Supporting Information

Acoustics/Thermo Responsive Hybrid System for Advanced Doxorubicin Delivery in Tumor Treatment

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1. Experimental Section

Preparation and characterization of RTSL. cRGD-DSPE-PEG_{2000} was synthesis by a previously described method.[50] DSPE-PEG2000-NHS (24 mg), cRGD (15 mg) and catalytic triethylamine (0.051 mg) were dissolved in 2 mL N,N-Dimethylformamide (DMF). The mixture was stirred overnight at room temperature, and then evaporated to remove DMF. RTSL composed of DPPC:MSPC:cRGD-DSPE-PEG_{2000}: DSPE-PEG_{2000}-biot in the molar ratio of 82:8:8:2 was prepared by a thin lipid film hydration method.[7] Briefly, phospholipids formulation was dissolved in 2 mL chloroform and evaporated at 35 °C to obtain a lipid film. The film was hydrated with 300 mM pH 4. 0 Citric Acid buffer (final concentration of 80 mg mL^{-1} lipid) at 55 °C and emulsified with ultrasonic method for 5 min. The liposomes were extruded 10 times through 100 nm polycarbonate filters with an extrusion (LiposoEasy LE-1 extruder, MORGEC, America).

Encapsulation of DOX into RTSL was performed according to a citrate-based pH-gradient DOX-loading protocol. Initially, the external buffer of the extruded liposomes pH was adjusted to pH 7.4 with 0.5 M Sodium Carbonate buffer (pH 11). Then DOX (5 mg mL^{-1} in water) was added to the liposomes with the initial DOX/lipid ratio at 1:20 (w/w), followed by gently shake and incubation in a 37 ^{o}C water bath for 20 min to allow drug encapsulation. Finally, the cooled RTSLs were passed through Sephadex G-50 columns (prepared in HEPES) to remove free DOX. The size distribution and polydispersity index (PDI) were determined by dynamic laser light scattering (DLS, Zetasizer Nano-ZS, Malvern, UK). Morphology of RTSL was characterized by Transmission Electron Microscope (TEM, Hitachi HC-1, Tokyo, Japan) using a negative staining method. The amount of cRGD peptide was measured by bicinchoninic acid (BCA) assay using an enzyme-labeled instrument (Biotek ELX800, USA). DOX concentration was determined using UV-vis analysis at a wavelength of 480 nm *via* UV-vis spectrophotometry (UV-2600, Shimadzu, Japan). The drug loading content (DLC) was calculated according to the following formula: DLC (%) = ((Mass of loaded drug in RTSL)/(Total mass of RTSL))×100%.

Characterization and photothermal profile of RTSL-IMBs under low-intensity ultrasound and laser exposure. Characteristically changes of RTSL-IMBs before and after low-intensity ultrasound and laser exposure were detected *via* laser scanning confocal microscope, DLS and TEM. Size, PDI and zeta-potential of samples were detected by a Malvern zetasizer device (ZEN3600, UK). To confirm the conjugation between RTSLs and IMBs and visualize the collapse of RTSL-IMBs under US and laser exposure, a drop of the prepared samples was deposited on a slide with a coverslip and observed *via* a Nikon A1 laser scanning confocal microscope (Tokyo, Japan) through TRITC filter (Ex 544 nm/Em 572 nm). To observe morphology of RTSL-IMBs after US and laser exposure, the prepared samples using 2% (v/v) phosphotungstic acid was detected *via* a Hitachi

HC-1 TEM (Tokyo, Japan). The drugs loading content of RTSL-IMBs were determined using a previously described method.[15] *In vitro* temperature change of RTSL-IMBs upon laser irradiation was detected by a thermocouple needle (OMEGA, USA). To assess stability of RTSL-IMBs at 4 ^{o}C, the size at different time points were determined *via* DLS. To determine the fluorescence properties of RTSL-IMBs, samples of RTSL-IMBs and free IR780 (with the same concentration of IR780) were placed in a 24-well plate and tested by In-Vivo Imaging System Fx Pro (Bruker, USA).

Cell culture. Human breast adenocarcinoma cell line MCF-7 was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in dulbecco's modified eagle medium (DMEM; Gibco, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco). Cells were cultured at 37 ^{o}C in 5% CO_2.

In vitro cell attachment experiment. MCF-7 cells (1×10^5 per well) were seeded on the six-well cell culture plate and allowed to grow for 12 h. After medium removed out, 1 mL non-targeted MBs or RTSL-IMBs suspention (1.5×10^7 MBs mL^{-1} in PBS) was added to cells on the wells respectively. Slightly shaking and incubation were necessary for MBs touching the cells. After 4 min' incubation, MCF-7 cells were washed by PBS for 3 times. Finally, 1 mL fresh PBS was retained on the wells to keep free MBs away from cells with the assistance of buoyancy. Cell-attached MBs were quantified *via* light microscope.

Immunohistochemical analysis. At the 15th day after first treatment, tumors were collected and prepared for immunohistochemical analyses of Ki67 and CD34 (Servicebio Technology CO., LTD, Wuhan, China). Microscopic images of the tissue slices were obtained using an optical microscope (Nikon Eclipse 80i, Tokyo, Japan). The ratio of Ki67 positive staining cells was counted at 400× magnification. Microvessel density (MVD), CD34 positive staining, was determined by the number of capillaries per microscopic field (five random fields).

2. Supplemental Table and Figures

Table S1. Characterization of LTSL, RTSL and RTSL-IMBs.

	LTSL	RTSL	RTSL-IMBs
Size（d.nm）	100.2±30.4	92.3±33.0	1436.0±283.4
PDI	0.1	0.1	0.1
Zeta potential（mV）	-12.5±7.2	-9.6±7.0	-
DOX loading	4.6%	4.6%	20.3μg per 10^8 MBs
cRGD content	-	2.2%	9.8ug per 10^8 MBs

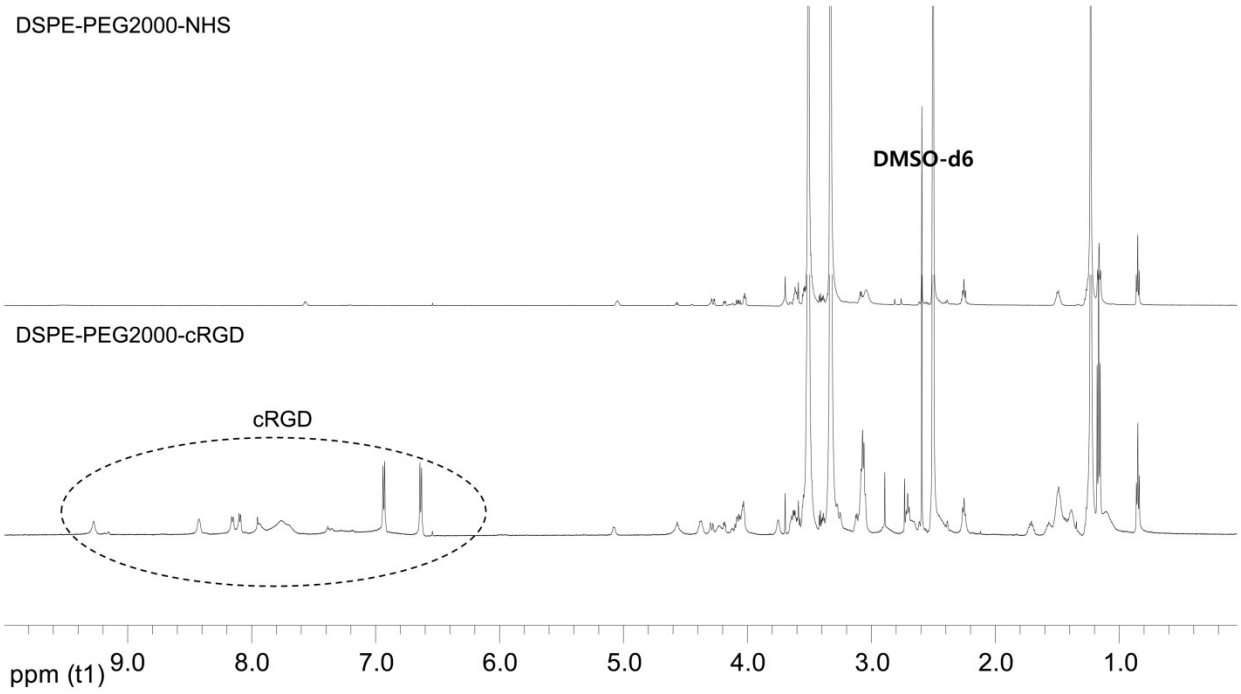

Figure S1. ^{1}H-NMR spectroscopy of cRGD-DSPE-PEG2000 and DSPE-PEG2000.

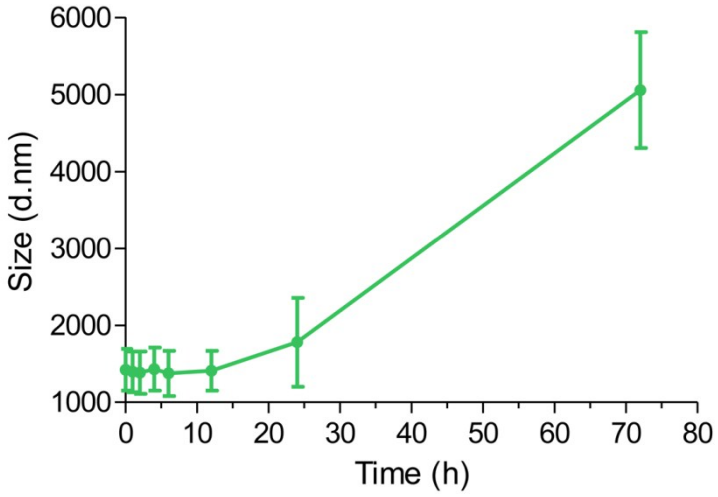

Figure S2. *In vitro* stability of RTSL-IMBs at 4 ^{o}C.

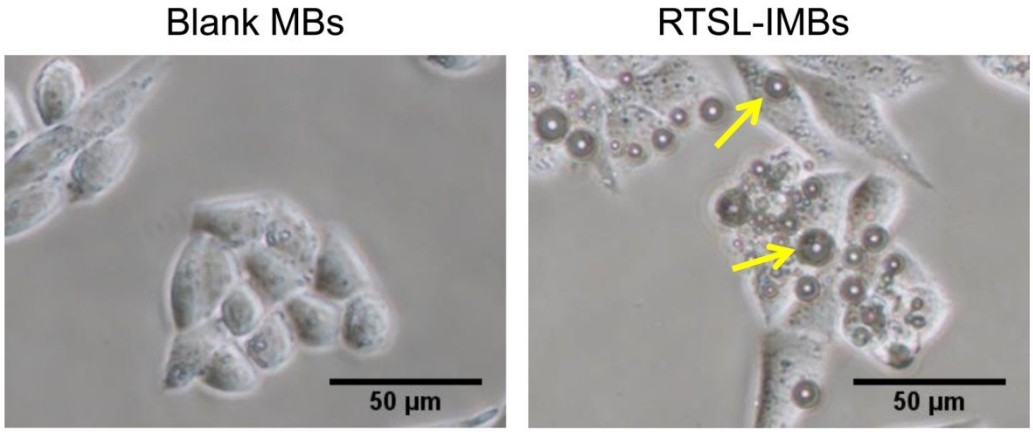

Figure S3. Bright-field micrographs of cell-attached microbubbles (Yellow arrows) in MCF-7 cells.

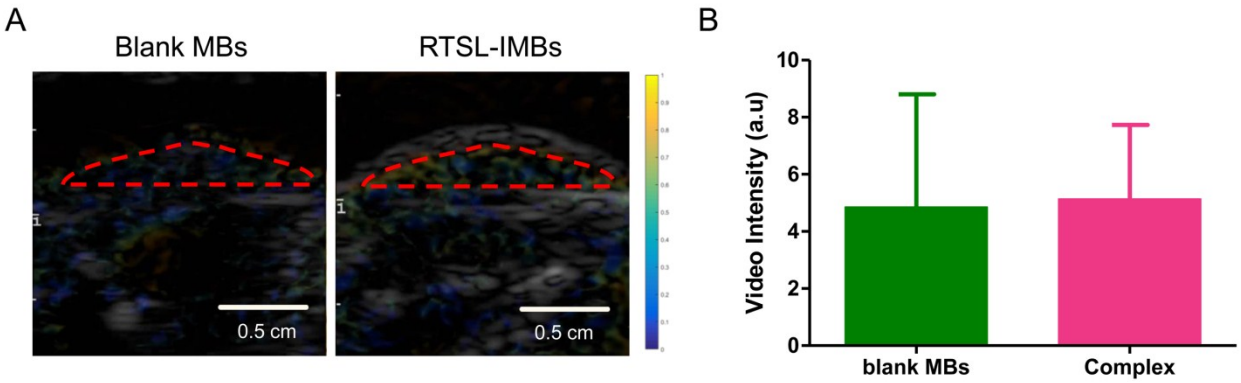

Figure S4. A). Color-coded ultrasound molecular images of normal skeletal muscle, showing difference in video intensity from subtraction of pre- and post-destruction signals by a color map

overlaid on B-mode image. Red dotted line represents the region of hind limb muscles. (Scale bar, 0.5 cm), and corresponding quantitative video intensity analysis of adherent microbubbles B).

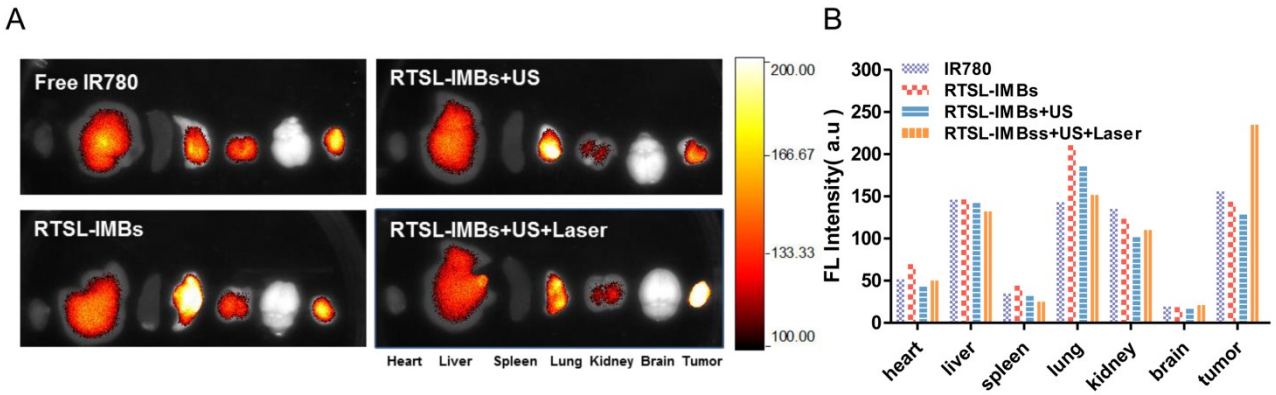

Figure S5. A) Biodistribution of Free IR780 and RTSL-IMBs upon US/US+laser irradiation in major organs and tumors, and corresponding quantification determined by average fluorescence intensity B).

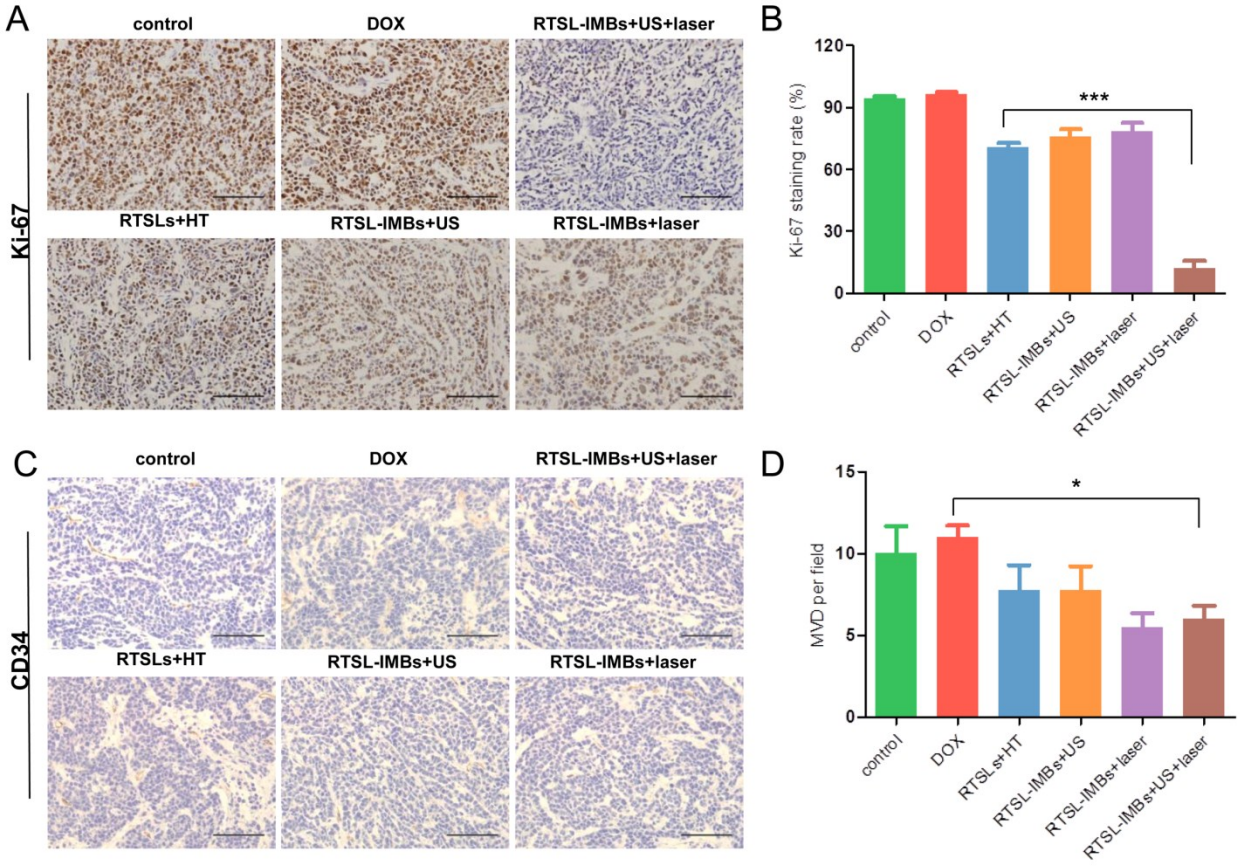

Figure S6. Immunohistochemistry analysis of tumors. A) Ki-67 and C) CD34 staining of the tumors harvested from different groups after 15 days of treatment. (Scale bar: 100 μm) The

corresponding quantification of Ki-67 B) and MVD D)

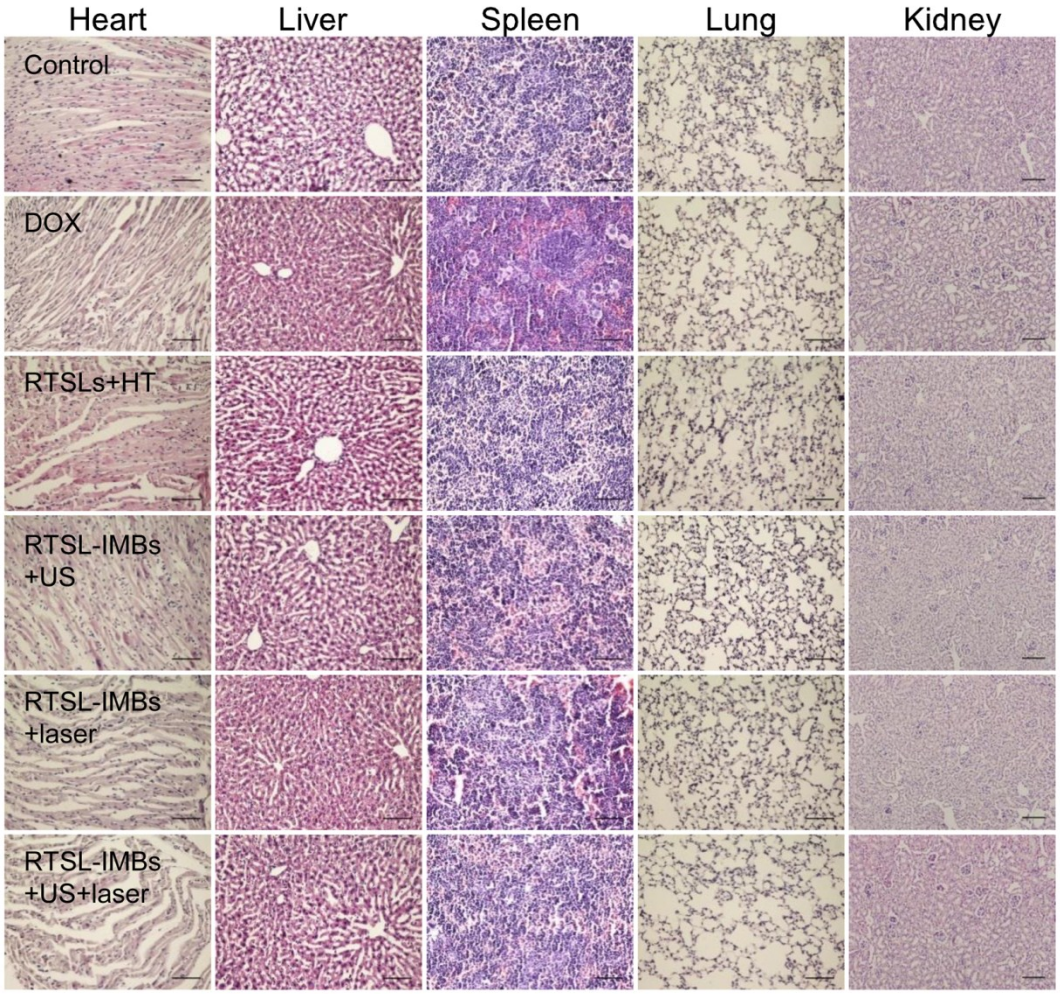

Figure S7. H&E stained imaging of major organs in different treatment groups. (Scale bar, 100μm)