Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Choline Phosphate Lipid as an Intra-cross-linker in Liposomes for Drug and Antibody Delivery under Guard

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1. Synthetic procedures

1.1 The synthesis of CP-lip

CP-lip was synthesized based on our previous method.[1] In brief, all glassware were flame dried and protected by argon before using. 2-ethoxy-1,3,2-dioxaphospholane 2-oxide[2] (0.012 mol, 1.82 g) and 3-(dimethylamino)propane-1,2-diyl dipalmitate (0.01 mol, 5.96 g) were added to 50 mL acetonitrile (dried via Na and distilled freshly before using). The reaction was conducted at 70 °C for 48 h, and then the solution was precipitated three times via THF, after removed the THF, the CP-lip was obtained with yield 63%. ^{1}H-NMR result and ^{31}P-NMR result were shown in Figure S2. ESI-MS result was shown in Figure S3. ^{1}H NMR (500MHz, $CDCl_3$): 5.67 (s, -$COOCH_2CHOOC$-), 4.50, 4.12 (d, -$COOCH_2CH(OOC$-), 4.35 (m, -PO_4^--CH_2CH_3-), 4.12 (t, -CH_2CH_2-PO_4^--), 3.90 (t, -$N^+CH_2CH_2$-), 3.79 (d, -$COOCH_2CH$-), 3.32 (d, N^+-$(CH_3)_2$), 2.28 (t, -CH_2CH_2COO-), 1.58 (t, -CH_2CH_2COO-), 1.26 (t, $CH_3(CH_2)_{12}CH_2$-, -PO-CH_2CH_3), 0.88 (t, $CH_3(CH_2)_{12}CH_2$-); ^{31}P NMR (500MHz, $CDCl_3$): δ (ppm) -0.65 (s). ESI-mass spectrum found 748.7 (M+H), 770.7 (M+Na), 1496.6 (M+M+H).

1.2 The synthesis of α-CP-lip

α-CP-lip was synthesized based on our previous method.[1] In brief, butyne CP-lip was synthesized via ring-opening reaction. All glassware were flame dried and protected by argon before using. Briefly, 2-(but-3-yn-1-yloxy)-1,3,2-dioxaphospholane 2-oxide (0.012 mol, 2.11g) and 3-(dimethylamino)propane-1,2-diyl dipalmitate (0.01 mol, 5.96g) were added to 50 mL acetonitrile (dried via Na and distilled freshly before using). The reaction was conducted at 70 °C for 48 h, and then the solution was precipitated three times via THF, after removed the THF, the butyne CP-lip was obtained with yield 57%. Azide-polyethylene glycol-carboxyl (1.2 mmol, 1.2 g), butyne CP-lip (1 mmol, 772 mg), copper sulfate pentahydrate (12.5 mg), sodium ascorbate (20 mg) and mixed solvent (5 mL, chloroform: methanol=4:1) were transferred to a 20 mL one-necked flask. The reaction mixture was stirred for 24h at room temperature. Finally, dialysis was used to remove the excessive azide-polyethylene glycol-carboxyl and the copper catalyst. After freeze-drying, PEG-CP-lip was collected with yield 35%. PEG-CP-lip (20 mg), NHS (5 mg) and DIC (5 mg) were dissolved in chloroform then stirred at room temperature for 12 h. Chloroform was removed under vacuum, then PD-L1 monoclonal antibody (20 mg) in water solution was added to the mixture and stirred for 5 h. Dialysis was used to remove the NHS and DIC to get the solution of α-CP-

lip. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) result of α-CP-lip was shown in Figure S12.

2. Isothermal Titration Calorimetry (ITC)

Typically, 17 consecutive injections of 2.5 μL each (PC-lips or CP-lips at a concentration of 13 mM) were made into the chamber (200 μL) filled with 1.625 mM PC-lips or CP-lips solution. Both solutions in the syringe and chamber were degassed under vacuum for 30 min immediately before use. Injections were made at 300-sec intervals. A constant stirring speed of 400 rpm was maintained during the experiments to ensure sufficient mixing after each injection. For calculation of the binding curve, the heat of dilution was measured in separate titrations and used as background to be subtracted.

3. QCM-D measurements

Quartz crystal microbalance with dissipation monitoring (QCM-D) measurements were carried out to quantify the adsorption of proteins on liposomes. Before the experiments, the sensors were carefully washed with piranha solution, followed by thee 5-min distilled water boiling cycles and, finally, airstream drying. The experiments were conducted in aqueous solutions; thus, the baseline sensor frequency was established for the Au-coated sensors in distilled water. The liposomes were deposited on the sensor surface, then pass in 10% FBS until the curve is stable, then the excess unbound protein adsorbed were washed out with PBS. Rinsing was carried out until the frequency and energy dissipation values reached a plateau. Variations in resonance frequencies and dissipations were monitored. All calculations were performed with QTools3 software (QSense, Biolin Scientific, Sweden).

4. Hemolysis assay

Fresh mouse blood was collected from heart, and the red blood cells (RBCs) were washed three times with PBS. After that, the RBCs were diluted and suspended with 10 mL PBS. First, 0.3 mL RBCs suspension was mixed with 1.2 mL PBS as negative control group, and with 1.2 mL water as positive control group. Various liposomes dissolved in 1.2 mL PBS with different concentrations were added to the RBCs suspension (0.3 mL), and then incubated for

2 h at 37 centigrade. Finally, the samples were centrifuged with 12000 r/min for 10 min, the supernatant was collected and measured the absorbance intensity at 541 nm with microplate spectrophotometer. The percent hemolysis was calculated according to the following equation:

$$Percent\ hemolysis\ (\%) = \frac{Absorbance\ intensity\ of\ samples}{Absorbance\ intensity\ of\ positive\ control} \times 100\%$$

5. In vitro drug release behavior

The Dox release behaviors from liposomes were conducted via dialysis method. In brief, 1 mL Dox loaded liposome (Dox@α-lipo) was transferred into dialysis bag (MCW:3500), and immersed in 9 mL PBS (pH 7.4, pH 6.8 and pH 5.0) with continuous stirring at 37 centigrade. At fixed time intervals, 1 mL dialysis solution outside the bag was collected to determine the Dox concentration via UV-Vis spectra, and 1 mL fresh PBS was added after determination.

Figures

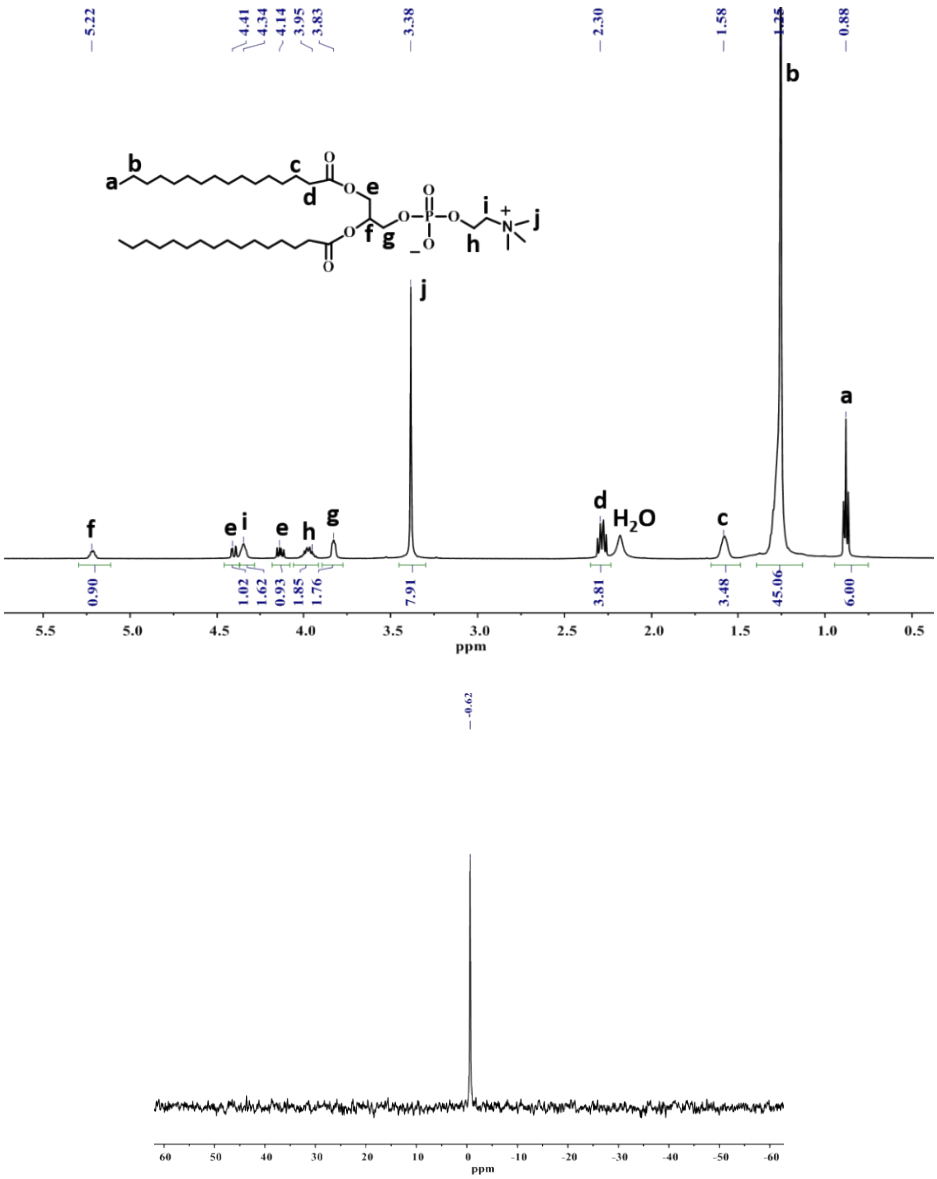

Figure S1. ^{1}H-NMR (top) and ^{31}P-NMR (bottom) of PC-lip in $CDCl_3$.

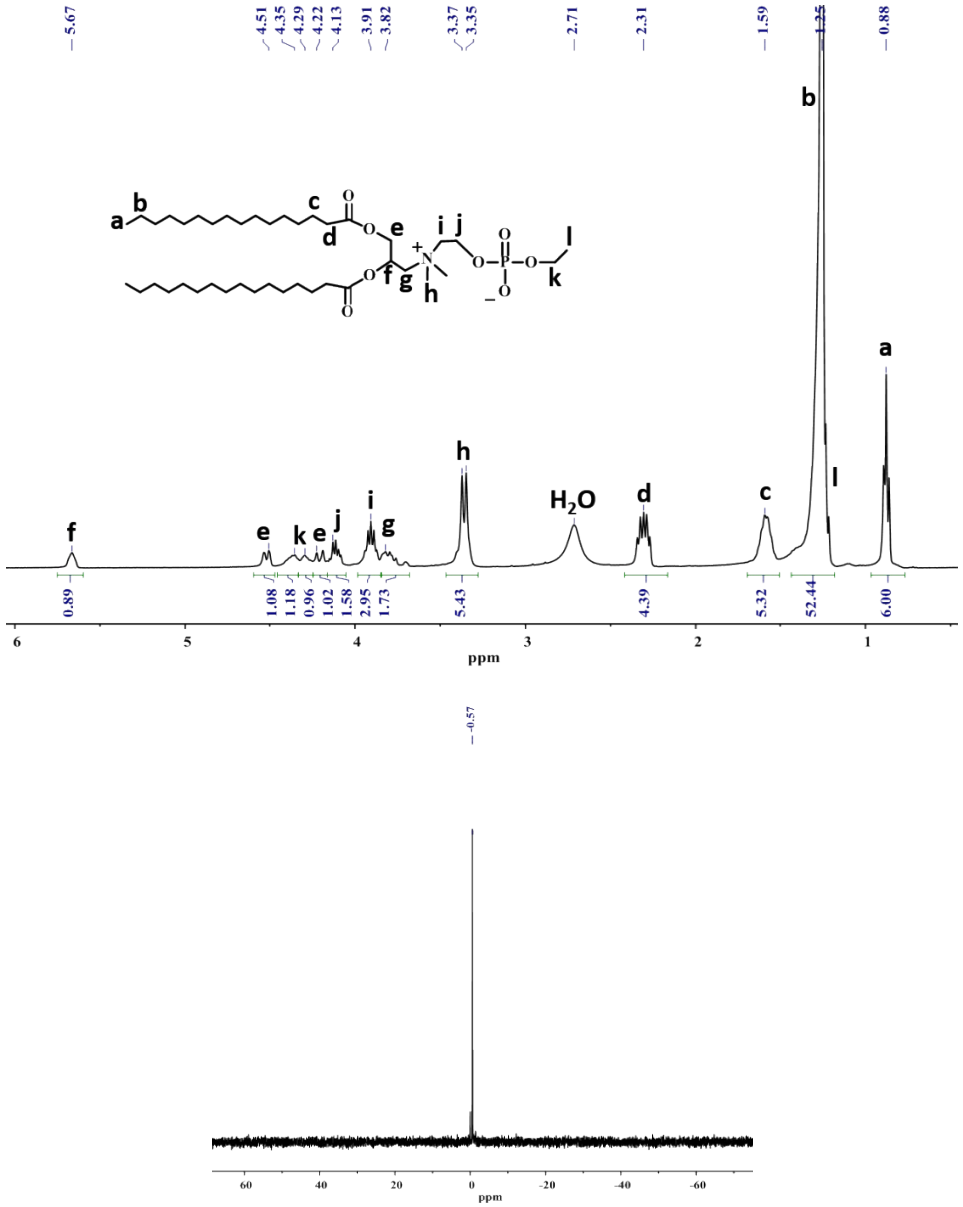

Figure S2. ^{1}H-NMR (top) and ^{31}P-NMR (bottom) of CP-lip in $CDCl_3$.

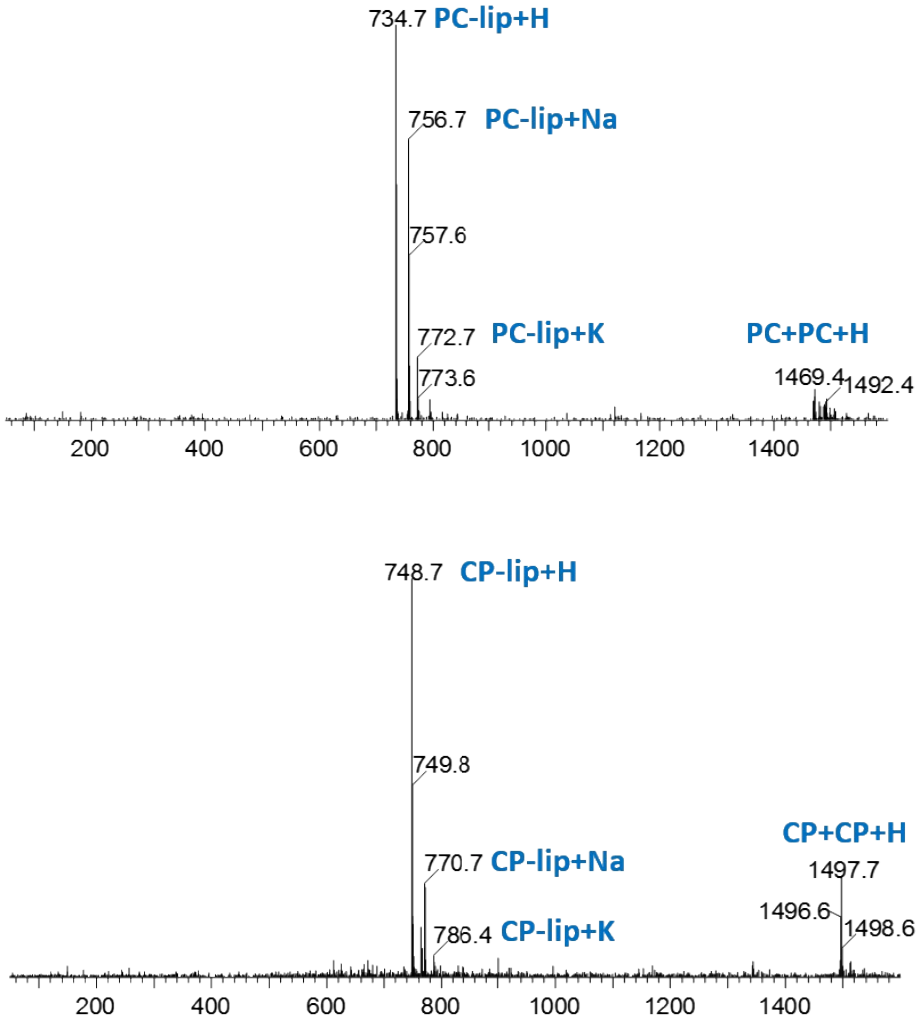

Figure S3. ESI-MS spectrum of PC-lip (top) and CP-lip (bottom).

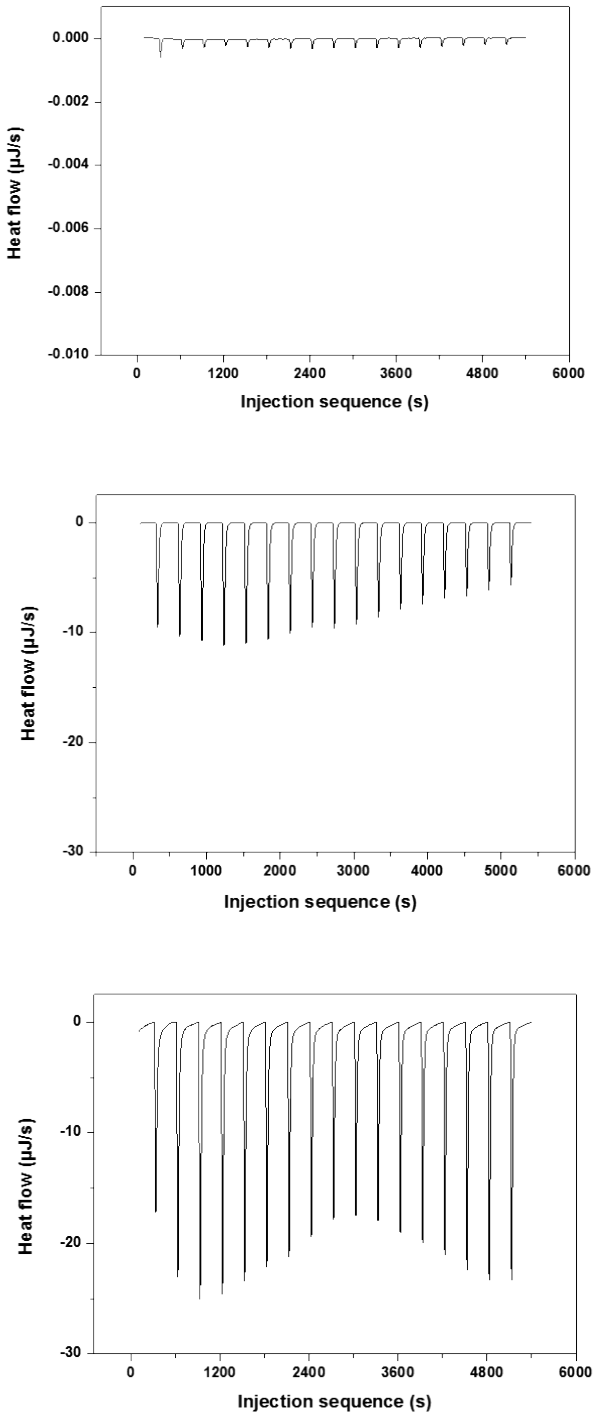

Figure S4. Isothermal titration calorimetry to test the exotherm of PC-lip to PC-lip (top), CP-lip to CP-lip (medium) and CP-lip to PC-lip (bottom).

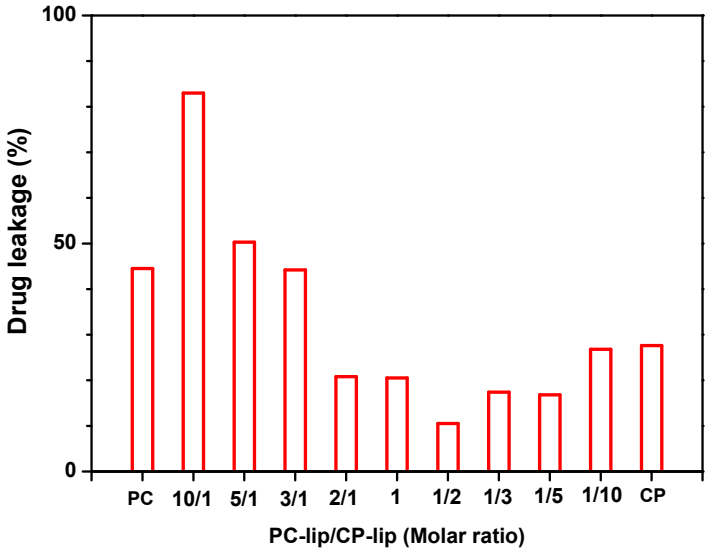

Figure S5. Drug leakage ratio of the liposomes formed by different ratios of PC-lip/CP-lip at 37 centigrade in serum detected by UV-Vis after 24 h.

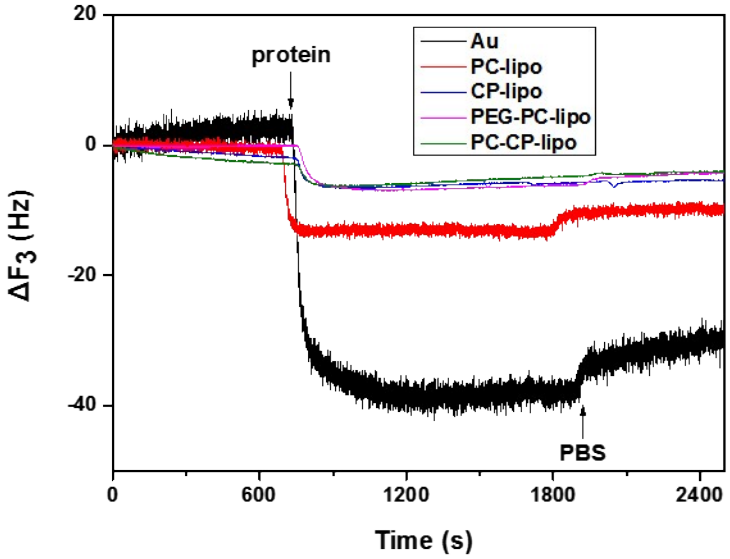

Figure S6. Quartz Crystal Microbalance to test the protein adsorption of different liposomes.

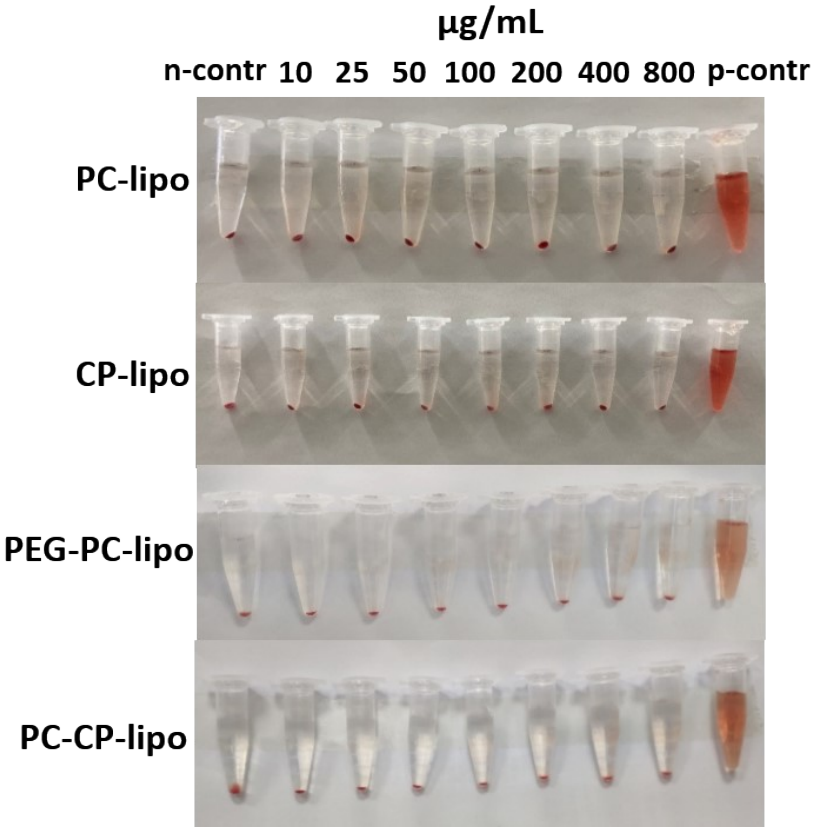

Figure S7. Hemolysis of different liposomes at different concentrations.

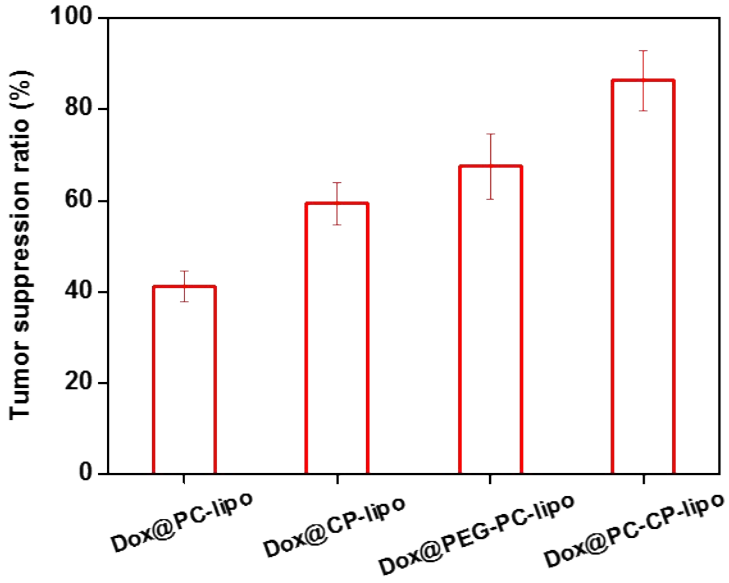

Figure S8. Tumor suppression ratio of different Dox loaded liposomes.

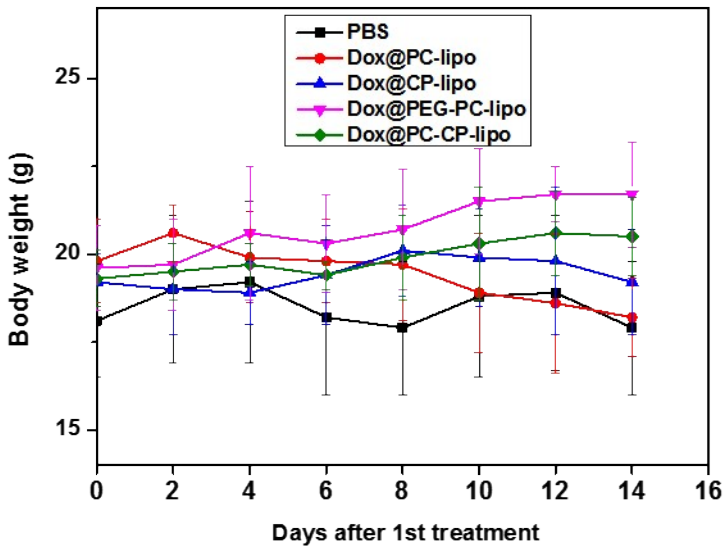

Figure S9. Body weight variation of mice during different treatments.

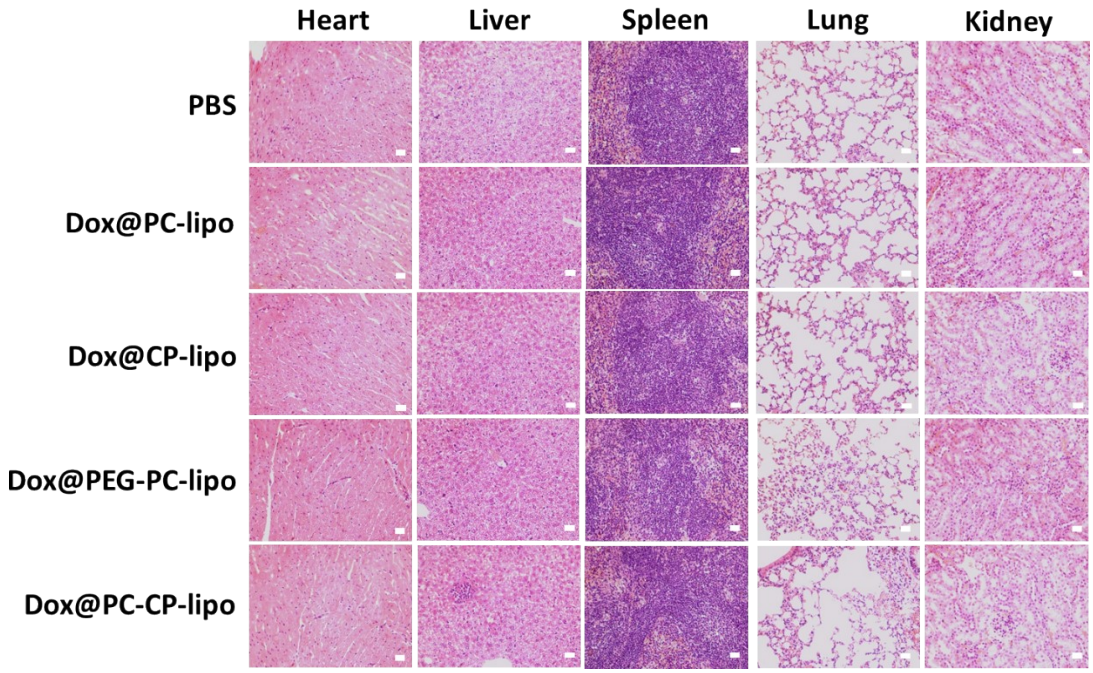

Figure S10. Hematoxylin and eosin staining of normal organs after different treatments. Scale bar: 50 μm.

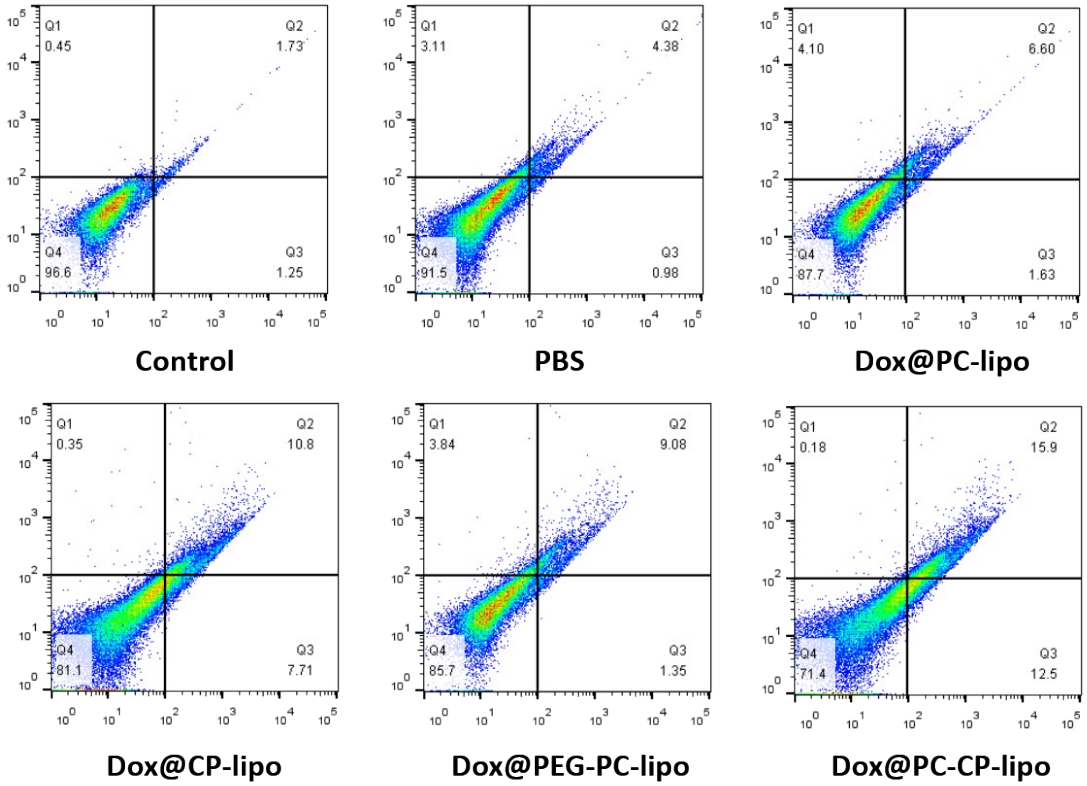

Figure S11. Representative images of proportion of matured dendritic cells ($CD11c^{+}CD80^{+}$) in tumors after different treatments detected by flow cytometry.

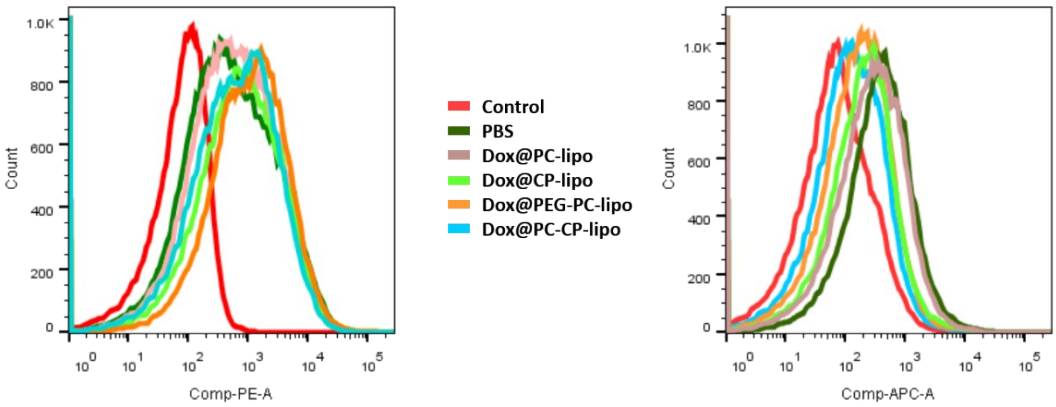

Figure S12. Representative flow cytometry test of CRT-positive cells (left) and Tregs ($CD25^+$, right) in tumors after different treatments.

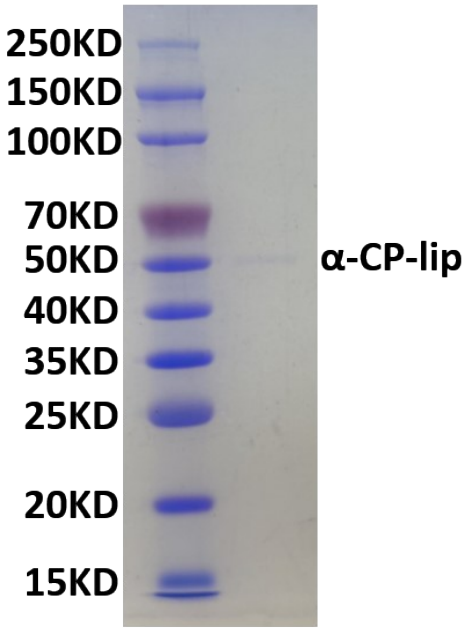

Figure S13. SDS-PAGE analysis of α-CP-lip.

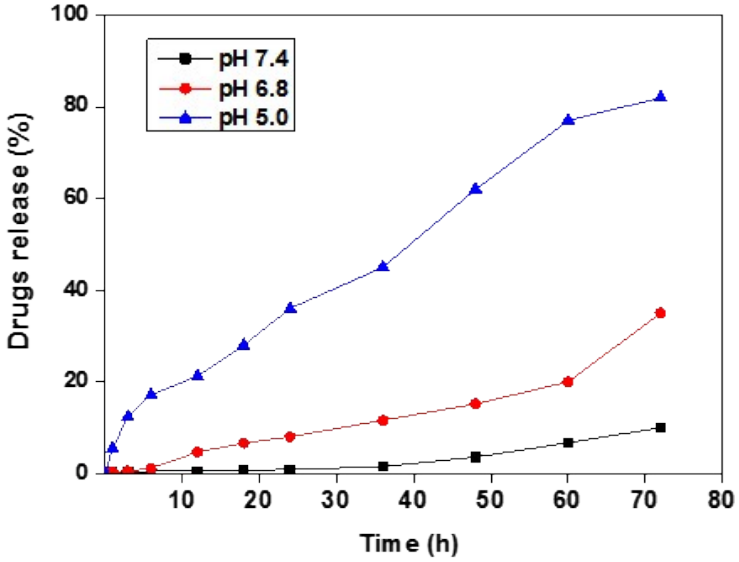

Figure S14. In vitro drug release from Dox@α-lipo at different pH in PBS buffer at 37 centigrade.

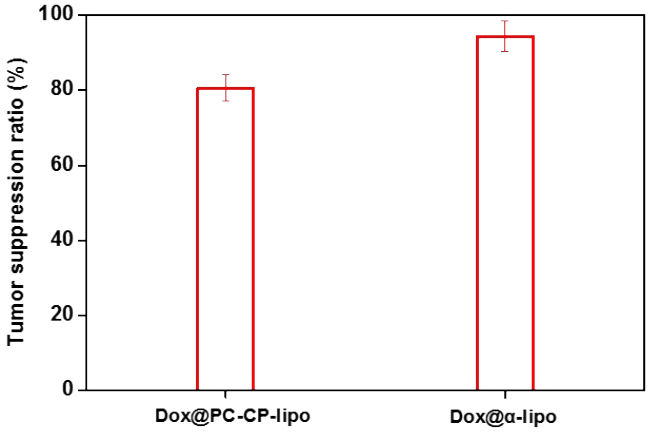

Figure S15. Tumor suppression ratio of Dox@α-lipo and Dox@PC-CP-lipo.

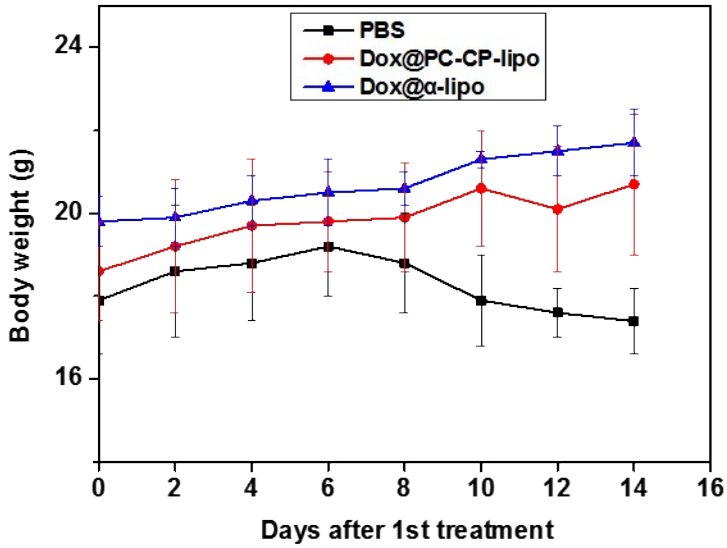

Figure S16. Body weight variation of mice during different treatments.

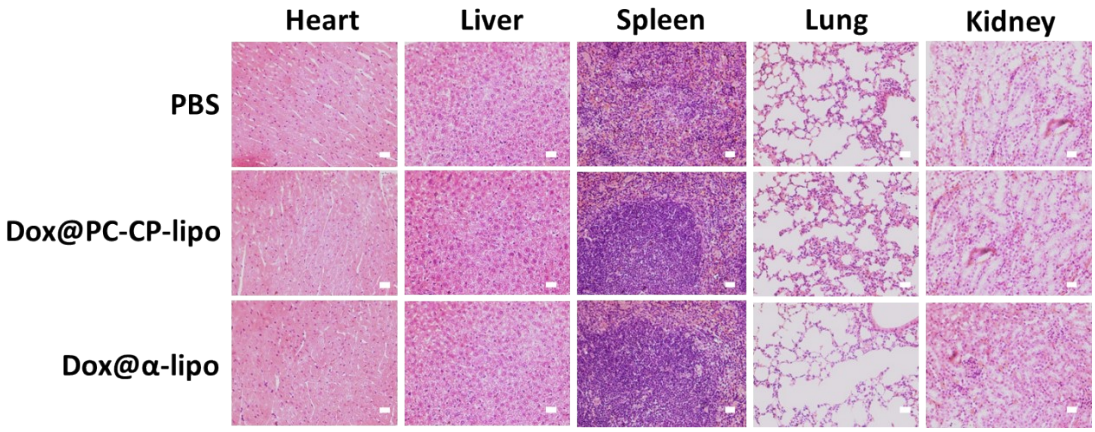

Figure S17. Hematoxylin and eosin staining of normal organs after different treatments. Scale bar: 50 μm.

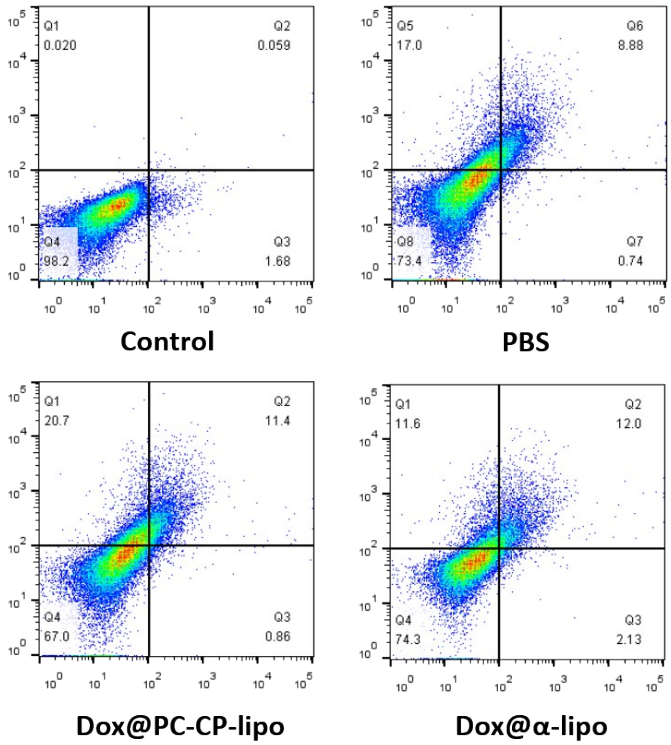

Figure S18. Representative images of proportion of tumor associated macrophages ($F4/80^{+}CD206^{+}$) in tumors after different treatments detected by flow cytometry.

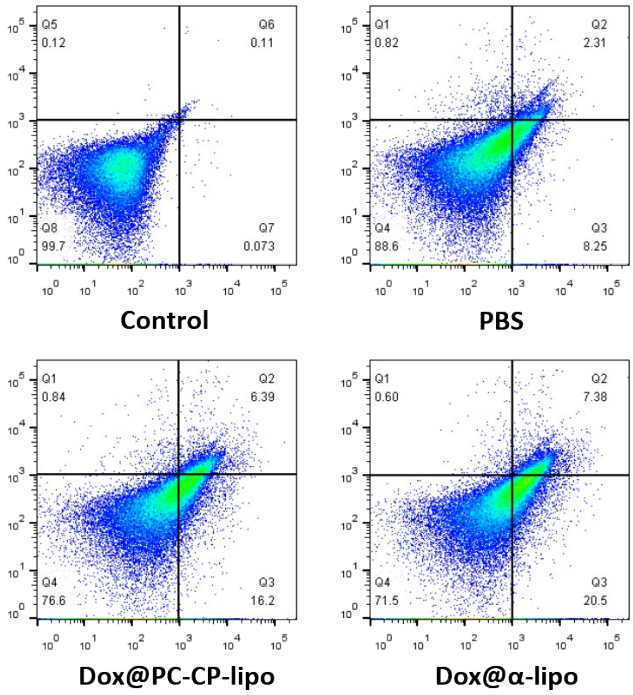

Figure S19. Representative images of proportion of T helper cells ($CD3^{+}CD4^{+}$) in tumors after different treatments detected by flow cytometry.

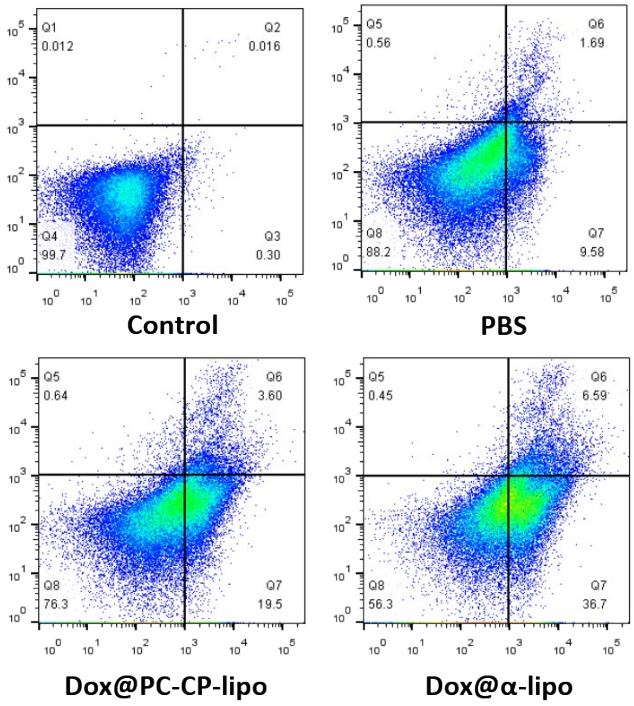

Figure S20. Representative images of proportion of cytotoxic T cells ($CD3^+CD8^+$) in tumors after different treatments detected by flow cytometry.

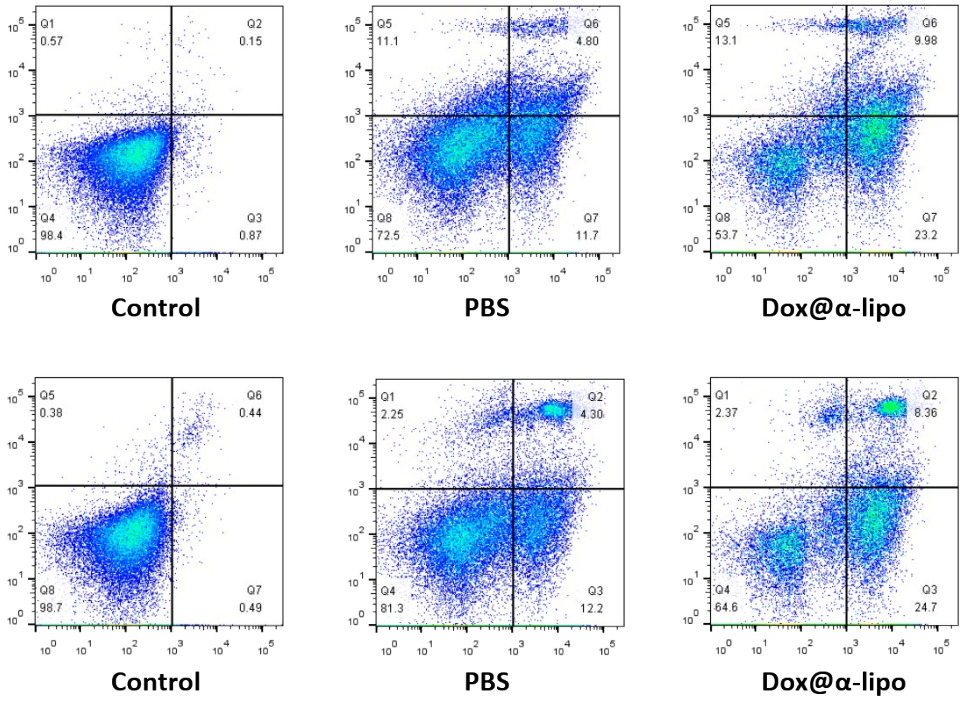

Figure S21. Representative images of proportion of $CD4^+$ central memory T cells ($CD4^+CD44^+CD62L^+$, top) and $CD8^+$ central memory T cells ($CD8^+CD44^+CD62L^+$, bottom) in the spleen detected by flow cytometry.

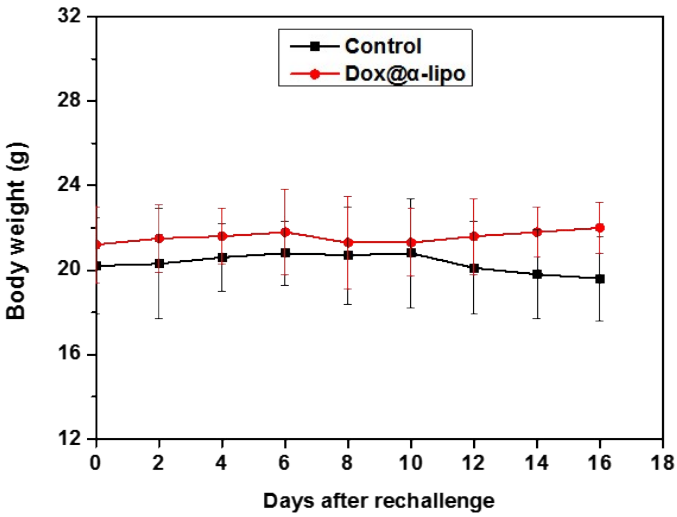

Figure S22. Body weight variation of mice during B16-F10 tumor rechallenge.

References

[1] S. Li, W. Mei, X. Wang, S. Jiang, X. Yan, S. Liu, X. Yu, *Chemical Communications* **2021**, *57*, 1372.

[2] W. Wang, S. Jiang, S. Li, X. Yan, S. Liu, X. Mao, X. Yu, *Chemistry of Materials* **2021**, *33*, 774.