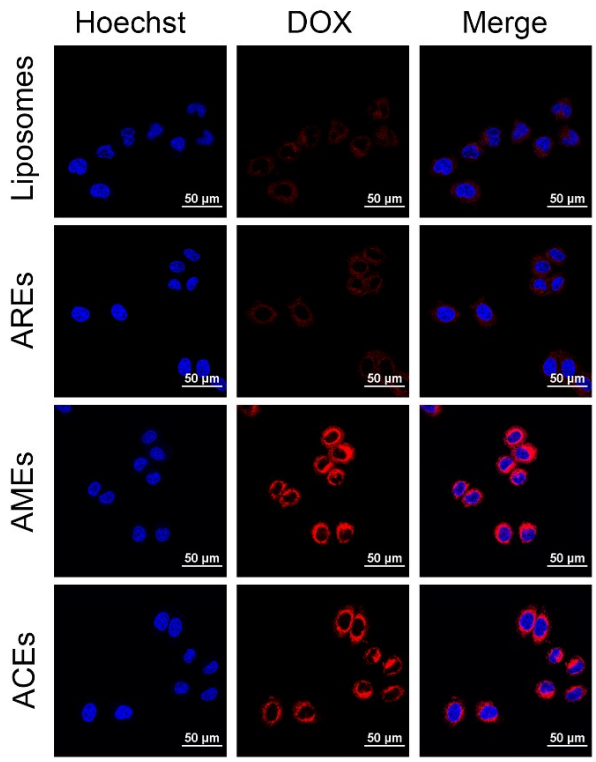

Fig. S7 *In vitro* DOX fluorescence imaging of liposomes, AREs, AMEs and ACEs in MCF-7 cells after 2 h incubation. The nucleus was stained with Hoechst 33342 (blue). The vesicles were loaded with DOX (red).

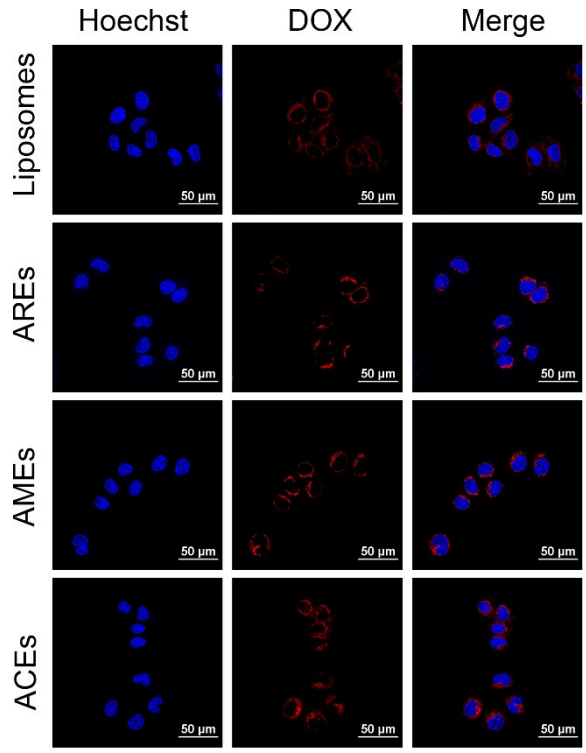

Fig. S8 *In vitro* DOX fluorescence imaging of liposomes, AREs, AMEs and ACEs in HeLa cells after 2 h incubation. The nucleus was stained with Hoechst 33342 (blue). The vesicles were loaded with DOX (red).

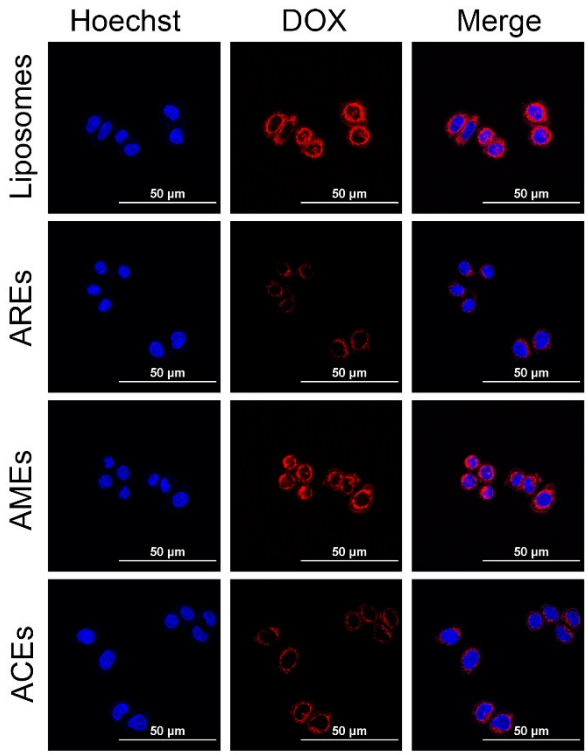

Fig. S9 *In vitro* DOX fluorescence imaging of liposomes, AREs, AMEs and ACEs in RAW 264.7 cells after 2 h incubation. The nucleus was stained with Hoechst 33342 (blue). The vesicles were loaded with DOX (red).

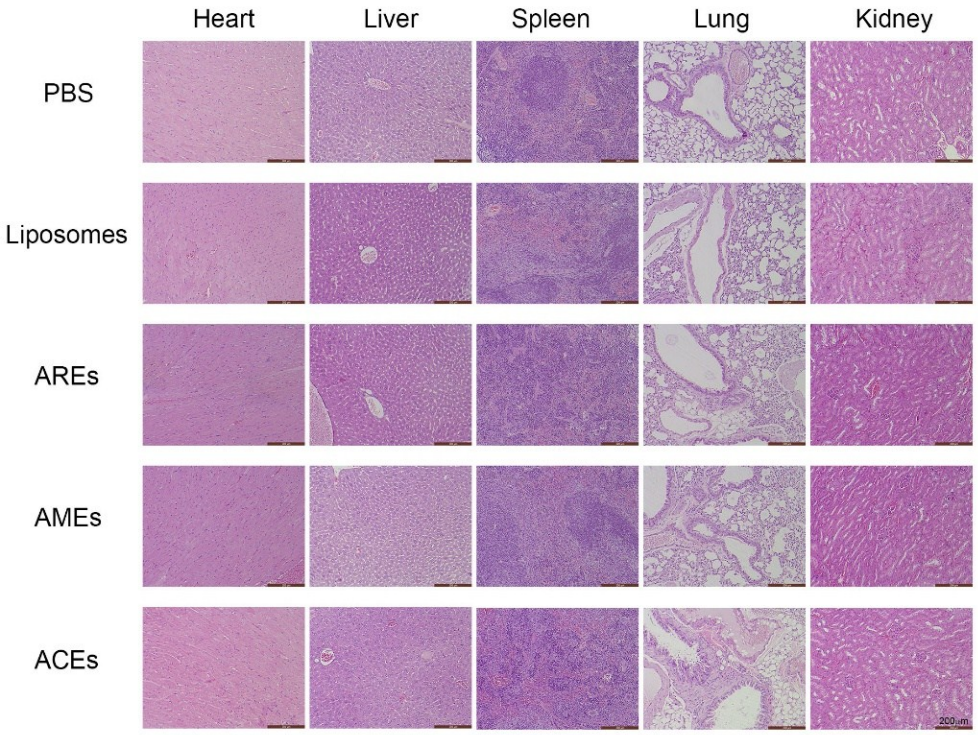

Fig. S10 H&E stained histological images of heart, liver, spleen, lung and kidney after treatment with PBS, liposomes, AREs, AMEs and ACEs. All images share the same scale bar. (Scale bar: 200 μm).