Electronic supplementary Information

Enhanced anti-metastatic therapy with down-regulation of heparinase expression by ROS-responsive micellar nanoparticles

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1. Materials.

Doxorubicin hydrochloride (DOX·HCl, > 98%) was purchased from Melonepharma (Dalian, China).

Polypropylene sulfide (PPS-COOH, MW 4500) was purchased from Xi'an ruixi Biological Technology Co.,Ltd (Xi'an, China).

Laminarin (LAM, MW 8000, containing sulfate groups) was purchased from Sigma-Aldrich. (Sulfate content can be increased by chlorosulfonic acid-pyridine method)

PEG2000 (>95%, MW 2000) was purchased from GuangZhou Tanshui Technology Co.,Ltd.(Guangzhou, China).

4-(Dimethylamino) pyridine (DMAP) (> 99%) was obtained from Chengdu Kelon Chemical Reagent Factory. (Chengdu,China).

Dicyclohexylcarbodiimide (DCC, >99%) was purchased from ChengDu Mixcy Chemical Co.,Ltd. (Chengdu, China).

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Biosharp (Shanghai,China).

1, 10-dioctadecyl-3,3,30,30-tetramethylindodicarbocyanine,

4- Chlorobenzenesulfonatesalt (DiD) was obtained from Biotium (Hayward, California, USA).

Mouse Heparinase Elisa Kit was purchased from cloud-clone (Wuhan, China).

Tetrahydrofuran (THF>99.5%, Extra Dry, with molecular sieves) and Dimethyl sulfoxide (DMSO>99.7%, Extra Dry, with molecular sieves) were purchased from Sun Chemical Technology Co.,Ltd (Shanghai, China).

Other reagents and chemicals were analytical level.

2. Cell Lines and Animals.

Mouse melanoma cells (B16F10) was purchased from the Shanghai Institutes for Biological Sciences, CAS (SIBS, Shanghai, China).

All cells mentioned above were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (ZhongQiaoXinZhou), 80 μg mL^{-1} streptomycin and 80 U mL^{-1} penicillin at 37 °C in a humidified atmosphere of 5% CO2 in air.

C57BL/6 female mice (about 6-week-old, 18-22 g, SPF) was purchased from Chengdu Dashuo Biological Institute (Chengdu, China).

All animal procedures for this study were in compliance with the Experiment Animal Administrative Committee of Sichuan University.

3. Synthesis and characterization of the LAM-PPS conjugate

To obtain the amphiphilic LAM-PPS conjugate, the carboxylic acid group of PPS-COOH and the hydroxyl of LAM were connected via ester bond formation. First, PPS-COOH (91mg, 0.02mmol), DMAP (8.5mg, 0.07mmol) and DCC (14.5mg, 0.07mmol) were dissolved in THF (6mL). Meanwhile, laminarin (160mg, 0.02mmol) was dissolved in DMSO (10mL) and added into PPS-COOH solution. The mixture was

stirred in an ice bath under an argon atmosphere for 30 min, then transferred to a 45°C oil bath and stirred for 24h under an argon atmosphere. Then the reaction solution was dialyzed against deionized water for 48h and lyophilized. The successful synthesis of the LAM-PPS conjugate was confirmed by ^{1}H-NMR spectroscopy and IR spectrum.

4. Synthesis and characterization of the PEG-LAM-PPS conjugate

In the same way, PEG-LAM was synthesized via ester bond formation.

PEG-COOH (60mg, 0.03mmol), DMAP (8.5mg, 0.07mmol) and DCC (14.5mg, 0.07mmol) were dissolved in THF (2mL). The mixture was stirred in an ice bath under an argon atmosphere for 30 min to activate the carboxylic acid groups in PEG-COOH, and then added slowly into the LAM-PPS reaction solution. The reaction mixture was stirred for 24h under an argon atmosphere at 45 °C and then dialyzed against deionized water for 48h and lyophilized. The successful synthesis of the PEG-LAM-PPS conjugate was confirmed by^{1}H-NMR spectroscopy and IR spectrum. Dextran (DEX) was chose as a control of LAM to synthesis PEG-DEX-PPS via the same method.

5. Preparation and characterization of DOX-loaded nanoparticles

DOX-loaded NPs (DOX-nPLPs) were prepared by ultrasonic emulsification. First, doxorubicin oleate (DOX-OA) was prepared to make it easier to be contained in the micelles. DOX hydrochloride (10 mg) was neutralized with sodium bicarbonate (100 μL, 50mg mL^{-1}) in water (4 mL), and oleic acid ethanol solution (10%) was added into the DOX solution and shaked vigorously to obtain DOX-OA complex. DOX-OA (3mg) was dissolved in dichloromethane (500μL) and mixed with PEG-LAM-PPS conjugate (80mg), and then stirred for 3h at 37°C in the dark. Then, the mixture was added to

deionized water (3mL) under ultrasonication 200 W, 5 s/5 s, 10 min) and vaporized by rotary evaporation at 37 °C to remove the dichloromethane. G-75 dextran Sugar gel chromatography column was used to remove free doxorubicin oleate that was not loaded into micelles. The control micellar nanoparticles (nPDPs and DOX-nPDPs) were generated by the same method. The mean sizes and zeta potentials of the nanoparticles in deionized water were measured by a Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). In addition, the morphologies of the nanoparticles were observed under a transmission electron microscope (Hitachi H-600, Japan). The micelle dispersion was disrupted by THF (5 volumes) and methanol (500 volumes) to measure the drug-loading capacities of the nanoparticles. The drug content was analyzed by a Varioskan Flash multimode reader (Thermo, USA) at Ex =480 nm and Em = 592 nm. Drug-loading capacity (%) = (weight of DOX in NPs) / (weight of DOX-loaded NPs) × 100.

6. Critical micelle concentration (CMC) of polymer

The CMC value of polymers was determined using pyrene as the hydrophobic fluorescence probe. nPLPs were diluted to different concentrations (1×10^{-5} mg mL^{-1}, $1 \times 10^{-4.5}$ mg mL^{-1}, 1×10^{-4} mg mL^{-1}, $1 \times 10^{-3.5}$ mg mL^{-1}, 1×10^{-3} mg mL^{-1}, $1 \times 10^{-2.5}$ mg mL^{-1}, 1×10^{-2} mg mL^{-1}, $1 \times 10^{-1.5}$ mg mL^{-1}, 1×10^{-1} mg mL^{-1}, $1 \times 10^{-0.5}$ mg mL^{-1} and 1 mg mL^{-1}) and added in tubes containing pyrene. After ultrasonication in water bath for 1.5h, the fluorescence intensity was analyzed by spectrofluorometer (SHIMADZU RF-600, JAPAN). Samples were emitted with 390 nm light, and the excitation spectra were recorded ranging from 300-350 nm. Plot of intensity ratio (I_{336}

/I_{333}) against the logarithms was obtained.

7. ROS-responsive properties of nPLPs

The ROS-responsive properties of nPLPs were evaluated by DLS and TEM. The nPLPs (2 mg mL $^{-1}$) are kept in water for 4h with or without 1M H_2O_2, which are dropped on copper meshes after DLS analysis. After 5 min, unnecessary liquid is removed and the Phosphotungstic acid solution (10 μL) is dropped on this copper mesh to stain the samples for 10 min. Finally, the copper meshes are washed with deionized water for 20 min, and dried at room temperature for observation.

8. Serum stability assay

Turbidity Method was used to evaluate serum stability. Micellar preparations was mixed with FBS (micellar preparations: FBS (v/v) = 1: 1). The concentration of micellar material was finally equal to 1 mg mL^{-1}. The mixtures was added to a 96-well plate and incubated in a shaking bed at 37°C for 24h. Varioskan Flash Multimode Reader (Thermo, USA) was used to measure the absorbance at 750 nm at different time points (0, 1, 2, 4, 8, 24 h).

9. *In vitro* drug release

Dialysis method was used to evaluate the *in vitro* DOX release behavior of DOX-nPLP. Free DOX-OA and DOX-nPLP solution (1mL, equivalent to the dose of DOX 3.5μg) were loaded into the dialysis bags (2kDa molecular weight cutoff). Dialysis bags were immersed in PBS (7 mL, Free DOX-OA: pH 7.4 PBS; DOX-nPLP: pH 7.4 PBS; pH 7.4: 100 μmol L^{-1} or 1 mmol L^{-1}, 5mmol $L^{-1}H_2O_2$ PBS) and incubated in a shaker at 37°C and 100 rmp. Release medium (1mL) was collected at different time points (1,

2, 4, 8, 11, 24, and 48 h) while release medium under the same conditions was rejoined the system. The amount of released drug was determined by the Varioskan Flash Multimode Reader (Thermo, USA) at Ex=480 nm and Em=592nm.

10. Cellular uptake in B16F10 cells

We conducted cell uptake assays in serum-free medium without or with H_2O_2. Briefly, B16F10 cells were seeded in 12-well plates (1×10^5 per well) and grown overnight before incubation with DOX-nPLP. After incubation with DOX-nPLP in H_2O_2-free, containing 25μmol L^{-1} H_2O_2 and 50μmol $L^{-1}H_2O_2$ medium for 2 h, the cells were harvested and washed with PBS. The cells were resuspended in PBS (300μL) after Centrifugation (2000 rpm for 3 minutes), and a flow cytometry (Beckman Beckman Coulter, Inc., USA, Cytomics FC 500) was used to quantify the mean fluorescence of DOX in individual cells.

We also used CLSM to detect the fluorescence signals of B16F10 cells in serum-free medium without or with H_2O_2. Briefly, B16F10 cells were seeded in confocal microscope dishes (1×10^5 per well) and grown overnight before incubation with DOX-nPLPs. After incubation with DOX-nPLPs in medium without H_2O_2, with 25 μmol L^{-1} H_2O_2 or with 50 μmol $L^{-1}H_2O_2$ for 2h, the cells were washed with PBS (pH 7.4) for three times, and were finally fixed in 4% (m/v) paraformaldehyde solution for 15 min. For nuclei labelling, the cells were stained with 0.5% DAPI for 5 min. After being washed five times with PBS, the cells were observed by CLSM (DOX: Ex = 488 nm, Em = 555 nm; DAPI: Ex = 340 nm, Em = 488 nm; Olympus Co., Ltd, FV1000, Japan).

11. ROS assay

B16F10 cells were cultured as a density of 1×10^5 in confocal microscope dishes. After incubation with or without ascorbic acid for 12h. The cells were stained with 10 μM 2', 7'-dichlorofluorescin diacetate (DCFH-DA) for 20 min and washed with PBS three times. CLSM and was used for analyzing the ROS level in B16F10 cells.

12. Intracellular drug release

To validate the intracellular drug release behavior of nPLPs, fluorescence resonance energy transfer (FRET) technique was used. A pair of FRET dyes, 3, 3'-dioctadecyloxacarbocyanine perchlorate (DiO) and 1, 1'-dioctadecyl-3, 3, 3, 3'-tetramethylindocarbocyanine per-chlorate (DiI) were encapsulated into nanoparticles. The fluorescence intensity was determined by spectrofluorometer (SHIMADZU RF-600, JAPAN). Fluorescence was excited by light at 460 nm, and the emission spectra were recorded ranging from 480-700 nm.

In addition, we further demonstrated the intracellular disintegration of nPLPs by confocal imaging of FRET signal after incubating DiO/DiI-nPLPs with B16F10 cells or ROS scavenged B16F10 cells. B16F10 cells were seeded in confocal dishes at a density of 1×10^5 cells per well and incubated with or without ascorbic acid for 12 h. Then, cells were washed and incubated with DiO/DiI-nPLPs for 2h and further incubated in fresh culture medium for 2h. FRET signal could be detected by Confocal Microscope.

13. *In vitro* cytotoxicity analysis

The MTT assay was used to investigate the toxicity of nPLP and DOX-nPLP. B16F10 cells were seeded in a 96-well plate (3×10^3 cells/well), and the plate was

incubated at 37°C and 5% CO2 for 12 hours. PBS, free DOX, DOX-OA, nPLPs, and DOX-nPLPs was diluted by serum-free medium to different concentrations and added to the wells after removing the culture medium. The plate was incubated for an additional 24h, and then, MTT solution (20μl, 5 mg mL^{-1} in PBS) was added to each well. The plate was incubated at 37°C and 5% CO2 for 4 hours. Finally, dimethyl sulfoxide(150μl) was added to each well after removing the culture medium. A microplate reader (Thermo, USA) was used to detect the absorbance of each group at 630 nm. The Cell viability (%) was calculated by the following formulas: Cell viability (%) = $(A_{test}-A_{DMSO})/ (A_{control}-A_{DMSO}) \times 100$.

14. *In vitro* inhibition effect of cell migration and invasion

Wound-healing and Transwell assays were used to evaluate the inhibitory effect of DOX-nPLP on the migration and invasion of B16F10 cells. For the wound healing analysis, B16F10 cells were seeded into a 6-well plate (5×10^5 cells/well), and cultured with complete medium until the cell density was above 95%. Scratch wounds were made by a 200μl pipette tip. The scratched cells were washed with PBS to form cell-free and straight scratches. PBS, free DOX (0.5μg mL^{-1}), LAM, nPLPs (100 μg mL^{-1}) and DOX-nPLPs was diluted by serum-free medium and the added into the plate. Images were obtained at 0 hours and 24 hours with an inverted microscope. Image J was used to count the scar area and calculate the healing rate.

The inhibitory effect of micelles on the migration of B16F10 cells was investigated by Transwell assay. For the cell transwell assay, 100μl cell suspension(1×10^5 cells, free serum) containing PBS solution, LAM, free DOX (0.5μg mL^{-1}), blank nPLP (40μg mL^{-}

^{1}), DOX-nPLPs，nPDPs or DOX-nPDPs respectively was loaded into the upper chamber of transwells (24-well insert; pore size, 8μm; Corning, USA) coated with Matrigel (BD Biosciences).The lower chamber were loaded with 600 μL of 20% FBS medium. After 48 h of incubation, the cells were fixed in 4% (m/v) paraformaldehyde solution for 10 minutes and stained with crystal violet. Finally, the bottoms of the chambers were observed under an inverted microscope and taken photos in five predetermined fields. Image J was used to count the number of cells and calculate the relative proportion of migration cells.

15. HPA expression detection *in vitro*

B16F10 cells were seeded in a 6-well plate (1.5×10^5/well) and grown overnight. After that, the cells were incubated with PBS, LAM, nPLP (100μg mL^{-1}), DOX-nPLPs, nPDPs or DOX-nPDPs for additional 36 hours. Mouse HPA ELISA kit was used to detect HPA expressed in culture medium and cells.

16. *In vivo* Tumor-targeting Assay and Metastasis-targeting assay

For solid tumor targeting assay, The B16F10 solid tumor-bearing mouse model was constructed by inoculating 1×10^6 B16F10 cells on the armpits of female C57BL/6 mice. When the tumors had grown to 200-300 volume, the tumor-bearing mice were injected with PBS, free DiD, and DiD-nPLP via tail vein (6μg DiD per mouse). The mice were sacrificed at 1h, 2h, 6h, 12h, and 24h, tumors and main organs were collected. An in vivo imaging system (IVIS Lumina Series III, PerkinElmer, USA) was used for *ex vivo* imaging.

Melanoma-bearing mice were randomly divided into two groups and i.v. injected

by free DOX and DOX-nPLPs (at an equivalent dose of 3 mg kg^{-1} DOX). The mice were sacrificed at 1h, 2h, 6h, 12h, and 24h. Tumors were collected and weighed. Two volume of physiological saline was added and the mixture was homogenate with the aid of homogenizer (Precellys 24, Bertin, France). The content of DOX in tumors was measured by LC-MS/MS.

For the metastasis targeting assay, B16F10 cells (2×10^5) were injected into C57BL/6 mice via the tail vein to generate a lung metastatic mouse model of melanoma. The mice were injected with PBS, DiD, DiD-nPLP (6μg DiD per mouse) on the 15th day after the injection of B16F10 cells. The lungs of the mice were collected at 12h and the ex vivo fluorescence images were obtained using the IVIS instrument.

17. Pharmacokinetic assay

For the pharmacokinetic assay, female C57BL/6 mice were divided into two groups (10mice/group). Free DOX and DOX-nPLPs were injected separately (at an equivalent dose of 3 mg kg^{-1} DOX) into the mice through the tail vein.5 mice were used for the first five time points, while the other 5 mice were used for the next five time points. At each time point (5min, 15min, 30min, 1h, 2h, 4h, 8h, 12h, 24h, 48h), approximately 150μL of blood was collected in 0.5mL tube (1% heparin sodium soaked) from the orbit sinus. The blood samples were centrifuged at 4 °C and 5500 rpm for 10 minutes. Then 50μL of supernatant plasma was obtained. 200μL of precipitant (acetonitrile: methanol=5:1) was added into the plasma. The samples were oscillated for 10 min, and then centrifuged at 4 °C and 12000 rpm for 10 min. After filtering through a 0.22μm filter, the supernatant was collected for LC-MS/MS analysis (AB

SCIEX OTRAP 5500). The chromatographic conditions were as follows: column-ACQUITY UPLC BEH C18 column (2.1X100mm, 1.7um); mobile phase: methanol: 0.1% formic acid water = 80: 20, flow rate: 0.5 mL min^{-1}, column temperature: 35 degrees. Injection volume: 1μL.The conditions of mass spectrometry were as follows: the electrospray ionization source (ESI) was used as the ion source; the multiple reaction monitoring (MRM) had transition of m/z 544.2→m/z 397.1 and the electrospray ionization in the positive mode was used to quantify DOX.

18. Antitumor efficacy

For the anti-tumor assay, B16F10 cells (5×10^4/mouse) were inoculated into the armpits of female C57BL/6 mice. Then the tumor-bearing mice were randomly divided into 3 groups (5 mice/group). The treatment was started on the 7th day after tumor implantation. PBS, free DOX, and DOX-nPLPs were injected into the mice every 2 days (DOX dose is 3 mg kg^{-1}) for a total of 4 administrations. And the tumor volume and mouse body weight were recorded, and tumor volume was calculated by the formula: $V= (L\times W^2/2)$, where W was the short diameter of the tumor and L was the long diameter of the tumor. The mice were sacrificed on the 18th day after tumor implantation and the tumor inhibition ratio (TIR) was calculated by the formula: TIR $(\%) = (1-V_t/V_c) \times100$ where V_c and V_t were the average tumor volumes at day 18 in the control and the treatment groups, respectively. Tumors and major organs were collected for *ex vivo* histological analysis.

19. Anti-metastatic assay

For the anti-metastatic assay, B16F10 cells (2×10^5/mouse) were injected into

C57BL/6 mice to establish the metastatic mouse model. Mice were randomly divided into 5 groups and the treatment was started on the 5th day. PBS, free DOX, free LAM, blank nPLPs (80 mg kg^{-1}) and DOX-nPLP (equivalent dose is 2.5 mg kg^{-1} DOX) were injected on mice every 2 days for a total of 4 administrations. On the 21th day, the mice were sacrificed, and the lungs were collected and imaged. And then the lungs were collected for *ex vivo* histological analysis.

20. Survival assay

B16F10 cells (5×10^4/mouse) were inoculated into the armpits of C57BL/6 mice to establish the tumor-bearing mouse model. The treatment was started on the 7th day after tumor implantation. PBS, free DOX, and DOX-nPLPs were injected into the mice every 2 days (DOX dose is 3 mg kg^{-1}) for a total of 4 administrations. B16F10 cells (2×10^5/mouse) were injected into C57BL/6 mice to establish the metastatic mouse model. The treatment was started on the 5th day. PBS, free DOX, free LAM, blank nPLPs (80 mg kg^{-1}) and DOX-nPLP (equivalent dose is 2.5 mg kg^{-1} DOX) were injected on mice every 2 days for a total of 4 administrations. The survival time of remaining mice in each group was recorded and the survival curve was obtained.

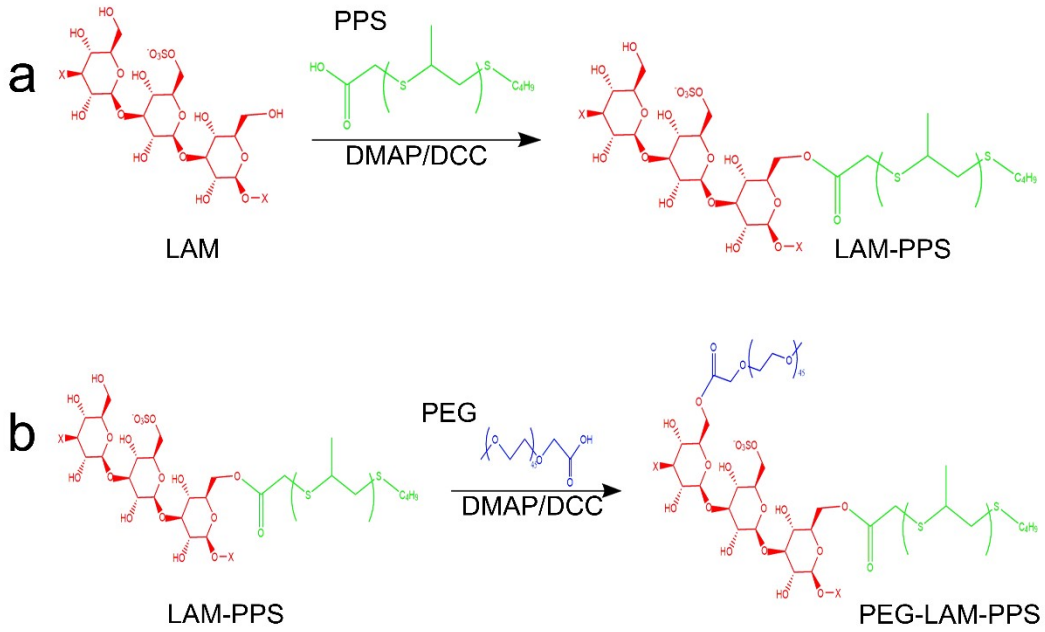

Figure S1. (a) The synthesis procedures and chemical structures of LAM-PPS conjugate and **(b)** PEG-LAM-PPS conjugate.

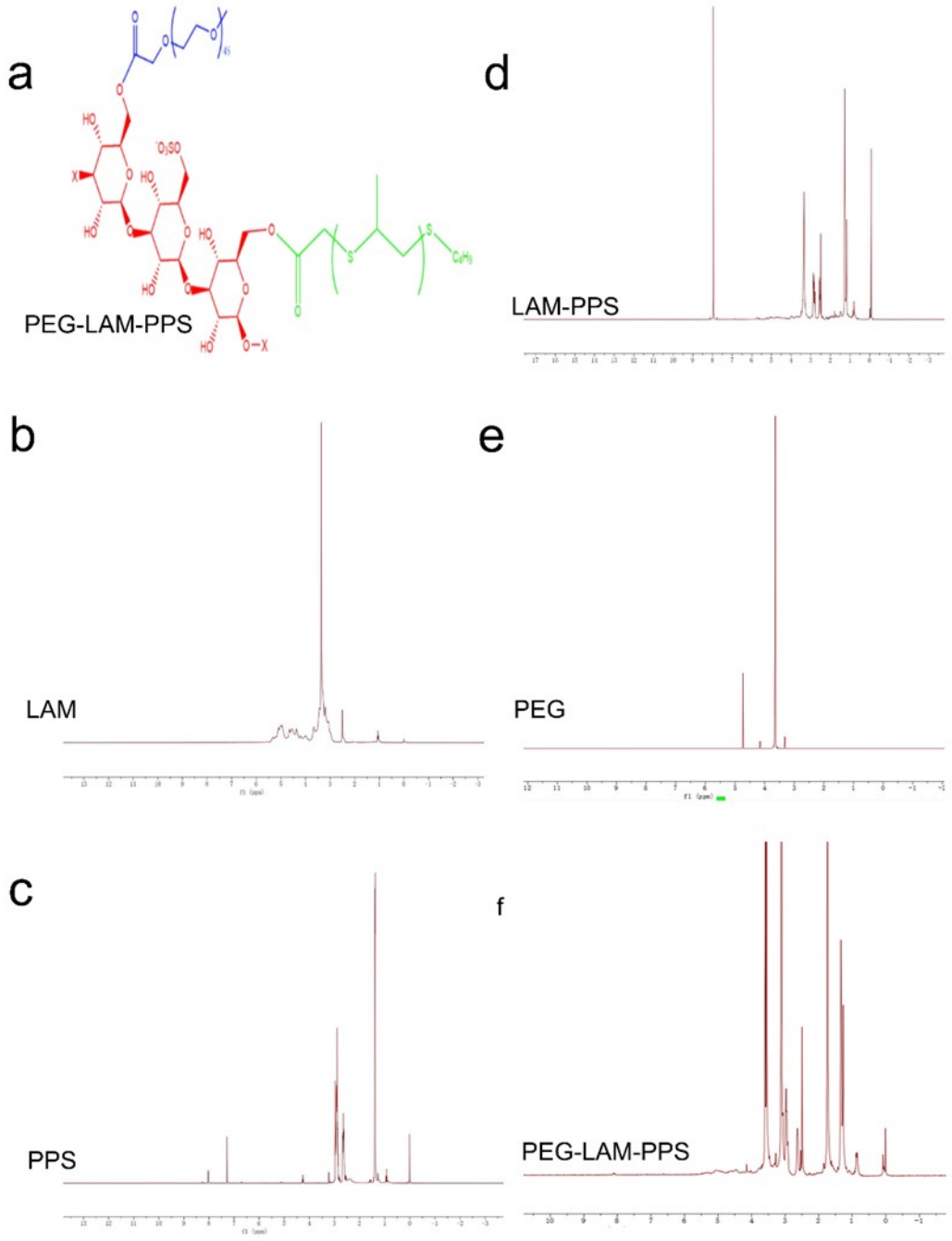

Figure S2. (a) Chemical structure of PEG-LAM-PPS conjugate. The 1H-NMR spectrums of **(b)** LAM (in D_2O), **(c)** PPS-COOH (in $CDCl_3$), **(d)** LAM-PPS (in $CDCl_3$ and DMSO-d_6) **(e)** PEG (in D_2O) **(f)** PEG-LAM-PPS (in THF-$d_8$and DMSO-d_6)

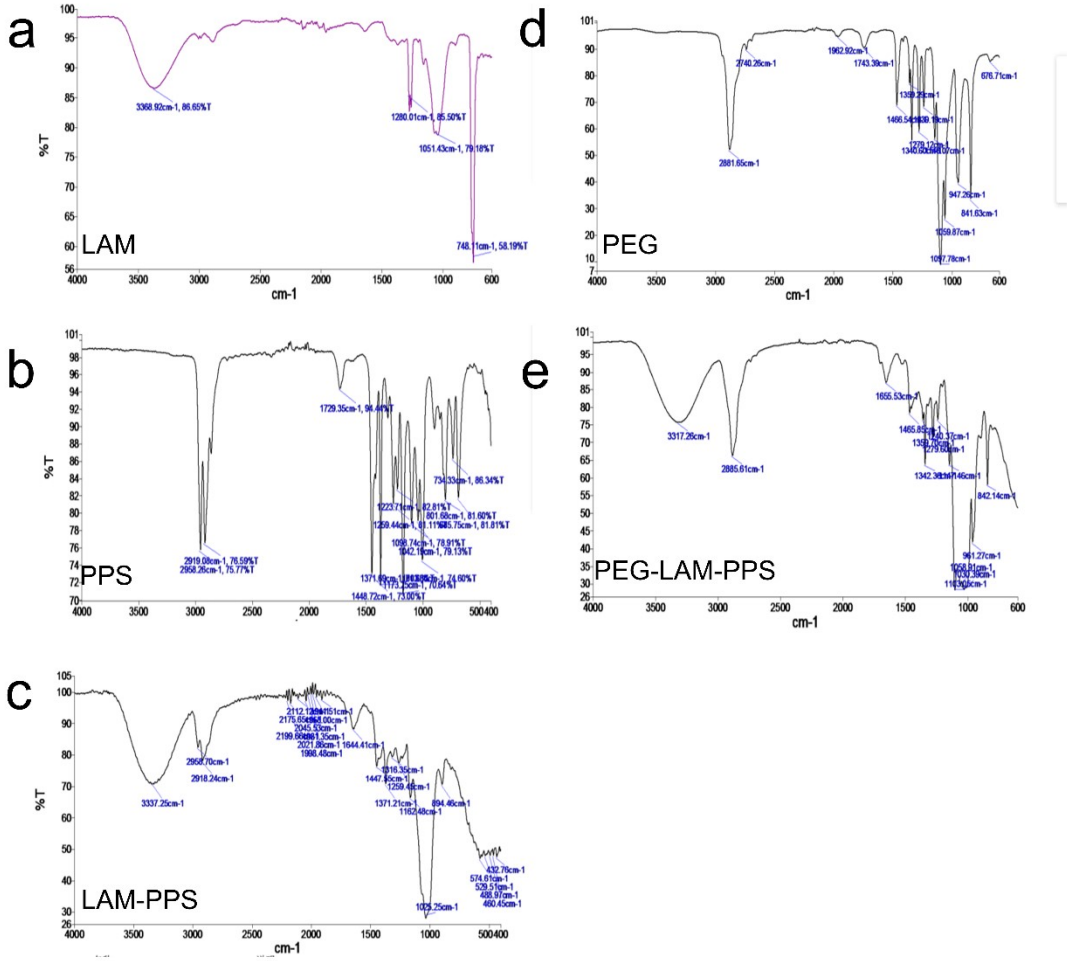

Figure S3. The IR spectra of **(a)** LAM, **(b)** PPS-COOH , **(c)** LAM-PPS conjugate **(d)** PEG-COOH and **(e)** PEG-LAM-PPS conjugate

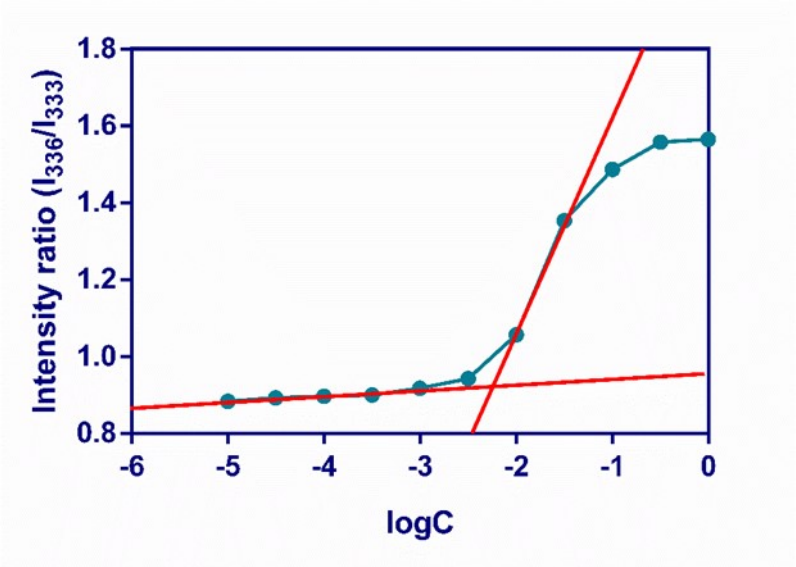

Figure S4. CMC curve of PEG-LAM-PPS.

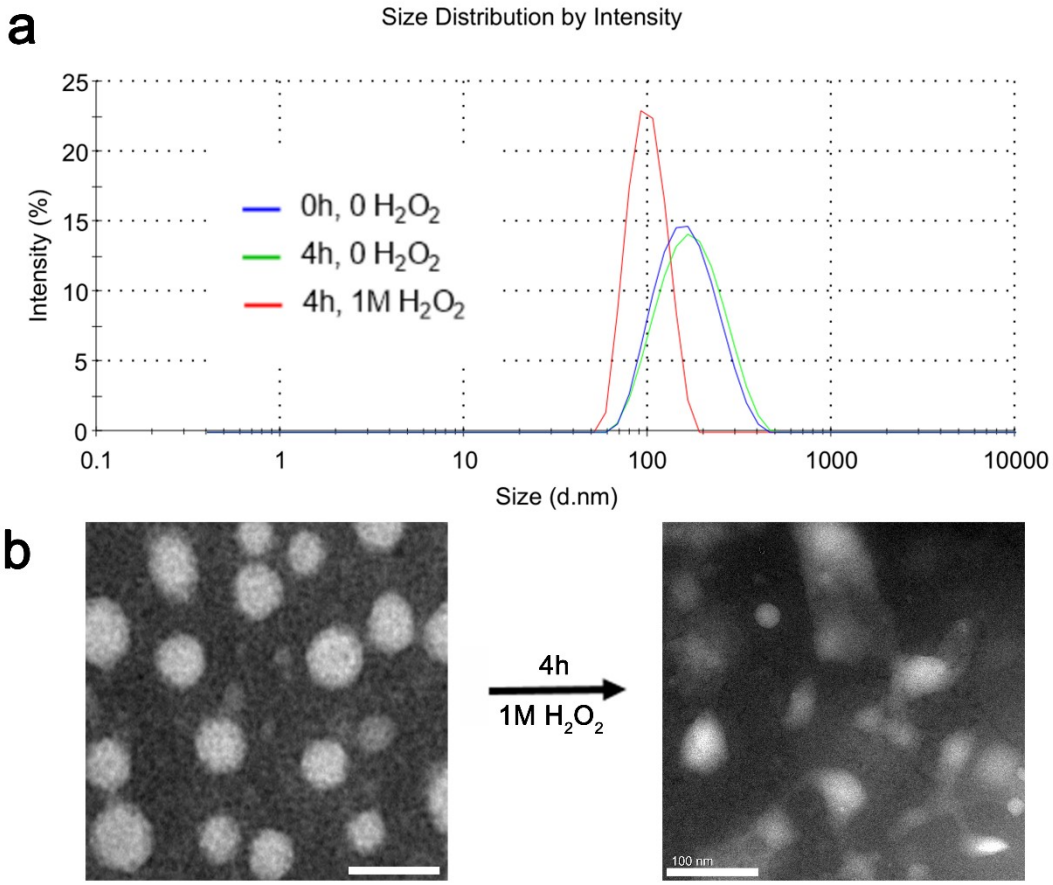

Figure S5. Changes in size distribution **(a)** and **(b)** TEM images of nPLPs after H_2O_2 treatment. Scale bar 100 nm.

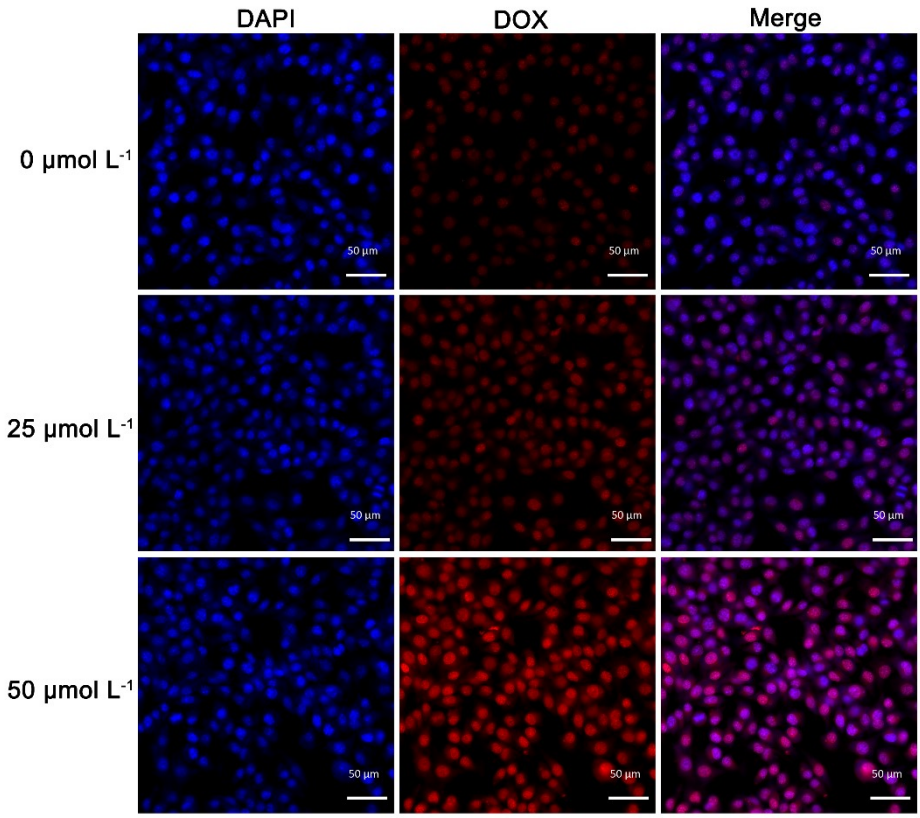

Figure S6. CLSM images for B16F10 cells after incubation with DOX-nPLPs for 2h under different H_2O_2 concentration respectively, including DOX channel (red) and DAPI-stained nucleus channel (blue). Scar bar 50 μm.

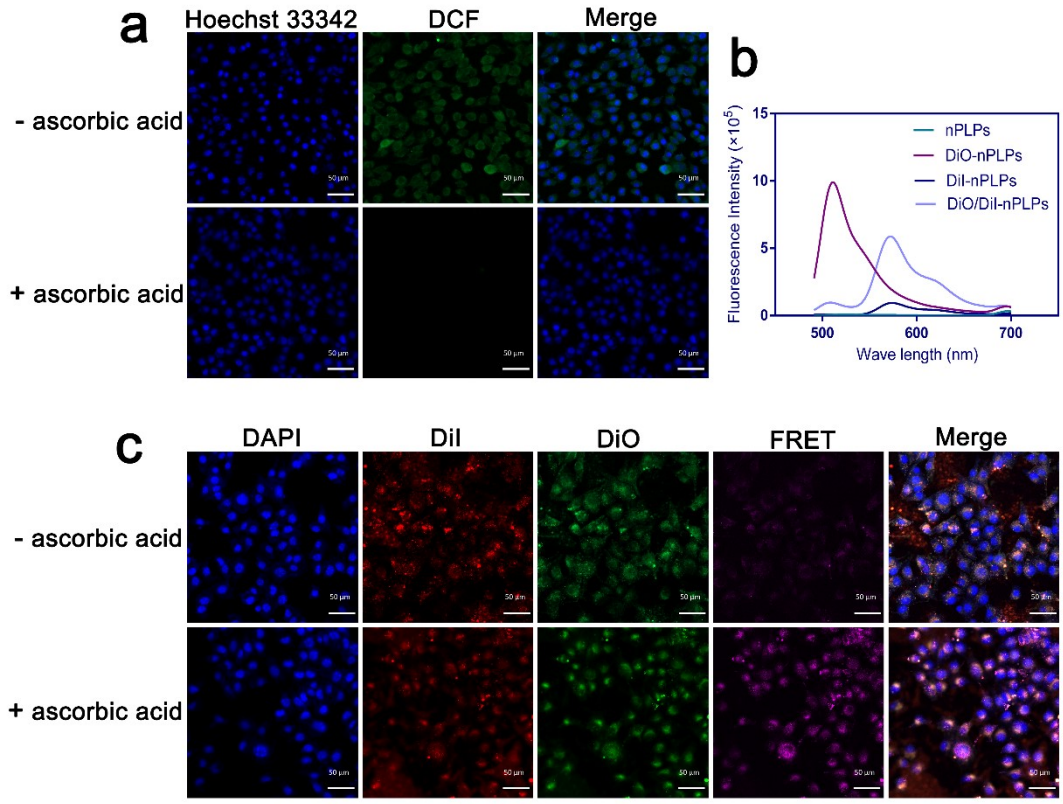

Figure S7. (a) CLSM images with ROS assay of B16F10 cells with or without ascorbic acid, including DCF channel (green) and Hoechst 33342-stained nucleus channel (blue). Scale bar 50 μm. **(b)** Emission spectra of nanoparticles containing DiO, DiI, or DiO/DiI, excited at 460nm. **(c)** Confocal images of B16F10 cells with or without ascorbic acid treated with DiO/DiI-nPLPs for 2h and further incubated in fresh culture medium for 2 h, including DiI channel (red), DiO channel (green), FRET channel (violet fluorescence, laser wavelength 488 nm, detection wavelength 540-700 nm) and DAPI stained nucleus channel (blue). Scar bar 50 μm.

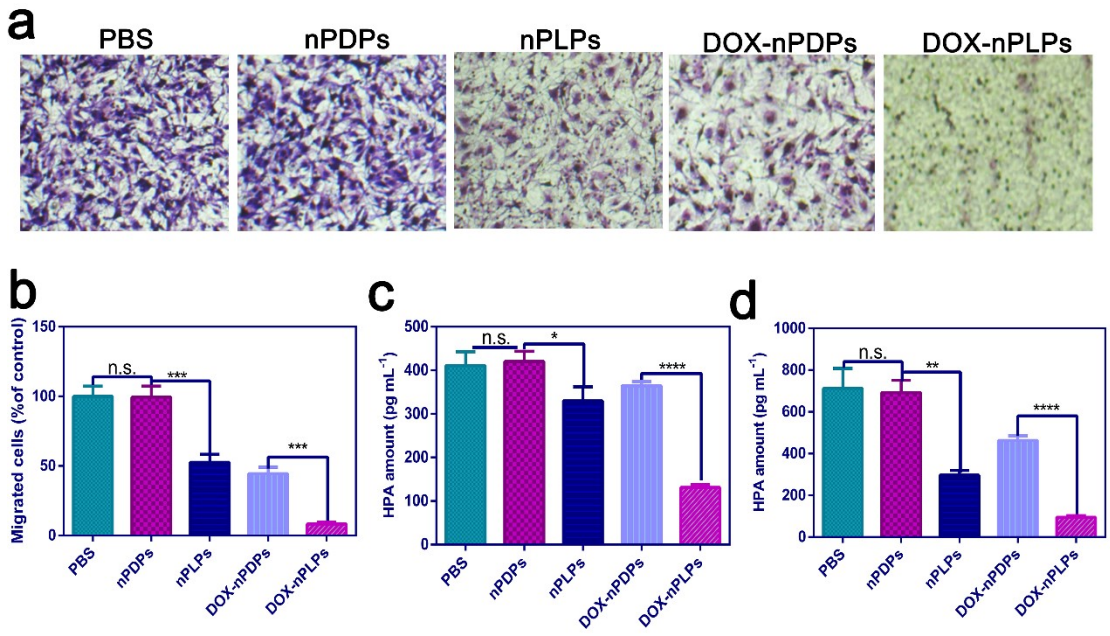

Figure S8 **(a)** Images and **(b)** quantitative analysis of invaded B16F10 cells after separate incubation with PBS, nPDPs, nPLPs, DOX-nPDPs and DOX-nPLPs for 48 h. Detection of HPA in B16F10 cells **(c)** and in B16F10 culture medium **(d)** by ELISA after separate incubation with PBS, nPDPs, nPLPs, DOX-nPDPs and DOX-nPLPs for 36h (means ± SD, $n = 3$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, *** indicates $p < 0.0001$).

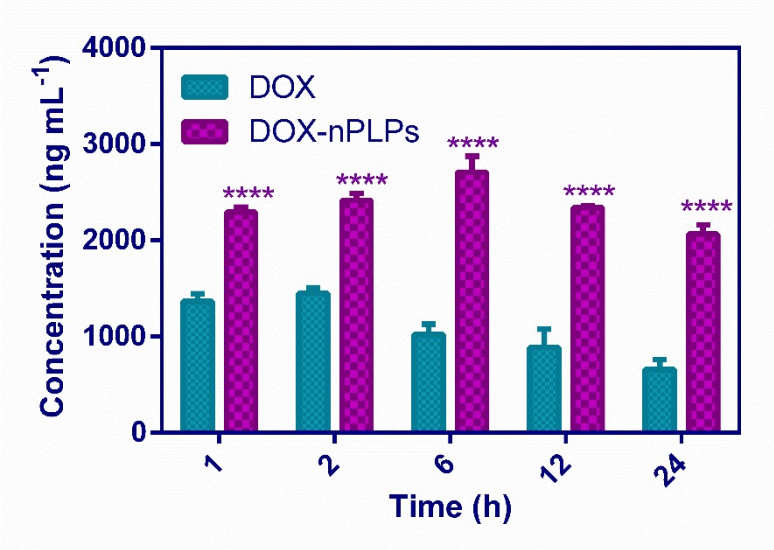

Figure S9. Quantification of *ex vivo* DOX levels in tumors at 1h, 2h, 6h, 12h and 24h after treatments measured by LC-MS/MS (mean ± SD, n = 5). **** $p < 0.0001$.

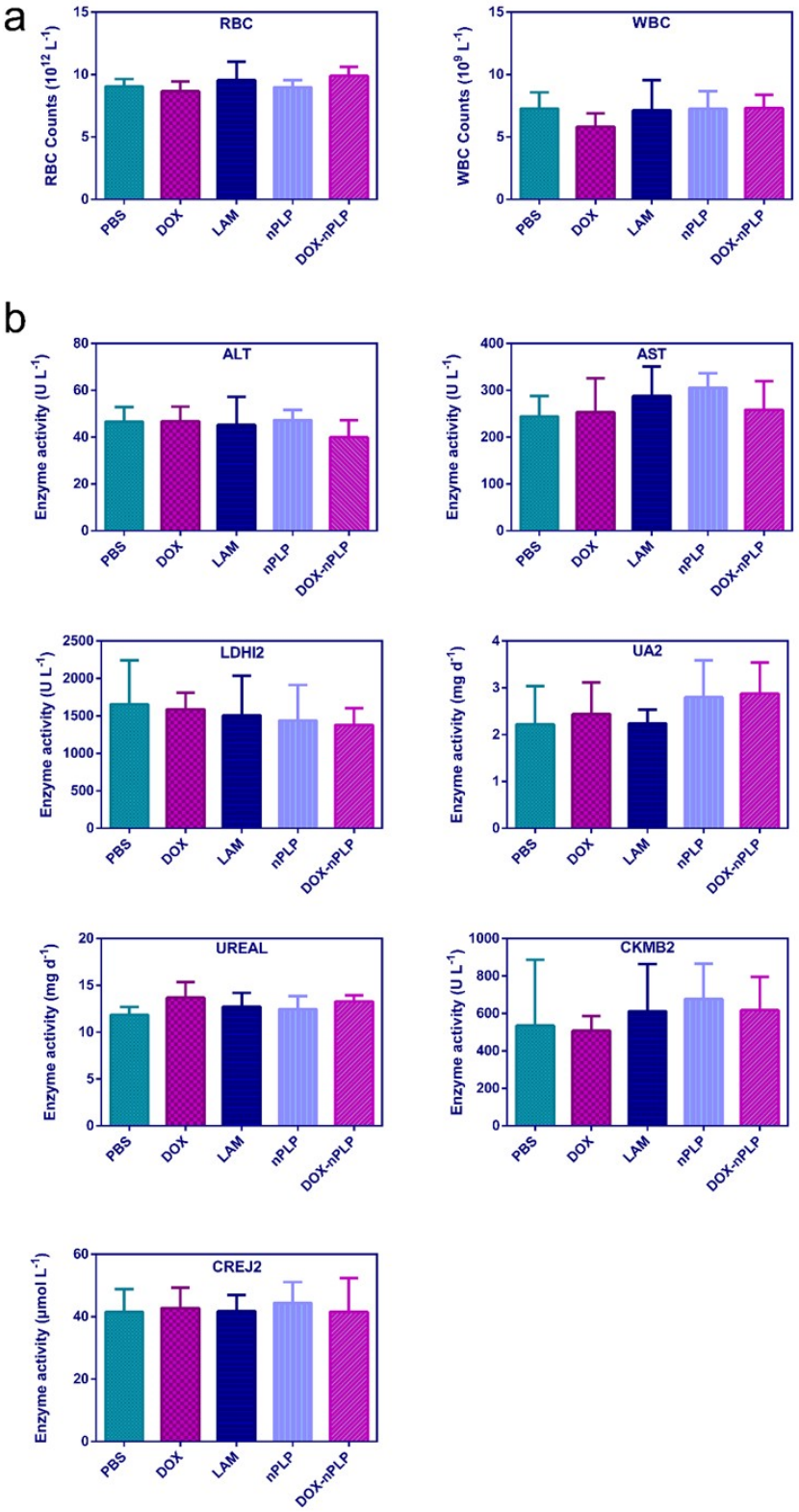

Figure S10. (a) Hematological analysis and **(b)** serum biochemistry data of B16F10 metastasis C57BL/6 mice treated with PBS, free DOX, LAM, nPLPs and DOX-nPLPs (at an equivalent dose of 2.5 mg kg-1 DOX) respectively in a total of 4 times. (n = 5, Means ± SD, * indicates $p < 0.05$).

Table S1. Average IOD value of Positive cell signal results from HPA analysis, showing DOX-nPLPs have lower HPA expression.

Groups	Average IOD value of HPA positive signal [/μm^2]	
	Melanoma tumor mice model	Melanoma metastatic mice model
PBS	89.51	221.93
LAM	-	151.85
nPLPs	-	131.79
DOX	134.17	87.55
DOX-nPLPs	52.09	67.02