Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Supplementary Material

cRGD-modified Nanoparticles of Multi-bioactive Agent Conjugate with pH-sensitive Linkers and PD-L1 Antagonist for Integrative Collaborative Treatment of Breast Cancer

Authors

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1. Experimental

1.1. Materials

Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing HVSF United Chemical Materials Co., Ltd., China. ADD was purchased from Biochempartner Biotech Co. Ltd. (China). TPGS was purchased from Sigma Aldrich. 4-formylbenzoic acid (TPA), dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), Triethylamine (TEA), trifluoroacetic acid (TFA) were purchased from Aladdin Bio-Chem Technology Co. Ltd. (Shanghai, China). N, N-dimethyl formamide (DMF), dichloromethane (DCM), triethylamine (TEA), methanol (MeOH) and ethyl acetate (EA) were analytical grade, and were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). 1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[maleimide(polyethylene glycol) -2000] (DSPE-PEG2000-Mal) were purchased from Avanti Polar Lipids (AL, USA). Hank's and RPMI-1640 medium, penicillin–streptomycin, fetal bovine serum (FBS) and trypsin were purchased from Gibco (Thermo Fisher ScientificTM, USA). 4,6-diamidino-2-phenylindole (DAPI), ATP assay kit (S0026) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Beyotime Biotechnology Co. Ltd. (Shanghai, China). Dir probe (1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine) and ROS probe (DCFH-DA) were purchased from Meilunbio Co. Ltd. (Dalian China). High mobility group box 1 protein (HMGB-1) ELISA kit was purchased from Shanghai Enzyme-linked Biotechnology Co. Ltd. (China). The primary antibody of calreticulin (CRT, ab2907) was purchased from Abcam Co. Ltd. (China) and Alexa Fluor 488-labeled goat anti-rabbit IgG (H+L) was purchased from Yeasen Biotechnology Co. Ltd. (Shanghai, China). ^{D}PPA-1 (NYSKPTDRQYHF) and cRGD (cyclo (Arg-Gly-Asp-D-Phe-Cys)) peptides were synthesized from Bioyeargene Biosciences Co. Ltd. (Wuhan, China). The antibodies for flow cytometry were purchased from BD PharmingenTM.

Female BALB/C mice (18−22 g) were provided by Shanghai JSJ bio-science Co. Ltd. (China) and bred under specific pathogen-free (SPF) condition (certificate: 20180004058054). All the animal experiments were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University (Case number: A2019028).

1.2. Synthesis of TDA conjugates

Synthesis of TDA was divided into three steps. First step was the synthesis of TPGS-TPA. 150 mg TPGS, 30 mg TPA, 41.2 mg DCC and 18.3 mg DMAP were dissolved into 10ml anhydrous DMF and then stirred for 24 h at room temperature. Afterwards, this reaction mixture was dialyzed with dialysis bag (MWCO: 1000) in DMF for 2 days and ultrapure water for another 2 days. Subsequently, the dialyzed aqueous solution was lyophilized to obtain TPGS-TPA. The second step was the conjunction of DOX and ADD (DOX-ADD). 232 mg DOX was dissolved into 60 mL mixture of methanol and DCM(4/1, v/v) with 1% TFA to obtain the DOX solution. 400mg ADD was dissolved into 40mL mixture of methanol and DCM (4/1, v/v) to obtain the ADD solution. The ADD solution was dropped slowly into the DOX solution and kept stirring under darkness for 24 h at room temperature. Afterwards, the reaction mixture was evaporated with a rotary evaporator to remove solvent. The obtained crude product was dissolved in MeOH and then was precipitated in cold EA for two times, washed with EA for three times to obtain the pure DOX-ADD product. The third step was the conjugation of TPGS-TPA and DOX-ADD. About 90 mg DOX-ADD was treated with 3-fold amount of TEA in 10 mL anhydrous DMF for 2 h at room temperature to obtain the DOX-ADD solution. 80 mg TPGS-TPA and 120 μL glacial acetic acid was dissolved in 5 mL anhydrous DMF to obtain the TPGS-TPA solution. The TPGS-TPA solution was dropped slowly into the DOX-ADD solution and kept stirring under N_2 protection at 35 °C for 24 h. Finally, the crude product solution was dialyzed in DMF with dialysis bag (MWCO: 2000) for 48 h. the dialyzed solution was evaporated with a rotary evaporator to remove the solvent and obtain the TDA product. The conjugate of TPGS and DOX (TD) was synthesized as our previous

report.[43] The obtain products above were analyzed by ^{1}H-NMR spectra (Bruker AVANCE III 400 MHz NMR spectrometer, solvents: d_6-DMSO or D_2O), fourier transform infrared spectroscopy (FTIR, PerkinElmer Spectrum Two, 32 replicate scan setting) and UV-Vis spectroscopy (HITACHI, U-2910 spectrophotometer). The molecular weight of TDA conjugate was characterized by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (Shimadzu 7090, Japan).

1.3. Preparation and characterization of NPs

TD and TDA NPs were prepared by simple nano-precipitation method. Briefly, 8 mg TD or 8 mg TDA and 0.8 mg DSPE-PEG_{2000} were added and dissolved in 1.5 mL DMF to obtain the DMF solution of TD and DSPE-PEG_{2000} or TDA and DSPE-PEG_{2000}. Then the DMF solution was slowly dropped into 10 mL ultrapure water and kept stirring for 5 min and then dialyzed in ultrapure water with dialysis bag(MWCO: 1000) to remove DMF and obtain the aqueous solution. The aqueous solution was centrifuged at 3000 rpm for 5 min to obtain the supernatant which was the TD or TDA NPs solution. The cRGD-TDA NPs solution was prepared by using the similar method above with 10% (w/w) DSPE-PEG_{2000}-Mal and then with 1 mg cRGD peptide. The Dir loaded TDA NPs solution was prepared as the similar method as that of the TDA NPs solution by dissolving Dir, TDA and DSPE-PEG2000 in DMF.

The prepared NPs solution were characterized by using dynamic light scattering (DLS, Zetaplus, Brookhaven, USA), transmission electron microscopy (TEM, JEM-1230, Japan) at 120kV. The stability of the TDA NPs solution at room temperature was investigated by observing their size change with time. The pH sensitivity of the TDA NPs was investigated by observing their size change with time in acetate buffer (pH, 5.0).

1.4. *In vitro* drug release test

Drug release test was done at 37 °C by using a dialysis method. Briefly, a dialysis bag (MWCO: 10,000) containing 5 mL of the TDA NPs solution (contain 2.3 mg TDA) was shaken in 50 mL of the 1% SDS buffer solution at pH 7.4, 6.8 or 5.0 as release medium in an air bath shaker at 100 rpm. At designated time points (1, 2, 3, 4, 8, 12, 24, 48, 72, 96, 120 h), the release medium was replaced with a fresh buffer solution. the amount of DOX or ADD in release medium was quantitatively analyzed by HPLC (Hitachi L-2000, a reversed phase inertia ODS C18 column (150 × 4.6 mm, pore size 5 μm, Agilent), mobile phase (methanol/0.02 M acetate buffer at pH 3.8, v/v = 80/20), UV detector with wavelength 237 nm, a flow rate of 1.0 mL/min, 25 °C). Each sample was tested in triplicate.

1.5. Cellular uptake test

1 mL complete DMEM containing 1.5×10^5 4T1 cells was added and incubated in each well of one 12-well plate. When the cells reached about 90% confluence, the medium was removed and the cells were washed with cold PBS twice. The cells were incubated with 1 mL of the DMEM containing free DOX, TDA NPs, or cRGD-TDA NPs at an equivalent DOX concentration of 2 μg/mL for a certain time (1, 3, or 6 h). Afterwards, the cells were stained with DAPI and observed by laser scanning confocal microscopy (Leica SP8). For quantitative analysis, the cells were digested with trypsin and analyzed by flow cytometry (Fortessa, BD, USA).

1.6. MTT assay

100 μL complete DMEM containing 5×10^3 4T1 cells was added and incubated in each well of one 96-well plate at 37 °C. When the cells reached about 90% confluence, the medium was removed and the cells were washed with cold PBS twice. The cells were incubated for 24 or 48 h with 100 μL DMEM containing free

DOX, free ADD, free DOX + ADD (1:1, mole), free DOX-ADD, TD NPs, or TDA NPs at different concentrations of DOX from 0.02 μg/mL to 20 μg/mL. Afterwards, 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added into each well and the cells were incubated for 4 h. Then, the medium was removed and 150 μL DMSO was added in each well to dissolve formazan crystal for optical density (OD) measurement. The OD measurement was carried out by using a microplate reader (Biotek, Synergy LX) under the absorbance mode at 490 nm. Each test was done for 5 times.

1.7. Wound healing and transwell invasion assay

Wound healing assay was conducted to evaluate anti-migration capacity. 1 mL DMEM containing 2×10^5 4T1 cells was added and incubated in each well of one 24-well plate. When the cells reached about 90% confluence, the monolayer of cells was scratched with a 200 μl pipette tip and washed with PBS to remove floating cells. The cells were then incubated with 1 mL of the DMEM containing free DOX, free DOX + ADD, free DOX-ADD, TD NPs, or TDA NPs at an equivalent DOX concentration of 2 μM for 24 h. The scratch wound was photographed using inverted microscope (OLYMPUS IX73) at 0 or 24 h. The wound area (A) was calculated using Image J software. The degree of change in wound area $((A_0 - A_{24})/A_0\times100\%)$ is denoted as migration rate.

Transwell invasion assay was conducted to evaluate anti-invasion capacity. The Upper chamber of one 24-well transwell plate with 8 μm pores was coated with 50 μL matrigel and placed in the incubator for 30 min. Then, 300 μL of the serum-free DMEM containing 2.5×10^4 4T1 cells was added to the upper chamber with free DOX, free ADD, free DOX + ADD, free DOX-ADD, TD NPs, or TDA NPs at an equivalent concentration of DOX (2μM). 1 mL of complete DMEM was added to the lower chamber. The cells were incubated for 24 h at 37°C in the incubator. Afterwards, the invaded cells were immersed and fixed in methanol for 20 min and then stained with crystal violet for 20 min, observed by microscope (OLYMPUS BX53).

1.8. *In vitro* ROS production

1 mL complete DMEM containing 1.5×10^5 4T1 cells was added and incubated in each well of one 12-well plate. When the cells reached about 90% confluence, the medium was removed and the cells were washed with cold PBS twice. The cells were incubated with 1 mL of the DMEM containing free DOX, free DOX + ADD, free DOX-ADD, TD NPs, TDA NPs, free DOX + 2 μM TPGS, free 2 μM TPGS, free 4 μM TPGS, or free 8 μM TPGS at an equivalent DOX concentration of 2 μM for 24h. Afterwards, the cells were incubated with DCFH-DA solution (10 μM) at 37°C for 30min. Then the cells were washed with cold PBS twice and the fluorescence intensity was measured by flow cytometry.

1.9. *In vitro* CRT, HMGB-1, ATP assay

1 mL complete DMEM containing 1.5×10^5 4T1 cells was added and incubated in each well of one 12-well plate. When the cells reached about 90% confluence, the medium was removed and the cells were washed with cold PBS twice. The cells were incubated with 1 mL of the DMEM containing free DOX, free DOX + ADD, free DOX-ADD, TD NPs, TDA NPs, free DOX + 2 μM TPGS, free 2 μM TPGS, free 4 μM TPGS, or free 8 μM TPGS at an equivalent DOX concentration of 2 μM for 24h. Afterwards, the cells were fixed by 4% paraformaldehyde and subsequently stained with anti-CRT primary antibody, secondary antibody of Alexa Fluor 488-labeled IgG (H+L), then rinsed two times with cold PBS and counterstained with DAPI for 20 min. CRT translocation on the cell surface was observed by microscopy. For quantitative analysis of CRT exposure, the cells were stained by antibodies and analyzed by flow cytometry. The amount of HMGB-1 or ATP in the medium was measured by ELISA assay kit or ATP assay kit.

1.10. Biodistribution

To evaluate the targeting ability of cRGD-TDA NPs, Dir loaded cRGD-TDA NPs (Dir@cRGD-TDA NPs) were constructed to check the biodistribution *in vivo*. 1×10^6 4T1 cells were inoculated subcutaneously in the second mammary pad of female BALB/c mice (18-22 g), after tumor volume reached 300 mm^3. The 4T1 tumor bearing mice were randomly grouped and treated by intravenous injection of free Dir, Dir@TDA NPs, or Dir@cRGD-TDA NPs at an equivalent Dir dose of 1 mg/kg. At designated time points (0, 1, 2, 4, 8, 12, 24, 48, 72 and 120 h), the mice were anaesthetized and imaged by IVIS imaging system, and euthanized to harvest the tumor, main organs and imaged. The mean fluorescent intensity was calculated by the analysis software image studio version 5.2.

1.11. *In vivo* ROS measurement

When the tumor volume reached 300 mm^3, the 4T1 tumor bearing mice were randomly grouped and treated by intravenous injection of saline, free DOX, free DOX + ADD, or cRGD-TDA NPs at an equivalent DOX concentration of 5mg/kg. 2 days after administration, 25 μL of DCFH-DA (10 μM) was intratumorally injected. After 30 min of DCFH-DA injection, the mice were euthanized and the tumor was collected. The tumor tissue was then counterstained with DAPI and photographed by microscopy.

1.12. *In vivo* CRT, HMGB-1 *assay*

When the tumor volume reached 50 mm^3, the 4T1 tumor bearing mice were randomly grouped and treated by intravenous injection of saline, free DOX, free DOX + ADD, or cRGD-TDA NPs at an equivalent DOX concentration of 5mg/kg every three days for 3 times. The mice were euthanized at 2 days after the final injection and the tumor was collected. The tumor tissue was stained with primary antibodies of anti-CRT, the second antibody of Alexa Fluor 488-labeled goat anti-rabbit IgG (H+L), counterstained with DAPI, and photographed by microscopy. The amount of HMGB-1 in tumor homogenate was measured by ELISA assay kit.

1.13. *In vivo* evaluation on maturation of DCs

When the tumor volume reached 50 mm^3, the 4T1 tumor bearing mice were randomly grouped and treated by intravenous injection of saline, free DOX, free DOX + ADD, free DOX-ADD, or cRGD-TDA NPs at an equivalent DOX concentration of 5mg/kg every three days for 3 times. The mice were euthanized at 2 days after the final injection and the inguinal lymph nodes were collected. The inguinal lymph nodes were homogenized into single-cell suspensions, then stained with antibodies (anti-CD45-FITC, anti-CD11c-PerCP-Cy5.5, anti-CD86-PE-Cy7 and anti-CD80-PE), were analyzed by flow cytometry.

1.14. Evaluation on anti-tumor efficacy in H22 tumor bearing mice

5×10^4 H22 cells were inoculated subcutaneously in the right front flank of female BALB/c mice (18-22 g), after tumor volume reached 50 mm^3, the H22 tumor bearing mice were randomly grouped and treated by intravenous injection of saline, free DOX, free DOX+ADD, free DOX-ADD, TD NPs, TDA NPs, or cRGD-TDA NPs at the equivalent DOX dose of 5mg/kg every two days for four times. The tumor volume and the body weight were measured every two days. The tumor volume was calculated as formula: $V = L\times W\times W/2$ (L: the length, W: the width). Once one of the mice was up to 2000 mm^3 in tumor size or 20 mm in maximum length of tumor, all the tested mice were euthanized and the tumor was collected. H&E and immunohistology analyses of the tumor were done.

1.15. Evaluation on anti-tumor and anti-metastasis efficacy in 4T1 tumor bearing mice

Luciferase expressed 4T1 cells were used to establish the luciferase expressed 4T1 tumor-bearing mouse model. When the tumor size reached 50 mm^3, the 4T1 tumor bearing mice were randomly grouped and treated by intravenous injection of saline, free DOX, free DOX + ADD,or cRGD-TDA NPs at an equivalent DOX concentration of 5mg/kg every three days for 5 times and/or peritumoural injection of the anti-PD-L1 peptides (100 μg per mice). The tumor volume and body weight were measured every two days. On 14 and 21 days, the mice were intraperitoneally injected with 3 mg luciferin, anaesthetized, and imaged by IVIS imaging system. Once one of the mice was up to 2000 mm^3 in tumor size or 20 mm in maximum length of tumor, all the tested mice were euthanized, the lung and tumor were collected. The lung was stained by Bousin's fixative buffer and photographed, and the metastatic nodules were counted.

The collected tumor was analyzed by H&E, immunohistology and flow cytometry. The infiltrating lymphocytes were isolated from the tumor by Ficoll separation medium and blocked by CD16/32 antibodies. Then, the lymphocytes were stained by fluorescent-labeled antibodies (anti-CD3-PerCP-Cy5.5, anti-CD4-PE-Cy7, anti-CD8-FITC, anti-CD69-PE, anti-IFN-γ-PE-Cy7, anti-NK1.1-PerCP-Cy5.5, anti-CD25-APC, anti-FoxP3-PE, anti-CD11b-FITC, anti-CD80-PE) according to the manufacturer's protocols and analyzed by flow cytometry.

1.16. *In vivo* evaluation on biosafety

Healthy BALB/c mice were treated by intravenous injection of saline, free DOX, free DOX + ADD, or cRGD-TDA NPs at an equivalent DOX concentration of 5mg/kg every three days for 5 times. Then the tested mice were euthanized. The main organs were collected and histologically analyzed by H&E staining. The blood biochemical analysis was conducted by automatic Celercare V5 analyzer (MNCHIP Co. Ltd., China).

1.17. Statistical analysis

All procedures were replicated at least three times and statistical analysis was completed by t-test using GraphPad Prism 6.01 software. Data was qualified as statistical significance if p value was less than 0.05.

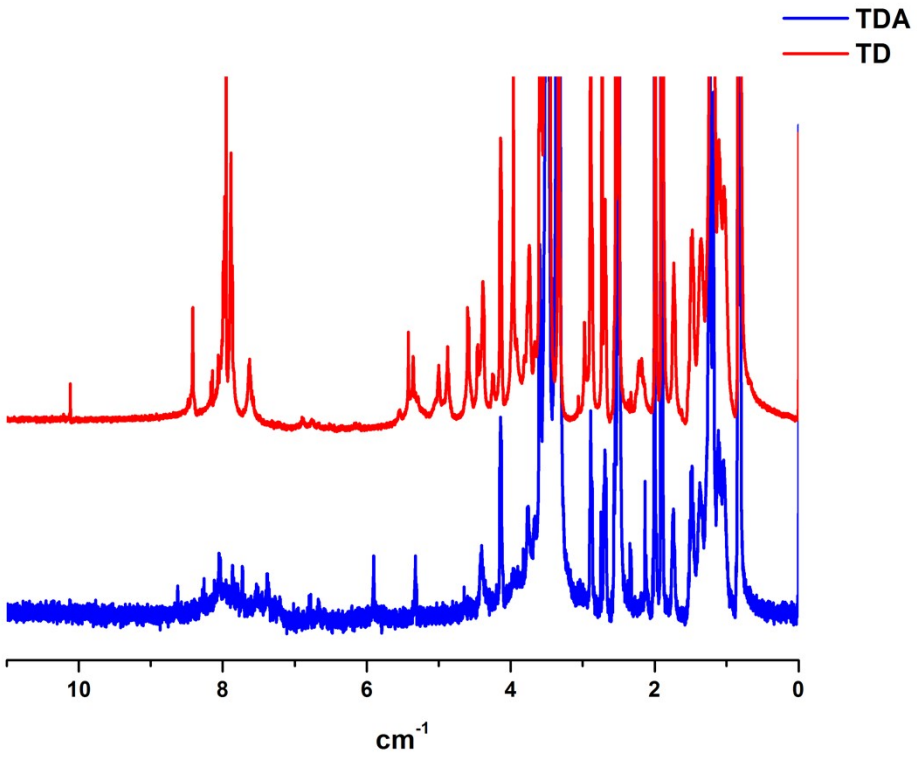

Fig. S1. The ^{1}H-NMR spectra of TD and TDA by method A (d_6-DMSO, 400 MHz).

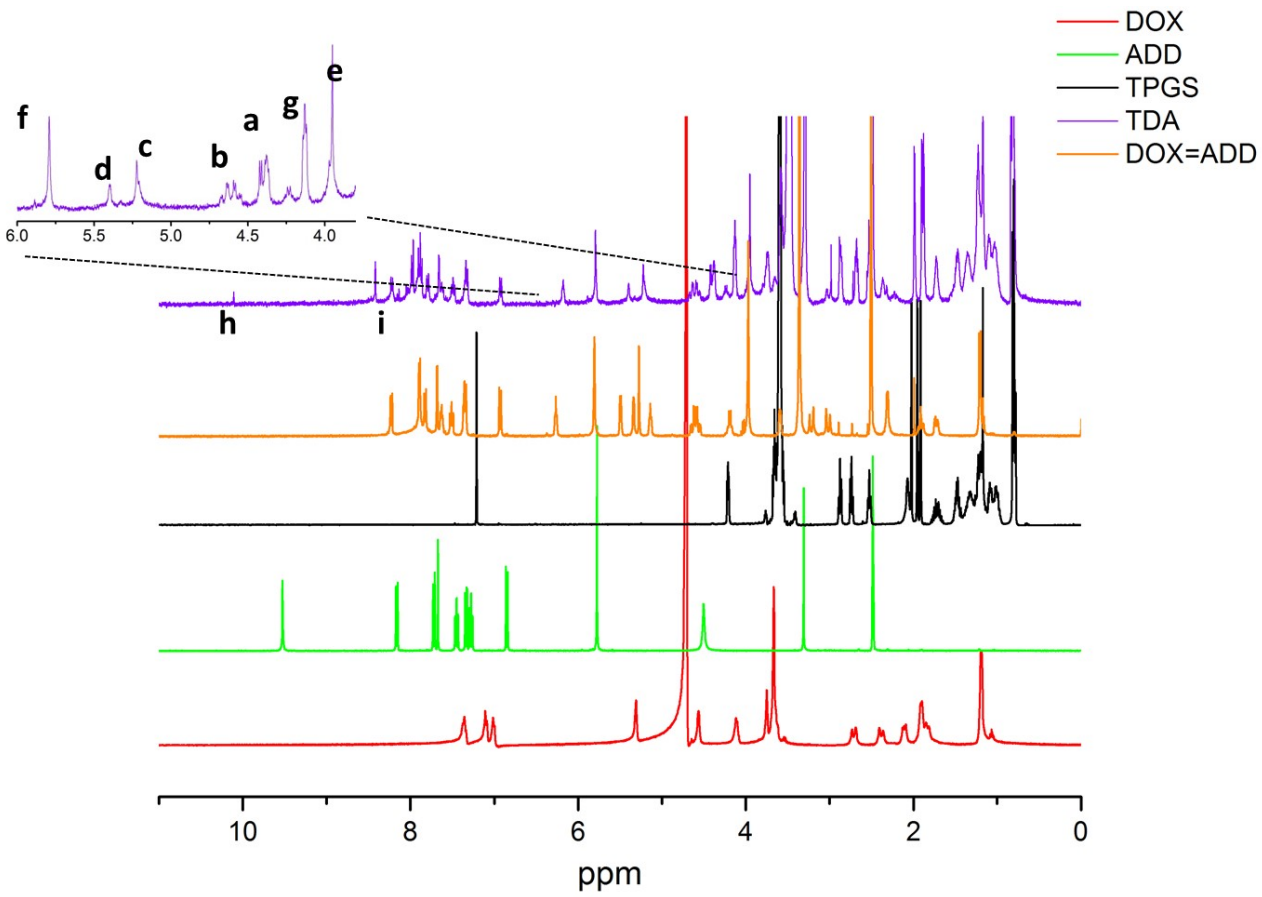

Fig. S2. ^{1}H-NMR spectra of DOX, ADD, DOX-ADD, TPGS, and TDA synthesized by method B. (DOX in D_2O, ADD in d_6-DMSO, DOX-ADD in d_6-DMSO, TPGS in $CDCl_3$, TDA in d_6-DMSO, 400 MHz).

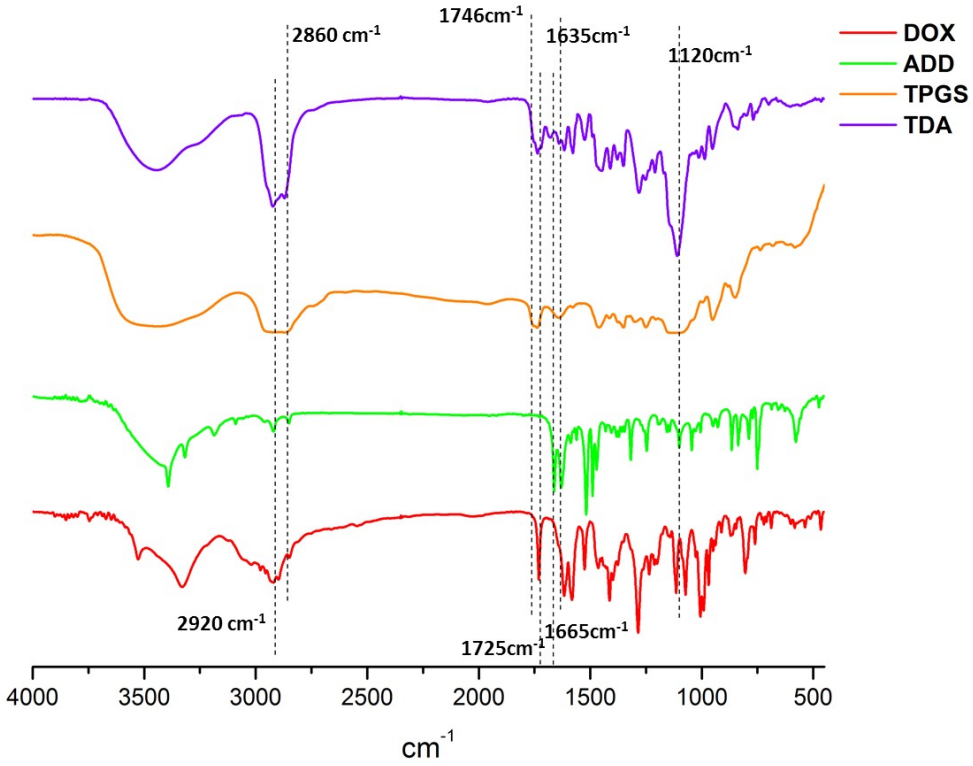

Fig. S3. FTIR spectra of DOX, ADD, TPGS and TDA.

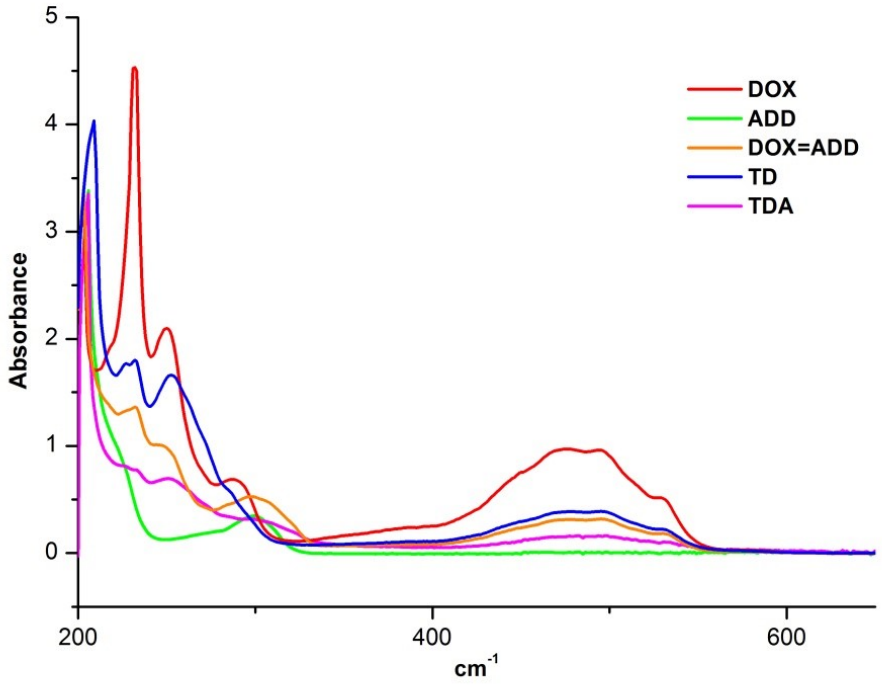

Fig. S4. UV scanning spectra of DOX, ADD, DOX-ADD, TPGS and TDA.

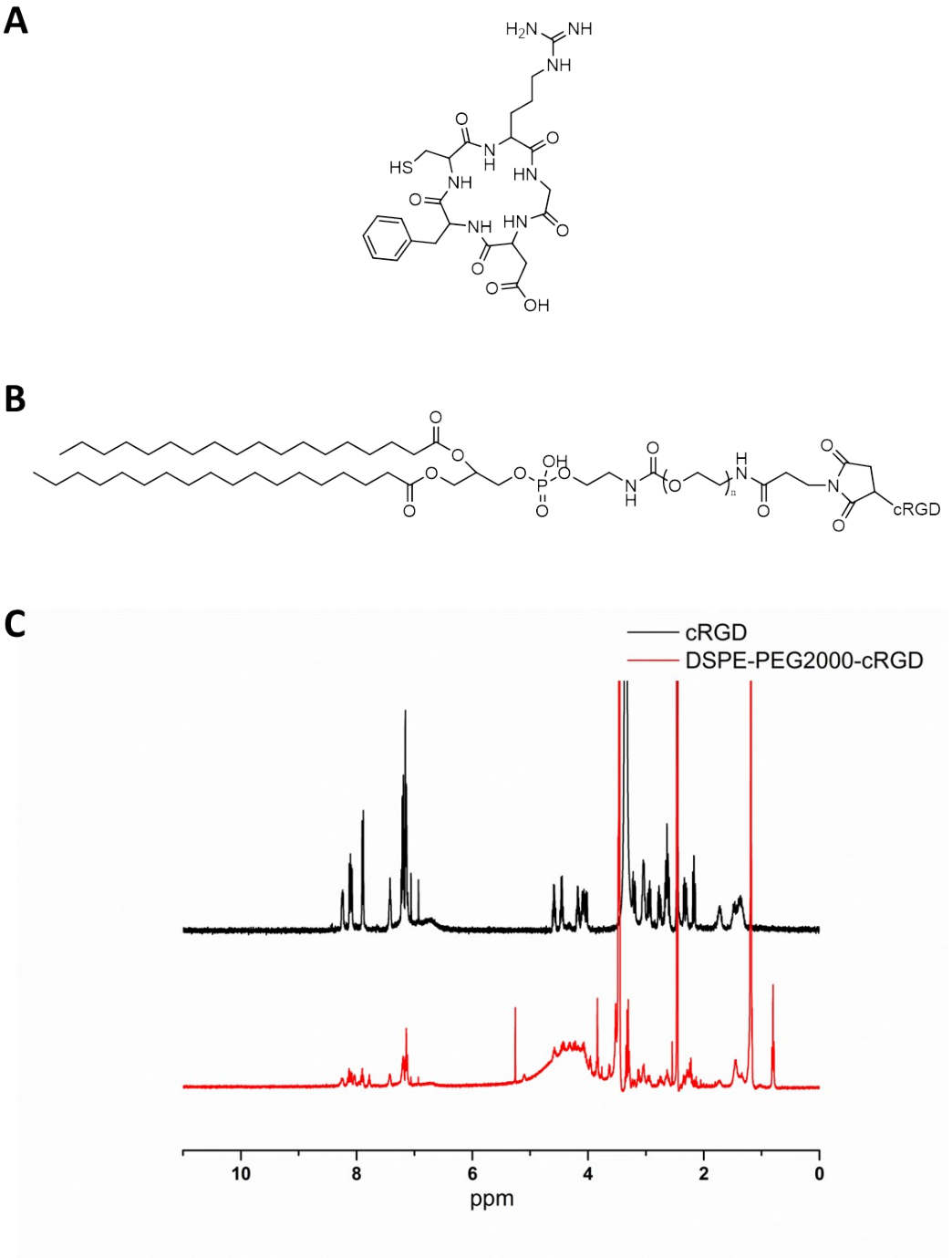

Fig. S5. Structures of (A) cRGD and (B) DSPE-PEG2000- cRGD conjugate; (C) ^{1}H-NMR spectra of cRGD and DSPE-PEG$_{2000}$-cRGD conjugate (d_6-DMSO, 400 MHz).

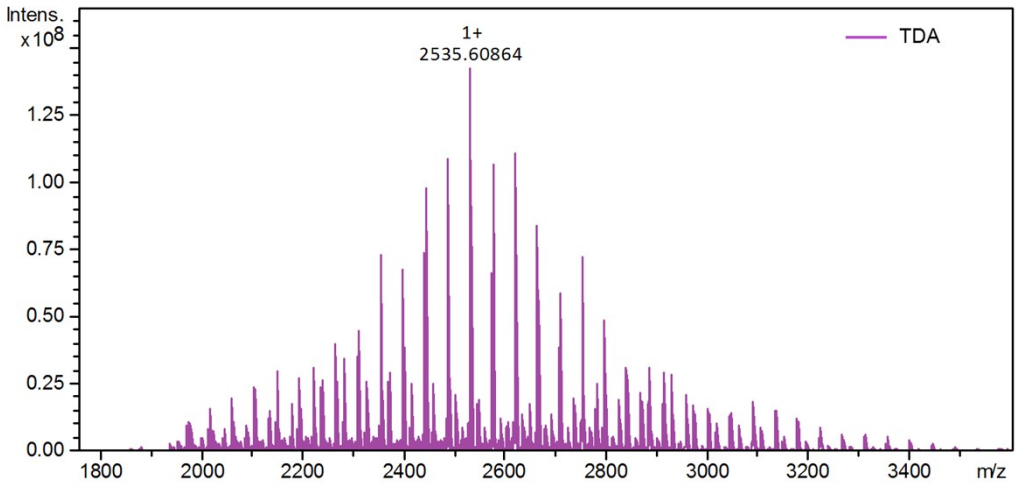

Fig. S6. MALDI-TOF-MS spectrum of TDA conjugate.

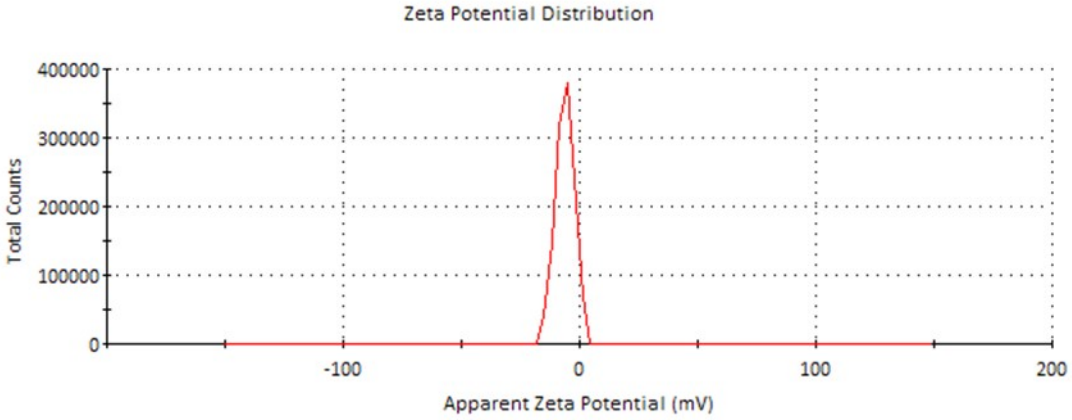

Fig. S7. Zeta potential distribution.

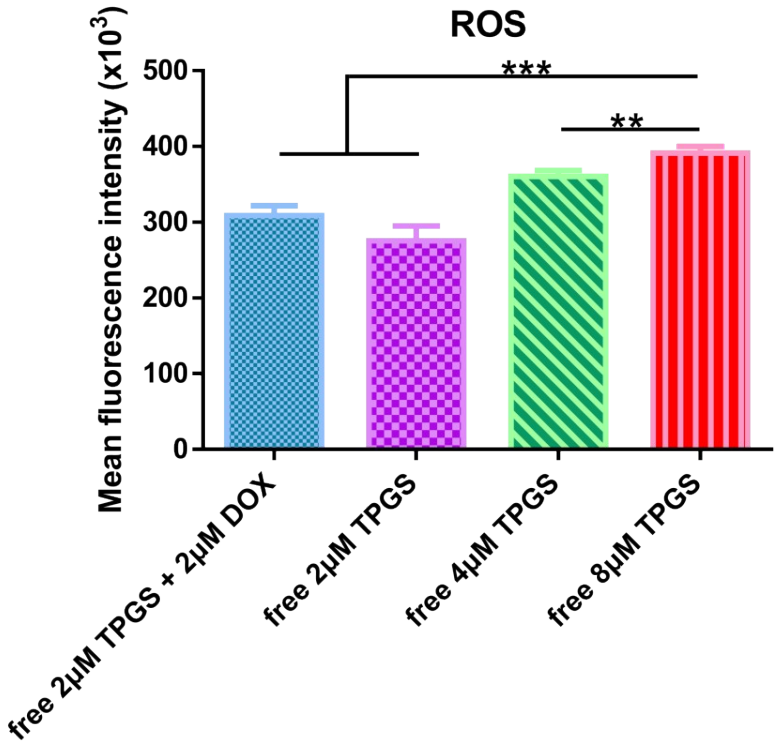

Fig. S8. *In vitro* ROS induced by TPGS in 4T1 cells. Quantitative measurement of ROS level by flow cytometry after incubated with free 2 μM TPGS + 2 μM DOX, free 2 μM TPGS, free 4 μM TPGS, or free 8 μM TPGS for 24 h (n = 3). (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; NS, no significant difference)

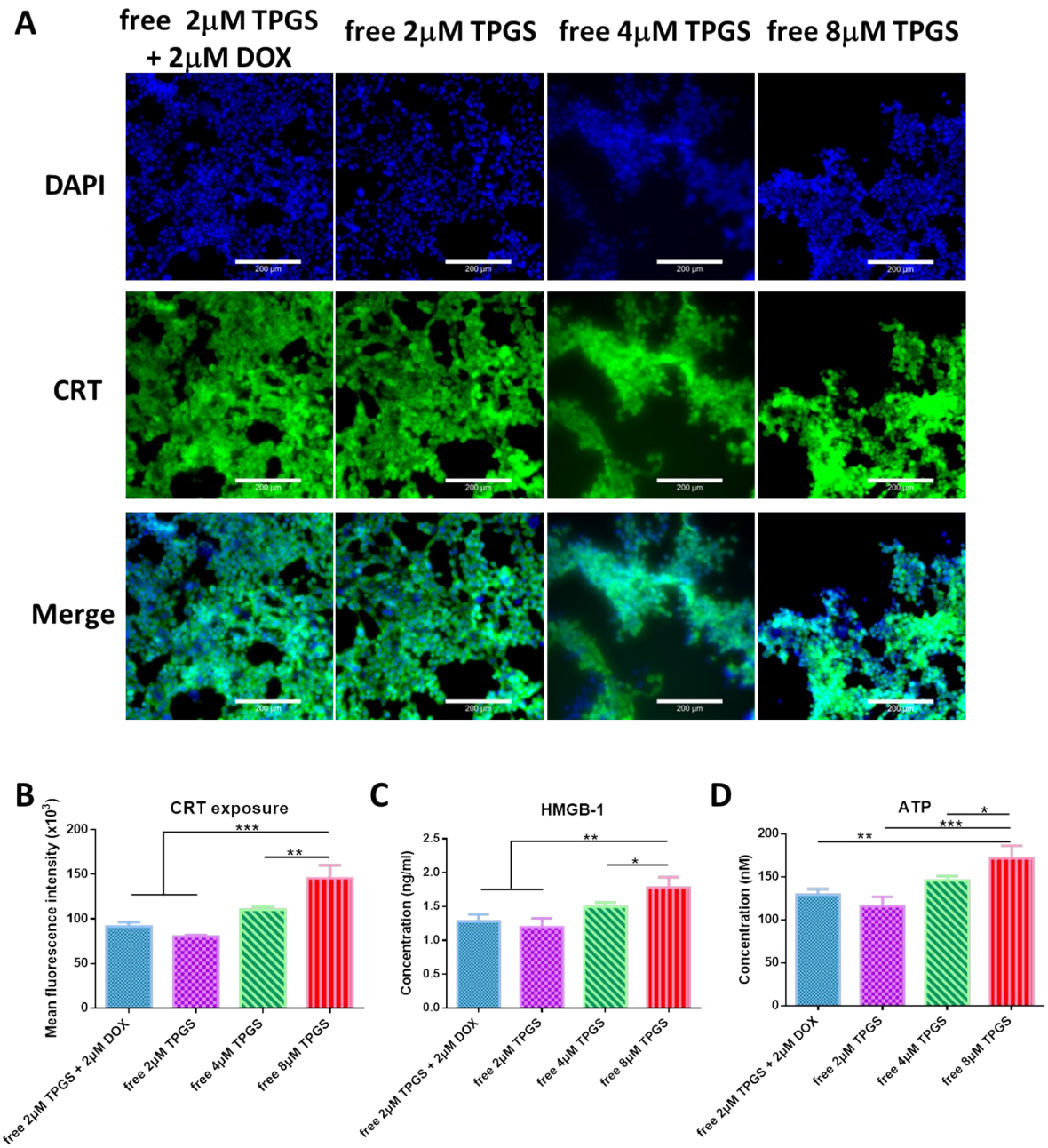

Fig. S9. *In vitro* ICD effect elicited by TPGS in 4T1 cells. (A) Microscopy images of CRT exposure (green signals) after incubated with free 2 μM TPGS + 2 μM DOX, free 2 μM TPGS, free 4 μM TPGS, or free 8 μM TPGS for 24 h (scale bar = 200 μm); (B) Quantitative measurement of CRT exposure by flow cytometry (n = 3); Concentration of HMGB-1 (C) and ATP (D) in medium after incubated with free 2 μM TPGS + 2 μM DOX, free 2 μM TPGS, free 4 μM TPGS, or free 8 μM TPGS for 24 h measured by

HMGB-1 ELISA kit or ATP assay kit (n = 3). (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; NS, no significant difference)

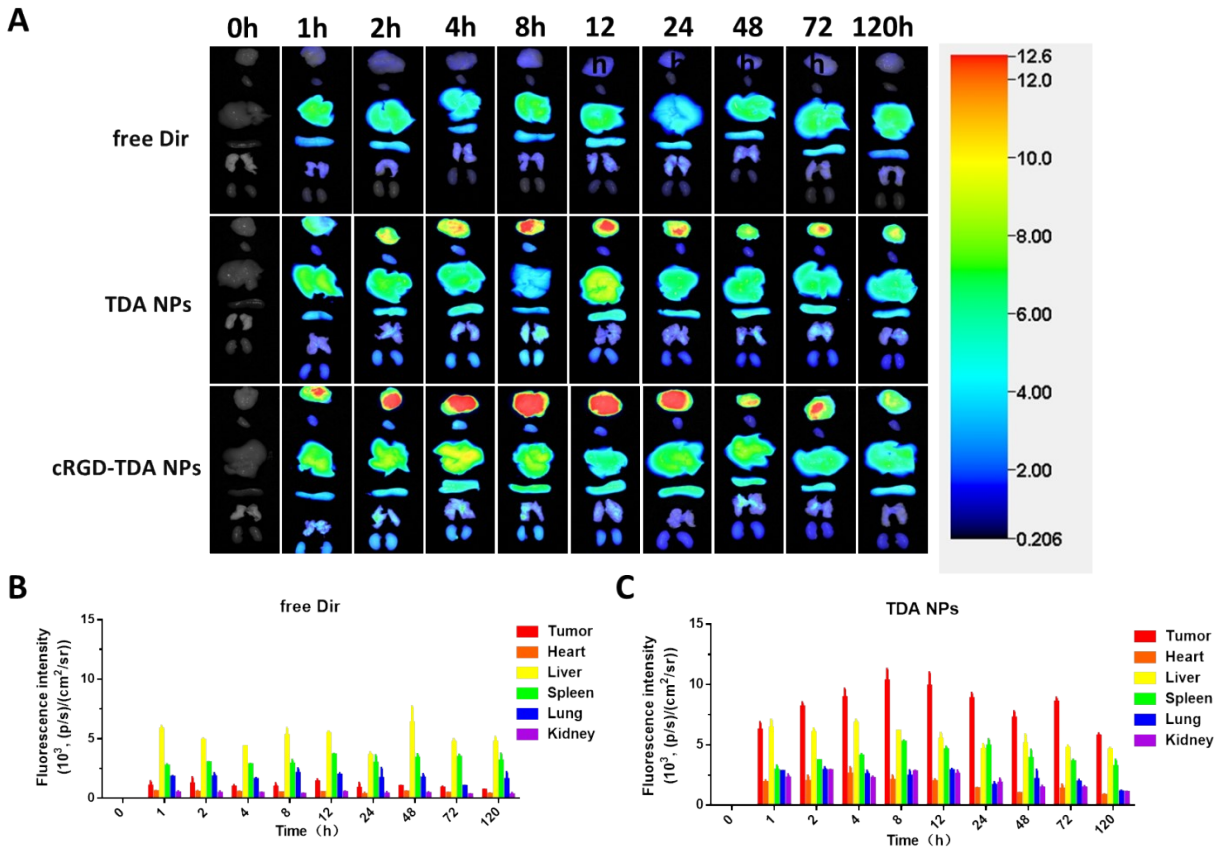

Fig. S10. *In vivo* tumor targeting property and biodistribution of cRGD-TDA NPs. (A) Fluorescent images of dissected tumor tissues and main organs (heart, liver, spleen, lung, spleen) at different time points (0, 1, 2, 4, 8, 12, 24, 48, 72, 120 h) after *i.v.* administration with free Dir, Dir@TDA NPs, or Dir@cRGD-TDA NPs; The quantitative mean fluorescent intensity of dissected tumor tissues and main organs at different time points (0, 1, 2, 4, 8, 12, 24, 48, 72, 120 h) after *i.v.* administration with (B) free Dir ,or (C) Dir@TDA NPs (n = 3) (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; NS, no significant difference)

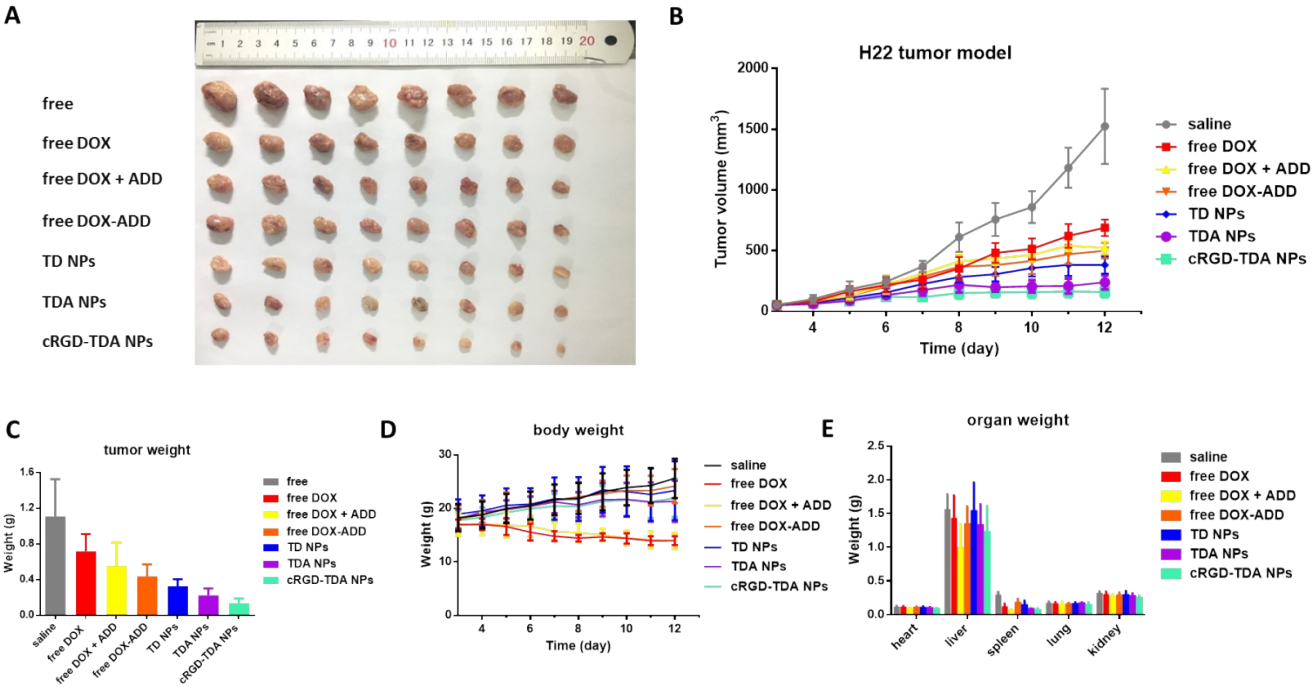

Fig. S11. Anti-tumor study in H22 tumor bearing mice. (A) The image of dissected tumors after sacrifice (n=8); (B) H22 tumor growing curves (n=8); (C) The weight of dissected tumor tissues after treatment with saline, free DOX, free DOX + ADD, free DOX-ADD, TD NPs, TDA NPs, or cRGD-TDA NPs, respectively (n=8); (D) The body weight curves in different groups; (E) The weight of main organs after sacrifice (n=8). (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; NS, no significant difference)

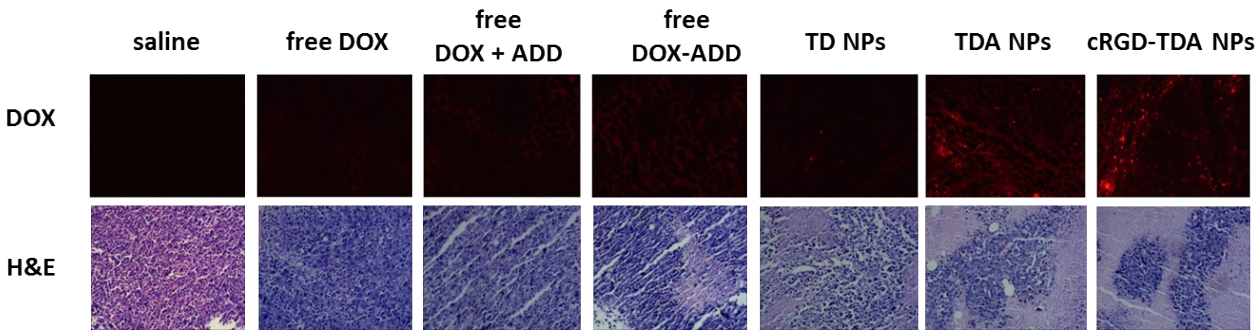

Fig. S12. Histology analysis in H22 tumor bearing mice. After treatments with different formulations in H22 tumor model, tumor sections of each group were collected for frozen sections to observe the accumulation of DOX in different groups (upper panel, scale bar = 200 μm), and were fixed in 4% PFA solution for H&E analysis (lower panel, scale bar = 200 μm).

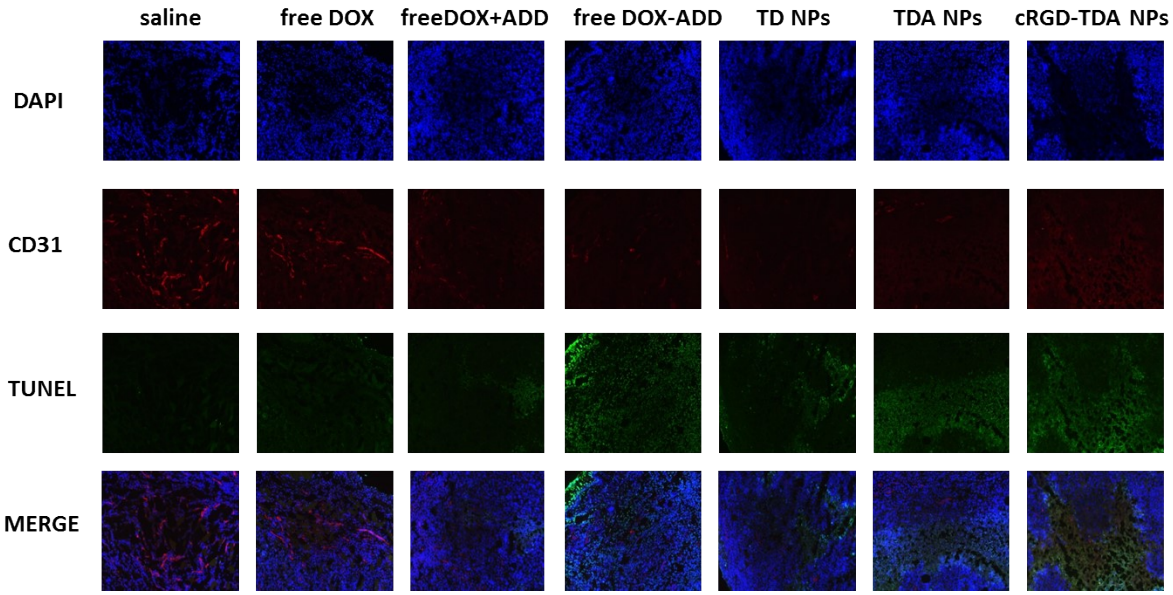

Fig. S13. Immunohistology analysis in H22 tumor bearing mice. After treatments with different formulations in H22 tumor model, tumor sections of each group were collected and stained with CD31, TUNEL and DAPI for immunohistology analysis (scale bar = 200 μm).

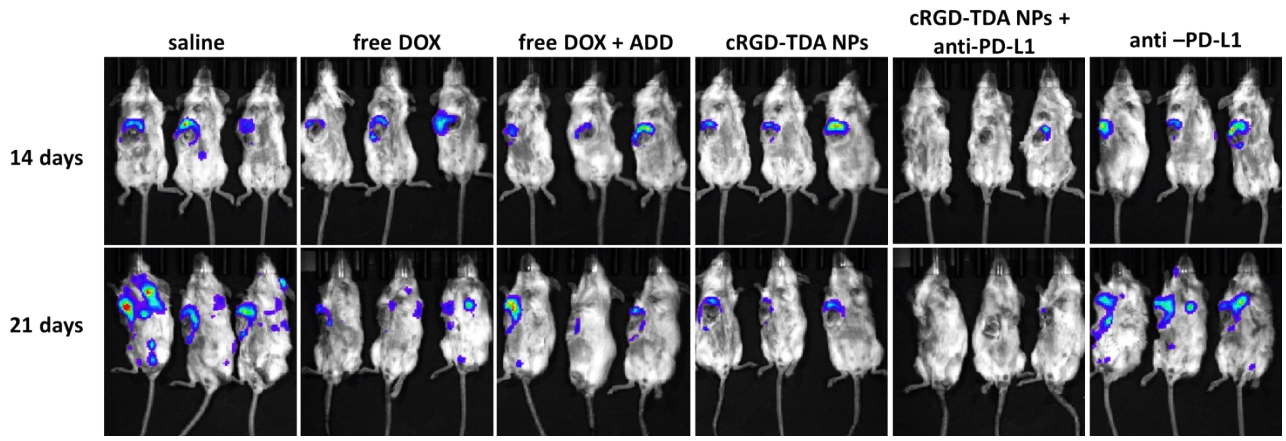

Fig. S14. Luminescence images of treatment with different groups 14 and 21 days after 4T1 cells inoculation to monitor the metastasis condition.

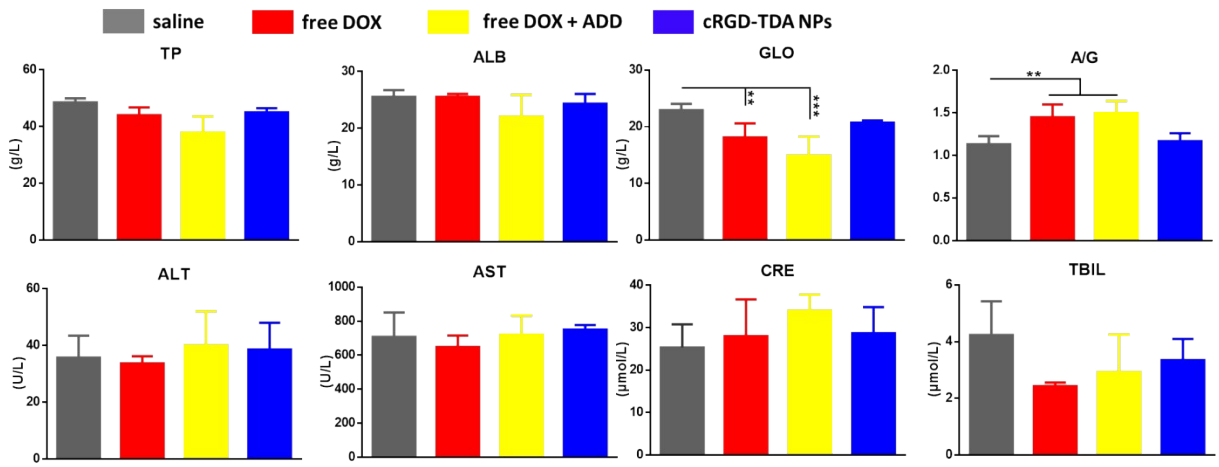

Fig. S15. Blood biochemical analysis. (total protein, TP; albumin, ALB; globulin, GLO; albumin/globulin, A/G; alanine transaminase, ALT; aspartate transaminase, AST; creatinine, CRE and total bilirubin, TBIL) in biosafety study.

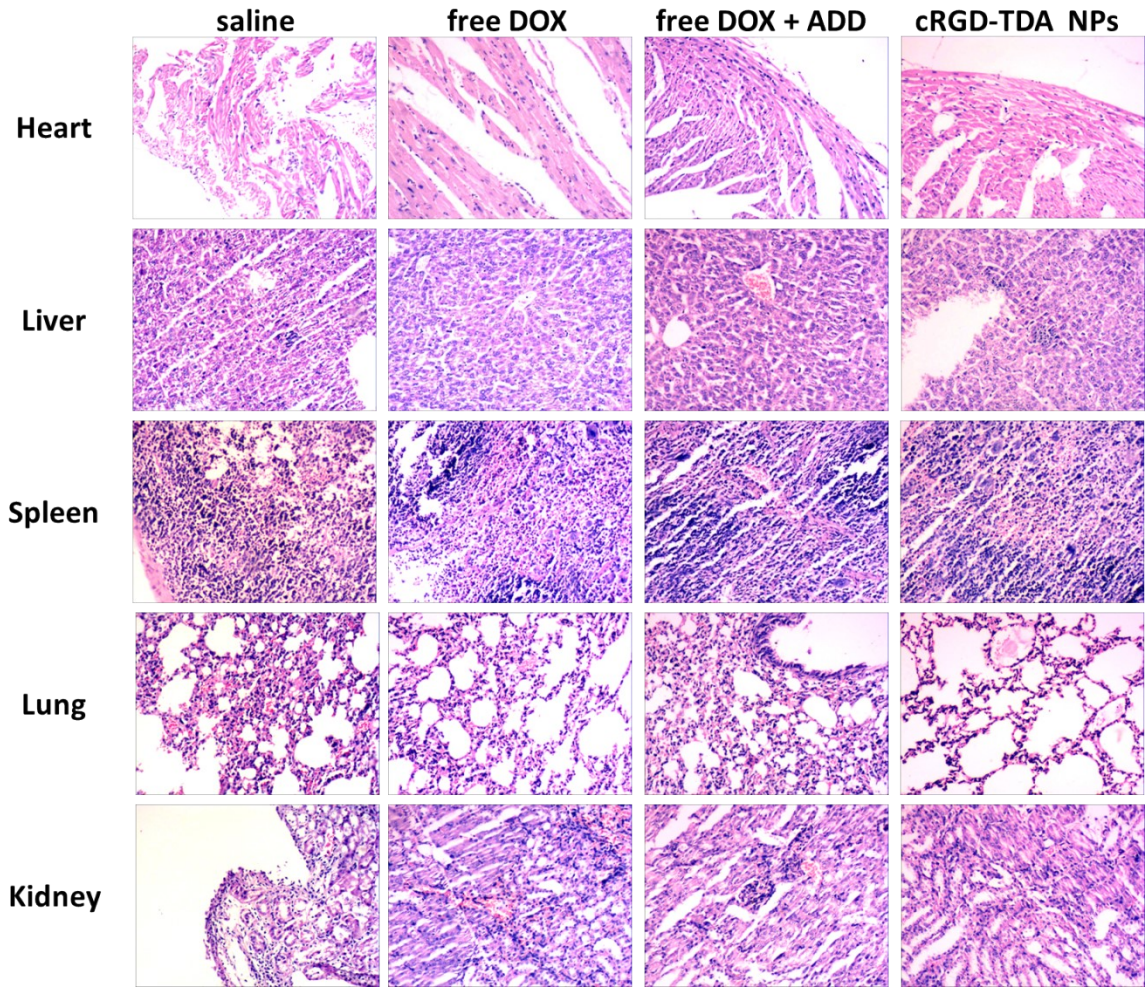

Fig. S16. The H&E staining sections of main organs in biosafety study (scale bar = 200 μm).