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Supporting Information

Tyrosinase-responsive tumor-specific cascade amplification drug release system for melanoma therapy

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Materials

Acetaminophen (APAP), thiodiacetic acid, 4-Dimethylaminopyridine (DMAP), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), hydroxyethylmethacrylate (HEMA) were purchased from Energy Chemical Co., Ltd (Shanghai, China). Doxorubicin hydrochloride salt was obtained from Hisun Pharmaceutical Co., Ltd (Hangzhou, China). Poly (ethylene glycol)5k-OH (PEG5k-OH) was purchased from Sigma Aldrich (Shanghai, China). All other organic reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (China).

Synthesis of BDOX

The prodrug BDOX was synthesized according to our previous work. Simply, 4-(hydroxymethyl) phenylboronic acid pinacol ester (0.5 g, 2.1 mmol) and p-nitrobenzoyl chloride (0.47 g, 2.4 mmol) were dissolved in 20 mL of anhydrous DCM, 0.6 mL of triethylamine was dissolved in 10 mL of anhydrous DCM and added into the above solution dropwise under the ice-water bath. After 12 h, the product was purified by column chromatography (hexane: ethyl acetate = 3:1). The obtained product (0.2 g 0.5mmol) and doxorubicin hydrochloride (0.20 g, 0.34 mmol) was mixed with triethylamine (143 μL, 1.03 mmol) in 5 mL of anhydrous DMF. The reaction was conducted at room temperature for 24 h in dark. And the product was purified by column chromatography (DCM: methanol = 15:1) to obtain BDOX (0.12 g, 43%). ^{1}H NMR (400 MHz, DMSO) δ = 8.06 (t, 1H), 7.79 (d, 3H), 7.40 (d, 1H), 7.30 (d, 2H), 5.51 (d, 1H), 5.30 (s, 3H), 5.05 (s, 3H), 4.76 (s, 2H), 4.09 (s, 4H), 3.87 (s, 1H), 3.68 (s, 1H), 3.30 (d, 1H), 3.07 (s, 1H), 2.34 (d, 1H), 2.17 (dd, 1H), 1.93 – 1.85 (m, 1H), 1.78 (dd, 1H), 1.36 – 1.28 (m, 12H), 1.25 (d, 3H). ^{13}C NMR (101 MHz, DMSO) δ = 213.88 (s), 186.32(s), 160.66 (s), 156.05 (s), 155.18 (s), 154.44 (s),140.52 (s), 134.38 (s), 126.66 (s), 120.25 (s), 110.60 (s), 94.53(s), 83.58 (s), 74.86 (s), 69.80 (s), 66.59 (s), 64.80 (s), 63.68 (s),

56.46 (s), 47.13(s), 31.94 (s), 29.77 (s), 24.92 (s), 16.99 (s).

Synthesis of 2-(2-((2-(4-acetamidophenoxy)-2-oxoethyl) thio) acetoxy) ethyl methacrylate (HSAPAP)

APAP (10 g, 66.2mmol), thiodiacetic acid (10.9 g, 72.8 mmol), and DMAP (1.6 g, 13.2 mmol) were dissolved in 50 mL anhydrous DMF, EDC (19.0, 99.3 mmol) was dissolved in 30 mL anhydrous DCM and added into the above solution dropwise under the ice-water bath. After 12 h, HEMA (10.3 g, 79.5 mmol) was added into the solution, followed by the dropwise adding of EDC (19.0, 99.3 mmol) in 30 mL anhydrous DCM under the ice-water bath. After 24 h, the solvent was removed by rotary evaporation. The crude product was dissolved in 200ml DCM and washed with 1M HCl (50 mL*3) and saturated salt solution. The product was purified by column chromatography. (hexane: ethyl acetate = 1:1) to obtain HSAPAP (5.2 g, 20.0 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.51 (d, 2H), 7.06 (d, 2H), 6.13 (s, 1H), 5.63 – 5.56 (m, 1H), 4.44 – 4.34 (m, 4H), 3.61 (s, 2H), 3.48 (s, 2H), 2.16 (s, 3H), 1.94 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 169.63 (s), 168.56 (s), 167.13 (s), 146.52 (s), 136.00 (s), 126.35 (s), 121.72 (s), 120.87 (s), 63.21 (s), 62.17 (s), 33.56 (d), 24.47 (s,), 18.29 (s)。

Synthesis of poly (ethylene glycol)-poly (2-(2-((2-(4-acetamidophenoxy)-2-oxoethyl) thio) acetoxy) ethyl methacrylate) (PEG-PAPAP).

Block copolymer PEG-PAPAP was obtained via RAFT, and macromolecular chain transfer agent PEG_{5K}-PETTC was prepared according to the literature. PEG-PETTC (0.20 g, 0.037 mmol), HSAPAP (0.20 g, 0.5 mmol), AIBN (6.56 mg, 0.04 mmol), and DMF (2 mL) were charged into a Schlenk tube with a magnet. After removing the O_2 by N_2 for 0.5 h, the reaction was heated to 70 °C for 15 h. PEG-PAPAP was obtained by precipitating in diethyl ether. (0.31g 77.5%) ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.50 (24 H), 6.99 (24H), 4.47 – 4.02

(36H), 3.64 (439 H), 2.07 (72 H).

Determination of critical micelle concentration (CMC).

Empty micelles were dissolved in water with different polymer concentrations from 0.1 μg/mL to 1 mg/mL. Nile red was dissolved in acetone at 7 μg/mL and 0.1 mL of the solution was added into bacteria bottles. After evaporation of the acetone, 2 mL of micelle was added to the bacteria bottles and the solution was vigorously stirred for 12 h. Finally, the fluorescence intensity was plotted as a function of micelle concentration. The data formed two distinct lines, the concentration at which these lines intersect was calculated and determined to be the CMC.

Preparation and characterization of TR-CARN

TR-CARN was prepared through the co-precipitation method. Simply, PEG-PAPAP (25 mg) and BDOX (8 mg) were dissolved in 300 μL of DMSO, and the solution was added into 4 mL deionized water under vigorously stirring. And the DMSO was removed by dialyzing. The precipitated BDOX was removed by filtration. The loaded BDOX was determined by HPLC. The size and zeta potential of the TR-CARN was characterized on a DLS spectrometer (Nano series ZEN3600, Malvern Instruments Ltd., UK), and the morphology was characterized on a TEM (JEM-1200EX, Japan).

Loading content = (BDOX in TR-CARN) / (BDOX and PEG-PAPAP in TR-CARN) * 100%

Encapsulation efficiency = (BDOX in TR-CARN) / (Feeded BDOX in preparation process) * 100%

Stability test of the TR-CARN.

The micelles solution (10 mg/mL) was prepared and mixed with PBS with or without FBS. The mixture was incubated at 37 °C and tested at intervals using DLS. The Z-average diameters

from every test were recorded and plotted as a function of time.

DOX and APAP release experiment.

TR-CARNs (1 mL) fabricated as described above were sealed in a dialysis bag with a molecular weight cut off of 3500 Da and incubated in 37 mL PBS containing 2% Tween 80 with different concentrations of H_2O_2. 100 μL of the solution outside the dialysis bag was collected at certain intervals to determine the DOX concentration with HPLC. APAP release experiment was conducted at three H_2O_2 concentrations, 0, 0.1 mM, and 1 mM. The next steps were the same as the DOX.

Intracellular ROS measurement

B16F10 and 4T1 cells were cultured in glass-bottom Petri dishes at a density of 100 000 cell/plate for 24 h before treatment. Cells were exposed to APAP, BDOX, and TR-CARN, and the equivalent DOX dose was 0.1 μM, and the APAP dose was 1 μM. After 2 h, the cells were stained with DCFH-DA in serum-free medium for 30 min, and cell nuclei were stained with Hoechst 33342 (Molecular Probes, Carlsbad, CA, two drops per mL medium) for 15 min. After washing with PBS 3 times, cell images were obtained on a CLSM (Nikon-A1 system, Japan). The DCF fluorescence was excited and monitored at the wavelengths of 488 and 523 nm, respectively, according to the recommended procedure of the kit.

***In vivo* ROS evaluation.**

C57BL/6 mice were injected with 10^6 B16F10 cells subcutaneously. Once the tumor volume reached 160 mm^3, the mice were treated with APAP I.P. and PEG-PAPAP I.V. After 6 h, the mice were intratumorally injected with 100 μL 1 mg/mL dihydroethidium (DHE). The mice were sacrificed after 4 h, and the tumors were harvested for the frozen section. And the fluorescence of the DHE was observed with CLSM (Nikon-A1 system, Japan). The

fluorescence intensity was calculated by Image J.

Cell uptake experiment and DOX activation

Confocal images of the uptake and DOX activation. B16F10 cells were cultured in glass-bottom Petri dishes at a density of 100 000 cell/plate for 24 h before treatment. Cells were exposed to BDOX, APAP+BDOX, DOX, and TR-CARN at the equivalent DOX dose was 0.1 μM, and the APAP dose was 1 μM. After 2 h, cell nuclei were stained with Hoechst 33342 (Molecular Probes, Carlsbad, CA, two drops per mL medium) for 15 min. After three washes with PBS, cell images were observed using a CLSM (Nikon-A1 system, Japan). DOX fluorescence (Ex 510 nm, Em 550 nm) was expressed as red, Hoechst 33342 (Ex 405 nm, Em 460 nm) was expressed as blue.

Flow cytometry analysis of the uptake and DOX activation. B16F10 cells were seeded onto twelve well plates at 100 000 cells/well as described above, treated with DOX, BDOX, or TR-CARN. The equivalent DOX dose was 0.1 μM, and the APAP dose was 1 μM. Cellular uptake and DOX activation were analyzed in the FL2 channel on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser used to scan DOX fluorescence.

Cell culture and *in vitro* cytotoxicity assays

B16F10, 4T1, and NIH/3T3 cell lines were purchased from ATCC (American Type Culture Collection). B16F10 was maintained in RPMI 1640 (Gibco) medium supplemented with 10% FBS (Gibco). For activation of the Tyr expression, the medium was replaced with DMEM (Gibco, USA) medium. NIH/3T3 was maintained in DMEM (Gibco, USA) medium. 4T1 was grown in RPMI 1640 (Gibco) medium. All the mediums were supplemented with penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were maintained in a humidified

atmosphere of 5% CO_2 at 37 °C.

3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to assess the cytotoxicity of APAP, BDOX, APAP+BDOX, and the TR-CARN in B16F10, 4T1, and NIH/3T3 cells. Briefly, cells were seeded in 96 well plates at a density of 3500 cells per well and incubated overnight. Cells were exposed to serial dilutions of the drugs and cultivated for another 48 h, and then the medium was replaced by a fresh solution containing 0.75 mg/mL MTT. After a 3 h incubation, the yellow tetrazolium salt (MTT) was metabolized into dark blue formazan crystals, and the MTT medium solution was carefully removed. Finally, 0.1 mL of DMSO was added into the wells, and the plate was gently shaken to dissolve the precipitates. The absorbance in each well was determined at 562 nm and 620 nm using a microplate spectrophotometer (Molecular Devices, SpectraMax M2e, USA). Cell viability was calculated as the ratio of the absorbance of the wells incubated with the drug to that of the wells incubated with culture medium.

***In vivo* antitumor studies**

C57BL/6 mice were injected with 10^6 B16F10 cells subcutaneously. Once the tumor volume reached 60 mm^3, mice were randomly assigned to five treatment groups (n = 6): PBS, APAP, BDOX, DOX, TR-CARN. DOX-equivalent dose was 5 mg/kg and APAP equivalent dose was 1.5 mg/kg. APAP was implemented by I.P. injection, the other treatments were implemented by I.V. injection every 2 days 5 times. Tumor volume (mm^3) was calculated using the formula: tumor volume = $(\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$. The tumor growth curves were plotted using the average tumor volume versus days after the first treatment. All the mice were sacrificed 10 days after the first treatment, and their tumors and main organs were resected.

Plasma pharmacokinetic study.

In the plasma pharmacokinetic study, female ICR mice were randomly divided into 2 groups (n = 4) and intravenously injected with DOX or TR-CARN at a DOX-equivalent dose of 10 mg/kg. Blood samples were collected into heparinized tubes and 100 μL of the supernatant plasma was mixed with 900 μL of acetonitrile to precipitate all the proteins. After centrifugation, the organic layer was collected, concentrated, and subjected to HPLC to determine the DOX and BDOX levels. Previously constructed standard curves of DOX and BDOX were used to determine the exact plasma concentration.

Histological examination of tumor tissues

The excised main organ and tumor in each group were fixed using 4% paraformaldehyde buffer, embedded in paraffin, and sectioned into 5 μm thick slices. The sections were stained with H&E (Beyotime, Shanghai, China) for histological examinations and imaged under an inverted microscope.

Statistical analysis

Data were presented as the mean ± SD. Assignments to treatments and selections of fields of microscopic inspection were made at random. Statistical significance was evaluated using the Student t-test when the groups showed different variances. Differences were considered statistically significant at a level of $p<0.05$ and very significant when $p<0.01$

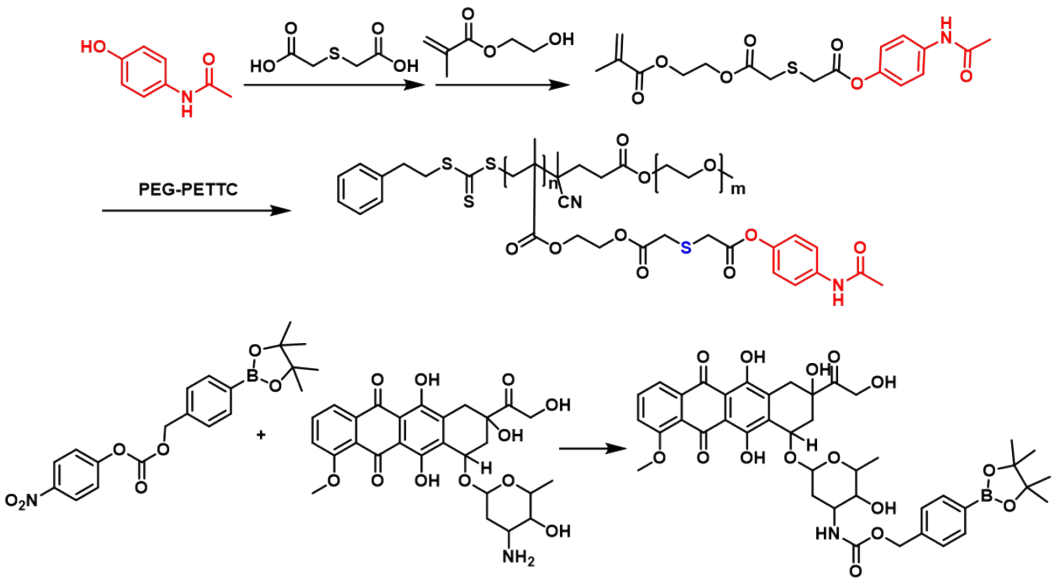


Figure S1. Synthesis routes of the PEG-PAPAP and BDOX.

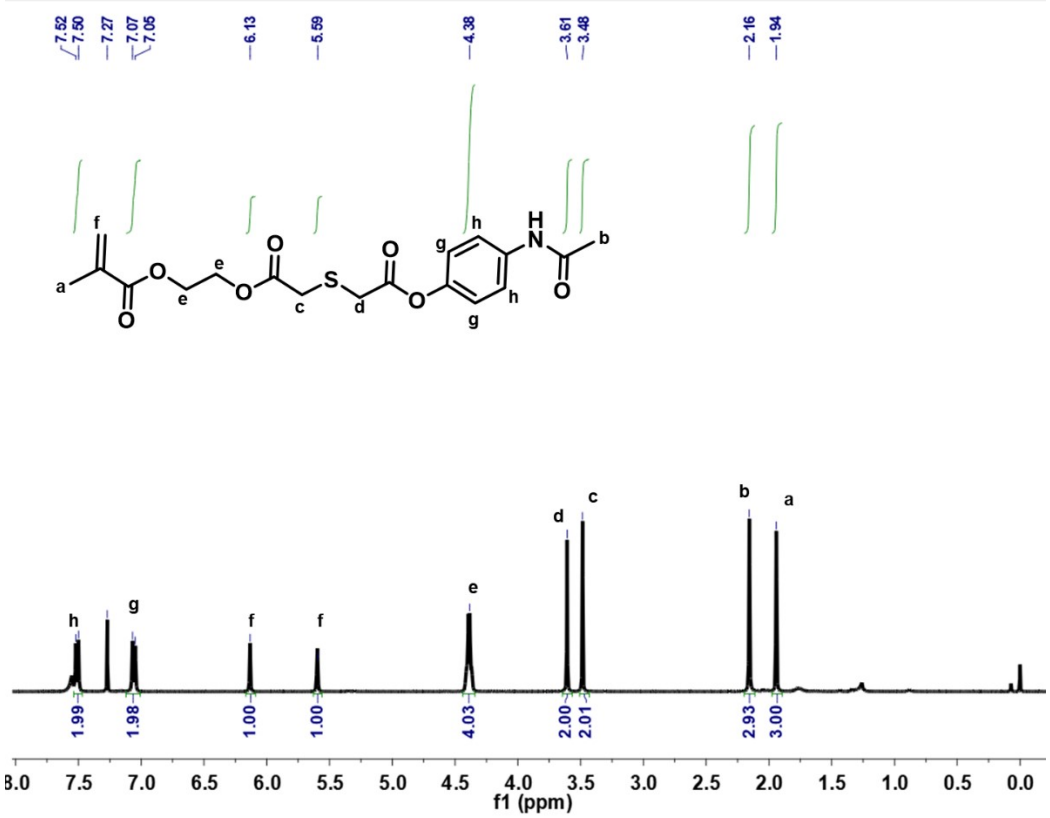


Figure S2. ^{1}H NMR spectra of HSAPAP in $CDCl_3$.

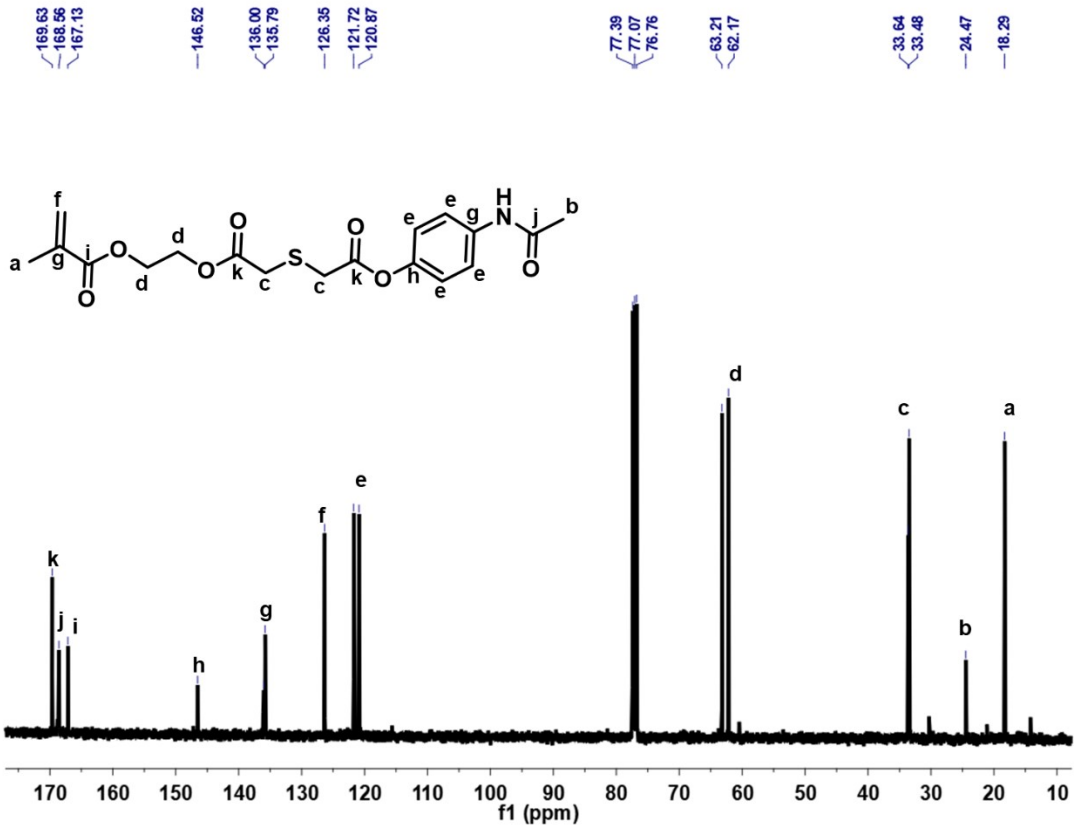


Figure S3. ^{13}C NMR spectra of HSAPAP in $CDCl_3$.

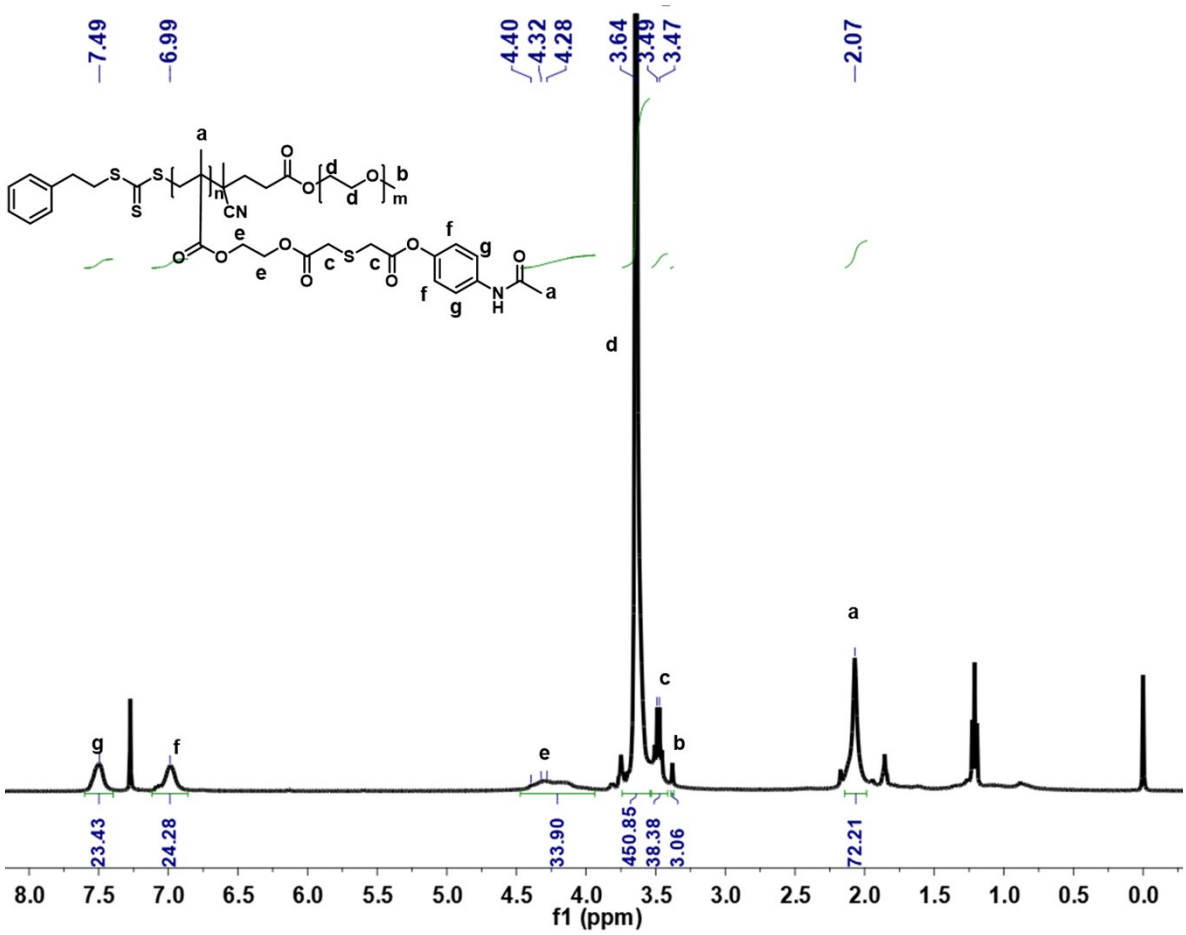
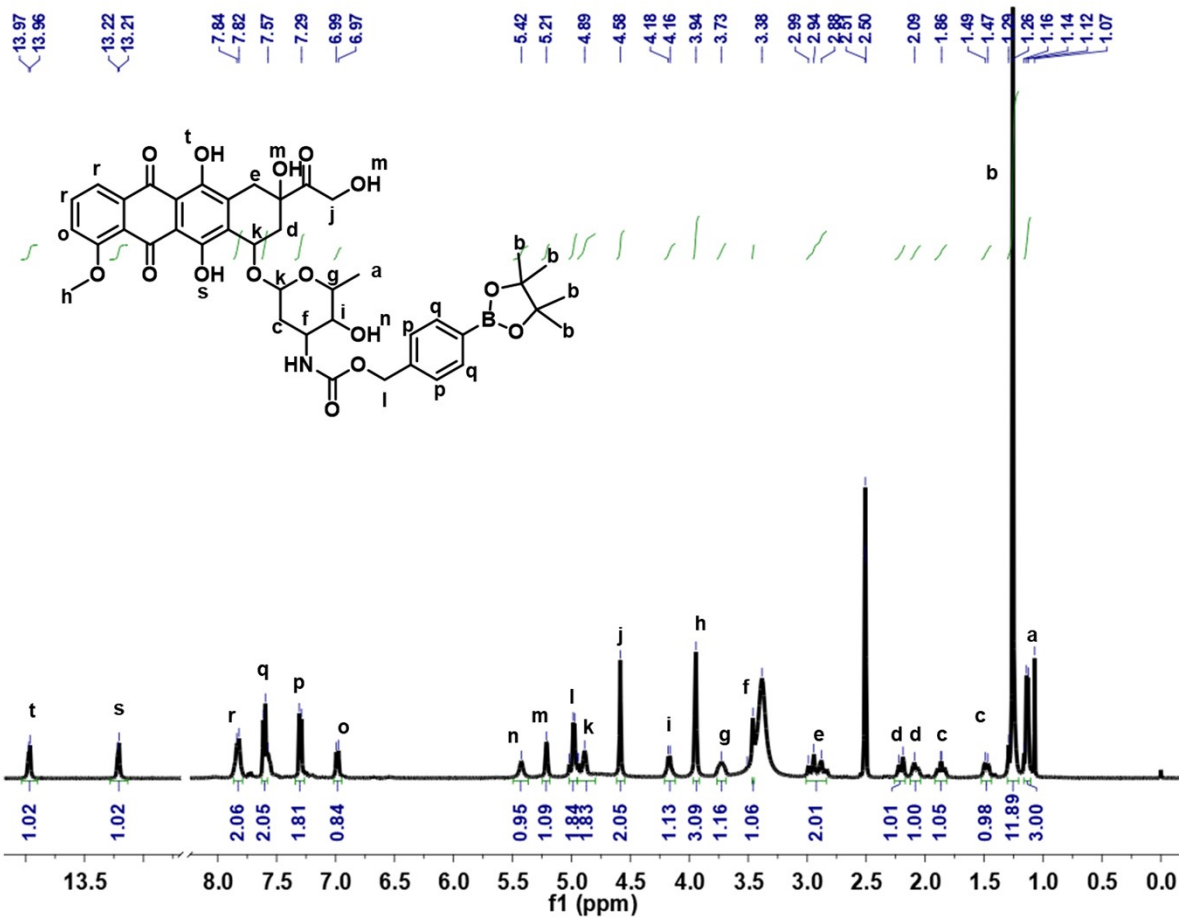


Figure S4. ^{1}H NMR spectra of PEG-PAPAP in $CDCl_3$.



Figur. S5. ^{1}H NMR spectra of BDOX in DMSO.

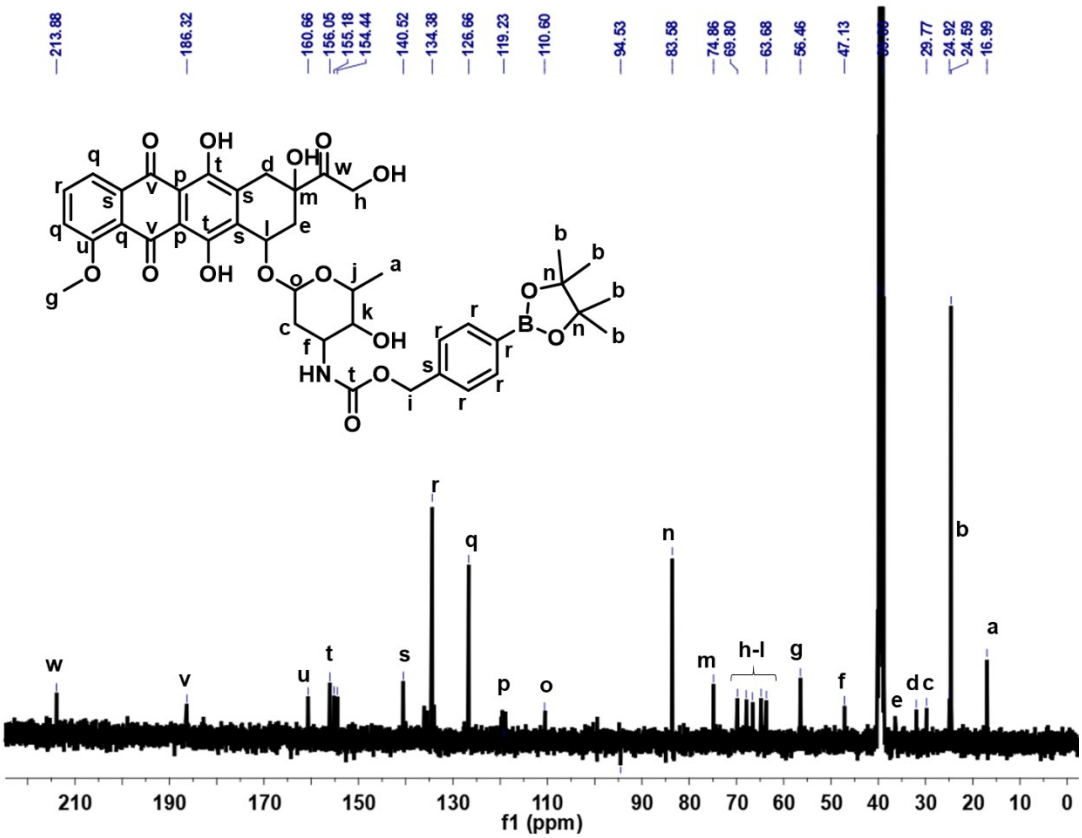


Figure S6. ^{13}C NMR spectra of BDOX in DMSO.

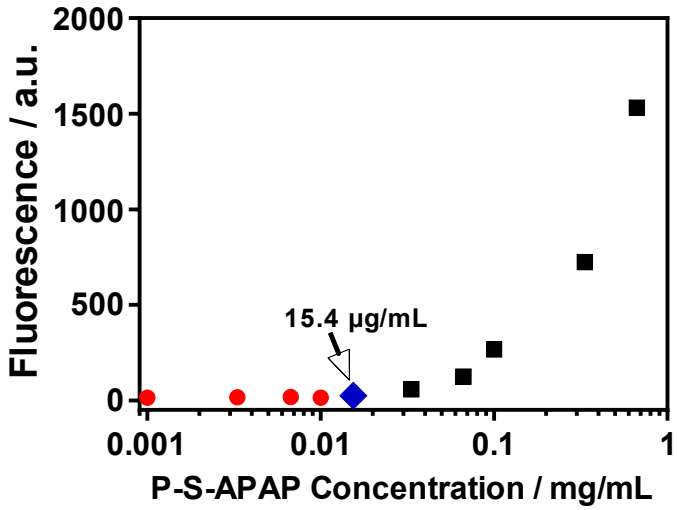


Figure S7. CMC of nanoparticles determined by Nile Red.

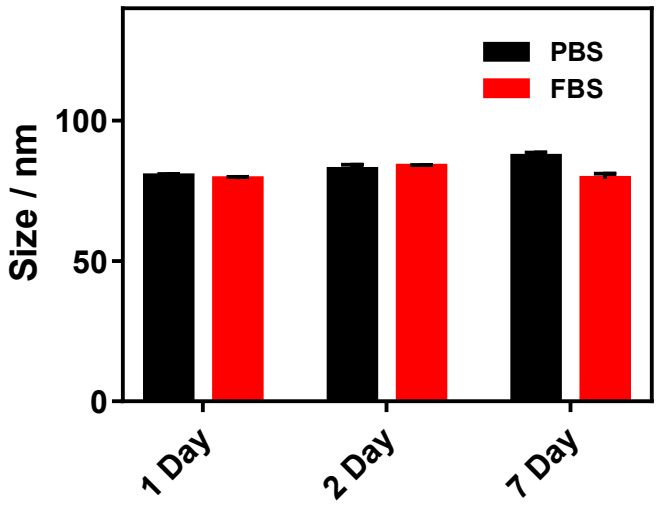


Figure S8. Stability of the micelles after days incubation in PBS or FBS.

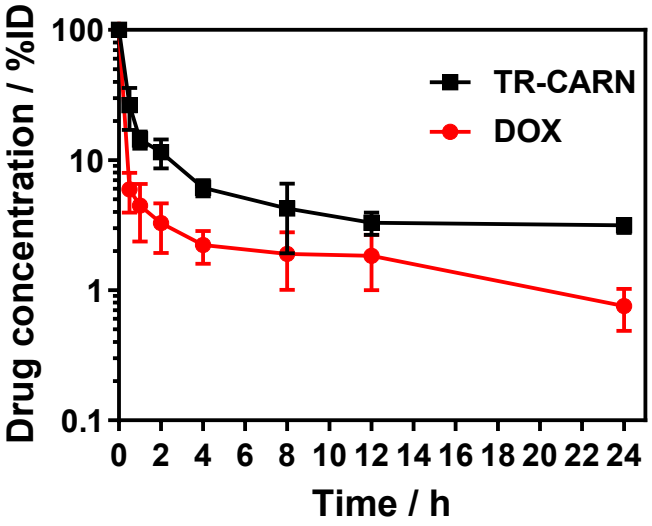


Figure S9. Percentage of the injected dose for DOX and TR-CARN in blood.

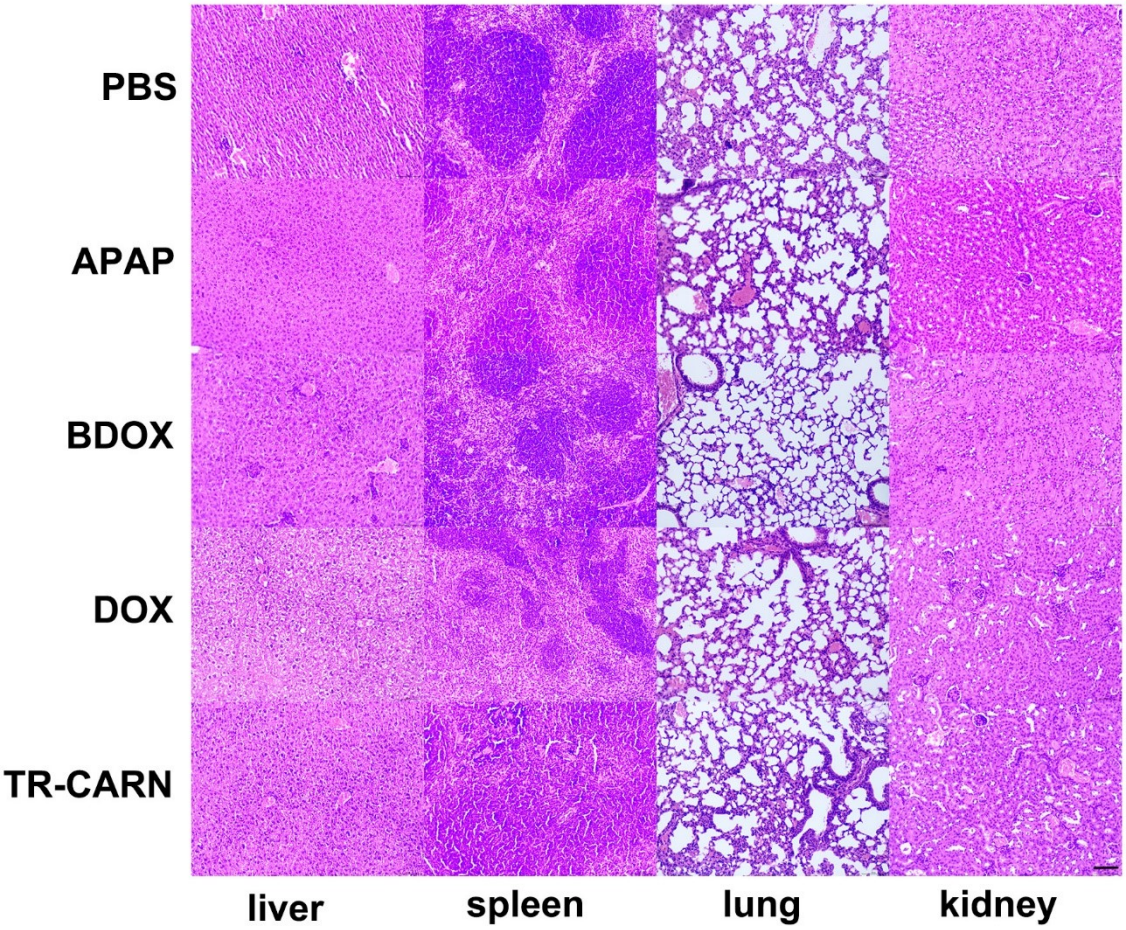


Figure S10. Histological images of the main organ from the treated mice, scale bars: 150 μm.