Supplementary Information

A Facile Strategy to Generate Polymeric Nanoparticles for Synergistic Chemo-photodynamic Therapy

Xin Deng, Yan Liang, Xinyu Peng, Ting Su, Song Luo, Jun Cao*, Zhongwei Gu and Bin He*

Synthesis and characterizations

1. Materials

1-ethyl-(3-dimethyllaminopropyl) carbodiie hydrochlide (EDC·HCl), 1-hydroxy-benzotriazole monohydrate (HOBT) were purchased from GL Biochem. Ltd. (Shanghai, China). Tetrakis(4-carboxyphenyl)porphyrin (Por) was purchased from TCI (Japan). Methoxy poly(ethylene glycol) (mPEG, Mw=2000 g/mol) and deuteron dimethyl sulphoxide (DMSO-d_6) were purchased from Sigma-Aldrich Co.. N,N-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA) were purchased from Asta Tech (Chengdu) Biopharm. Co. Ltd. (Chengdu, China). Doxorubicin hydrochloride (DOX·HCl, Shanghai Yingxuan Chempharm Co. Ltd. China) was deprotonated according to the method previously reported. N,N-Dimethylformamide (DMF) and CH_2Cl_2 were purchased from Kelong Chemical Co. (Chengdu, China) and distilled before used. All other solvents were purchased from Kelong Chemical Co. (Chengdu, China) and used without further purification. All the reagents and sovlent were used as received without further purification.

2. Mesurement

The structure of the amphiphiles was determined by GPC, MS and ^{1}H NMR. The mPEG-Por was dissolved in methanol, dropped on the stainless steel plate and measured by the MALDI-TOF spectrometer (Bruker, autoflex III smartbeam). The ^{1}H NMR measurement was performed on NMR spectrometer (Varian, 400 M.Hz). The mPEG-Por was dissolved in DMSO-d_6 and D_2O, respectively. Relative molecule weight of the amphiphile was performed on GPC(Waters 2414, USA) and analyzed with GPC-SEC data analysis software. Samples were analyzed at 25 ^{o}C with chloroform as eluent at a flow rate of 1.0 mL min^{-1}. The size of the nanoparticles was measured by dynamic light scattering (DLS, Malvern Zetasizer Nano ZS, UK). Atomic force microscopy(AFM, MFP-3D-BIO, USA) and transmission electron microscopy (TEM, JEM-100CX-JEOL, Japan) were employed to observe the morphology of the nanoparticles. The AFM sample was prepared by dropping the freshly prepared nanoparticles solution onto the mica plate while TEM samples were prepared by dipping a copper grid with Formvar film into the freshly prepared nanoparticles solution. The copper grid was dried overnight at room temperature. UV-vis absorption (Specord 200 PLUS) and Fluorescence spectra (F-7000, Hitachi, Japan) were used to measure the drug loading content, releasing profile and π-π interaction between drugs and carriers.

3. Synthesis of mPEG-Por Amphiphiles

The simple one-step synthetic process of the mPEG-Por amphiphiles was presented in Figure S1 in supporting information (SI). Tetrakis(4-carboxyphenyl)porphyrin (Por) (0.24 g, 0.30 mol), $mPEG_{2000}$ (0.60 g, 0.30 mol), EDC.HCl (0.12 g, 0.60 mmol) and HOBt (0.08 g, 0.60 mmol) were dissolved in 30 mL of DMF, DIEA (0.50 mL, 3.0 mmol) was injected into the solution. The reaction was stirred at room temperature for 48 h. The solvent was evaporated by the rotatory evaporator and the crude product was received. The crude product was washed with saturated

$NaHSO_4$ and saline solution three times in turn and dried by anhydrous Na_2SO_4. The product was purified by silica column chromatograophy twice with the eluents (MeOH:EtOAc:CH_2Cl_2=1:4:10 and MeOH:CH_2Cl_2=1:10). After precipitated in cold ether, the product mPEG-Por was dialyzed in a dialysis tubing (Spectra/Por MWCO = 1000) and freeze-dried.

4. Fabrication of the Bank and Drug Loaded Nanoparticles

The freeze-dried amphiphiles (mPEG-Por, 5 mg) were dissoved directly into 5 mL DD water at the condition of ultrasound, and then stirred overnight to get the mother solution. Other different concentration of blank nanoparticles were obtained through the diluting of the mother solution.

Drug-loaded nanoparticles were prepared according to the approach previously published[1] The freeze-dried amphiphiles (mPEG-Por, 10 mg) were dissolved in 1 mL of DOX solution in DMSO (2.5 mg/mL), the mixture was ultrasonicated for one hour. The solution was dropped into 10 mL of deionized water and stirred overnight. The mixture was transferred into a dialysis tubing (Spectra/Por MWCO = 1000) and dialyzed against deionized water at 4 °C for 12 h. The deionized water was changed every two hours. The solution in the dialysis tubing was subsequently lyophilized after centrifugation and filtration through the 0.45 μm filter. The whole procedure was performed in dark.

5. Characterization of the Bank and Drug Loaded Nanoparticles

5.1 CAC of the blank nanoparticles

Many methods were used to determine the critical aggregation concentration of the amphiphile such as fluorescence probe technique, conductivity method, surface tension method.[2] However, in our system the most used fluorescence probe method was not working due to the interaction between probe and amphiphile. Inspired by the theory of fluorescence probe method, we developped a new method mesuring CAC which used the Por moiety as the probe. We prepared different concentrations of blank nanoparticles and then mesured directly using fluorescence spectra. Also, we adopted the conductivity method to assure the novel method's results which also produced different concentration and directly mesured by conductivity meter. The processed results revealed in Figure S3 and Figure S4.

5.2 Morphology of the bank and nanoparticles

The size and zeta-potential of the nanoparticles was measured by dynamic light scattering, the samples were tested by triple times. The results were obtained of the average value of the three times.

The morphology of the nanoparticles were charactered by AFM and TEM. The AFM samples were got through dropping the prepared nanoparticles solution on the surface of the muca and then dried spontaneously. The TEM samples were prepared by dipping a copper grid with formvar film into the freshly prepared nanoparticles solution and dried spontaneously.

5.3 Drug-loading capacity of the drug-loaded nanoparticles

The content of DOX in drug-loaded nanoparticles was determined by UV-Vis measurement (data from the peak value at 485 nm) in DMSO using calibration curve obtained from DOX/DMSO solutions with different DOX concentrations. Drug loading content (DLC) and drug encapsulation efficiency (DEE) of the nanoparticles were calculated according to the following formulas:

DLC (%) = [weight of drug in nanoparticles/weight of drug-loaded nanoparticles] ×100%

DEE (%) = [weight of drug in nanoparticles/weight of drug in feeding]×100%

5.4 The release profile of the drug-loaded nanoparticles

The releasing curve of the drug-loaded nanoparticles was measured by the fluorescence spectra. DOX-loaded nanoparticles (0.66 mg) were dispersed in PBS (1 mL, ionic strength = 0.01 M, pH = 7.4). The mixture was transferred in dialysis tubings (Spectra/Por MWCO = 1000). The tubings were immersed in vials containing 25 mL of phosphate buffered saline (PBS) solution. The vials were put in a shaking bed at 37 °C. 1 mL of medium with released drug was taken out for fluorescence measurement and replenished with an equal volume of fresh medium in prescribed time intervals. The released DOX was detected by a fluorescence detector with excitation wavelength at 480 nm and emission wavelength at 550 nm. The release experiments were conducted in triplicate, and the mean value was presented.

6. The π-π stacking interaction between drugs and carriers

The π-π stacking interaction between fixed concentration DOX and different concentration carriers

We prepared the DOX solution with the fixed concentration firstly, and then took out the fixed volume of the solution and mixed with different volume of the specific concentration's mPEG-Por solution, and made the mixed solution's volume to one milliliter, at last we evlated the π-π stacking with the fluorescence spectra. This is the preparing process for the sample for evaluating the π-π stacking interaction between fixed concentration DOX and different concentration carriers. The sample preparing process for studying the π-π stacking interaction between fixed concentration carriers and different concentration DOX was the same as above.

7. Uptake of the drug-loaded Nanoparticles

The cell uptake assay were measured by the Laser Scanning Confocal Microscopy (CLSM, Leica TCS SP5, Germany) and Flow Cytometry (FACSAria, BD, USA). The HepG2 cells were cultured in the culture dishes containing drug-loaded nanoparticles at 37 ℃ for different periods of time (2 h, 6 h). The DOX concentration of the drug-loaded nanoparticles was 10 μg/mL. Before we observed the uptake assay by the CLSM, we firstly discarded the culture medium and then washed the cells with PBS three times and last keep some drops PBS on to the surface of the cell for testing. The quantitative experiment of the uptake was determined by Flow Cytometry. The process was much about same with the Laser Scanning Confocal Microscopy, the difference was that after washing with PBS three times we should harvest the cells to obtain homogeneous solutions using the trypsinization. After all the cells collected into the tube, we centrifuged and then discarded the supernatant and then used the PBS to make the cells suspending again and kept its situation until the results we obtained through the Flow Cytometry. The exticed spectra of DOX was 488 nm and collected the emssion spectra at the wavelength of 550 nm.

8. The distribution of the drug-loaded nanoparticles in vivo

Six week old female BALB/c nude mice were purchased from Chengdu Dashuo Company. We kept them at the experimental situation for one week. Then we injected the prepared suspending HepG2 cells(about two million per mL, 0.10 mL) into the dorsal right side subcutaneously. About two weeks later, the tumor formed and then made a label for every mice. After this we injected the different solutions(DOX concentration was 8 mg/kg) into the tumor-bearing nude mice intravenously. At the different time(0.5 h, 3 h, 6 h, 10 h, 24 h), we acquired the pictures through the Mastro EX in vivo fluorescence imaging system (CRi, Inc., USA). Another group we sacrificed the mice after the injection 10 hours and got the main organs for the pictures. All animals experiments were performed according to our institutional and NIH guidelines for care and use of research animals.

9. Anti-tumor effect of the drug-loaded nanoparticles in vitro and in vivo

9.1 Anti-tumor effect of the drug-loaded nanoparticles in vitro

The anti-tumor effect in vitro was assessed by the cell viability incubated with drug-loaded nanoparticles for 24 hours at the different situations(Dark and Light). The light was emitted by the diode (Saijia electric technology co., Ltd, China) which the wavelength was about 650 nm and the power was 200 mW. The dark group meant that we didn't use the diode to shine the cells while the light group meant that we illuminated the cells twice after incubuting cell with drug-loaded nanoparticles 4 and 10 hours later. We lighted the cells about 1 min every time and then wrapped them up with tinfoil for the continued incubation. When the time satisfied, we discarded the culture medium and washed the cells three times with PBS. And then with the method of CCK-8 detection(Dojindo Molecular Technologies, Inc., Japan), we obtained the results of the cell viability for the different groups through the enzyme micro-plate reader (Thermo scientific MK3, Thermo Fisher, USA).

9.2 Anti-tumor effect of the drug-loaded nanoparticles in vivo

The animal experimental permission was obtained from the ethics committee of Sichuan University. With the same methods in section 8, we used the 4T1 bearing female Balb/c mice which was about 4 week old obtained from Chengdu Dashuo, Inc.(Sichuan, China). When the tumor value reached about 100 mm^3, we injected the different solutions into the mice intravenously. We kept the same DOX dose(5 mg/kg) in each groups for the systemic injection. The injection was performed by every 4 days for a total of four times. About 6 hours later for every injection, we positioned the mice and illuminated the tumor three minutes with the diode as previously described in vitro. And 24 hours post-injection, we treated the mice with the same methods again. In the treatment process, we used the vernier caliper to measure the tumor volume for every two days while the body weight were recorded at the same time. We extracted the tumor tissues to study the anticancer efficacy on day 24 and the section were subjected to hematoxylin and eosin staining for the pathologic analysis.

The tumor volumes were calculated by the following formula: tumor volume V (mm^3) = 1/2 ∗ length (mm) ∗ width (mm) ∗ width (mm). Every group had 5 mice for the research. All procedures were performed according to our institutional and NIH guidelines for care and use of research animals.

10. Statistical analysis

All data are presented as means±S.D. Statistical significance ($p < 0.05$) was evaluated by using Student t-test when experiment groups were compared.

Reference

[1] a) X. Deng, X. Xu, Y. Lai, B. He, Z. Gu, Journal of Biomedical Nanotechnology 2013, 9, 1336; b) J. Cao, S. Zhai, C. Li, B. He, Y. Lai, Y. Chen, X. Luo, Z. Gu, Journal of Biomedical Nanotechnology 2013, 9, 1847.

[2] a) P. Alexandridis, J. F. Holzwarth, T. A. Hatton, Macromolecules 1994, 27, 2414; b) I. Garcia-Mateos, M. Mercedes Velazquez, L. J. Rodriguez, Langmuir 1990, 6, 1078; c) S. Alexander, W. M. de Vos, T. C. Castle, T. Cosgrove, S. W. Prescott, Langmuir 2012, 28, 6539.

Results

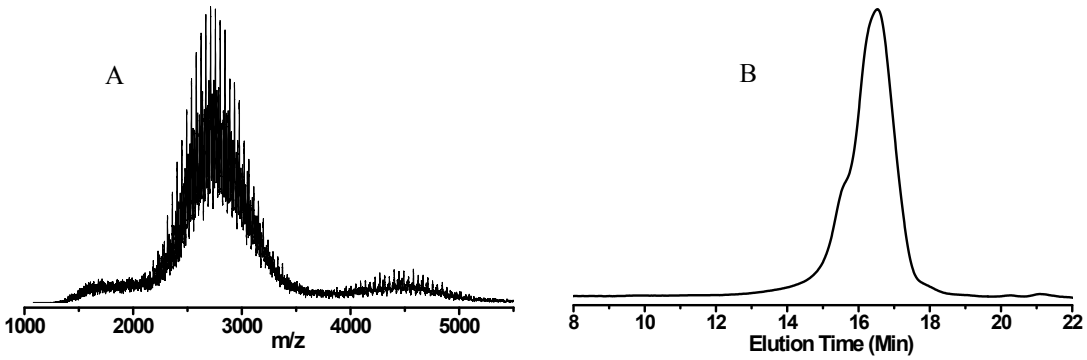

Figure S1. MS (A) and GPC (B) spectra of mPEG-Por conjugates.

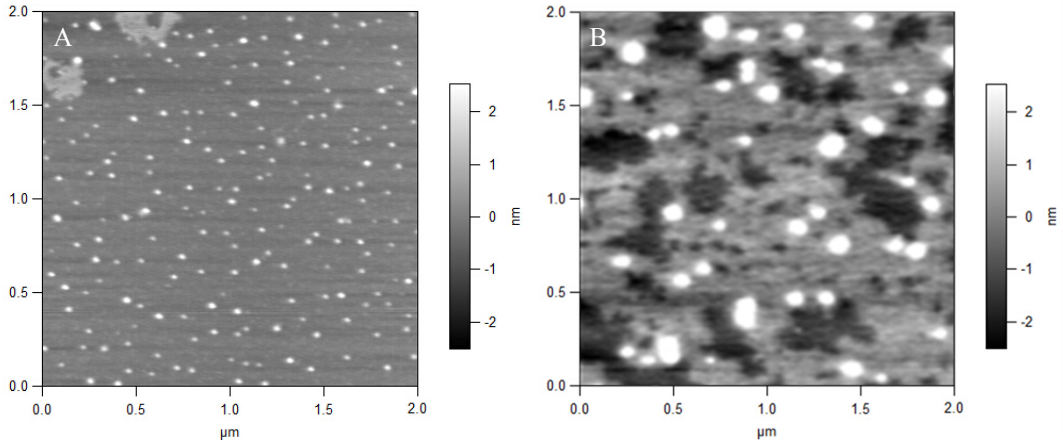

Figure S2. AFM images of blank NPs (A) and DOX/NPs (B).

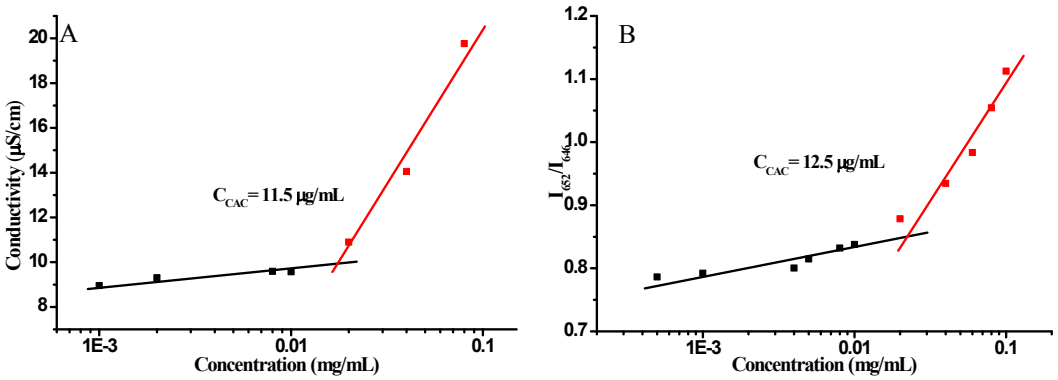

Figure S3. CACs of the NPs measured by self-fluorescence (A) and conductivity (B) of mPEG-Por conjugates.

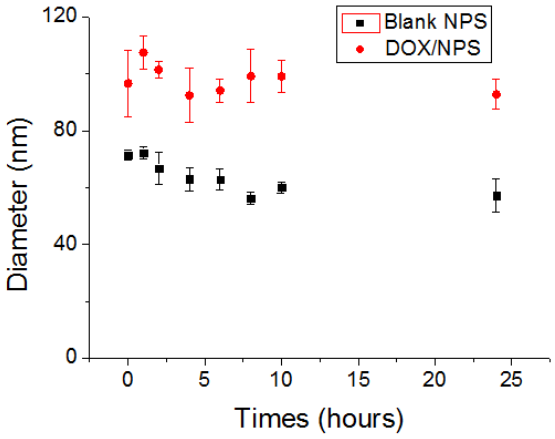

Figure S4. The stability of blank and drug loaded nanoparticles.

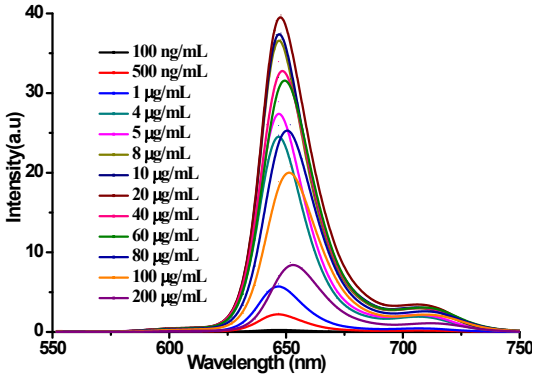

Figure S5. The fluorescence spectra of mPEG-Por conjugates with different concentrations.

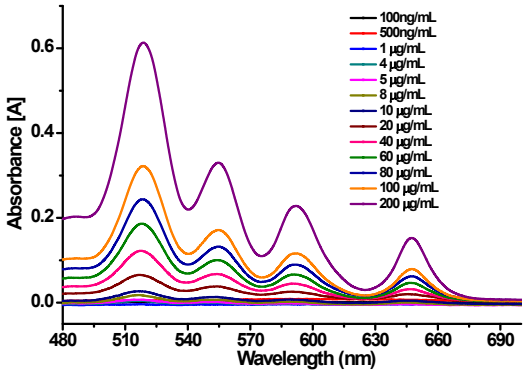

Figure S6. The UV-VIS spectra of mPEG-Por conjugates with different concentrations.

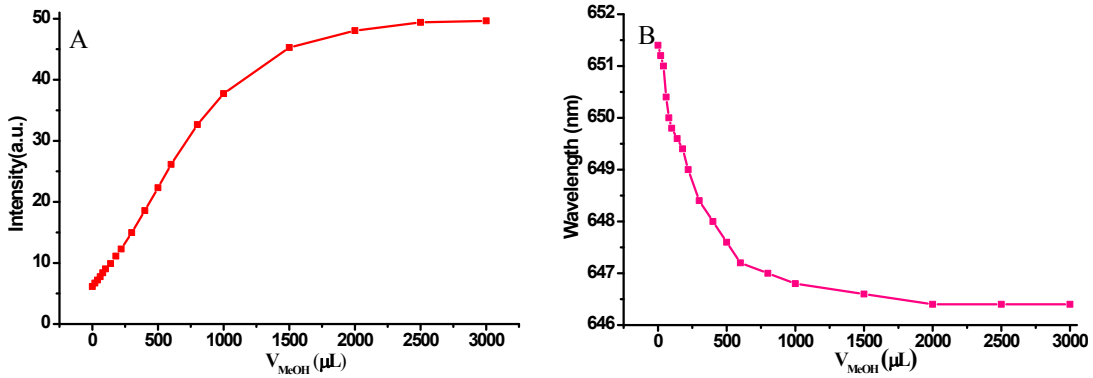

Figure S7. The intensities at 550 nm (A) of the DOX fluorescence and the blue shifts (B) of Por fluorescence of the DOX/NPs with the addition of different volumes of methnol.

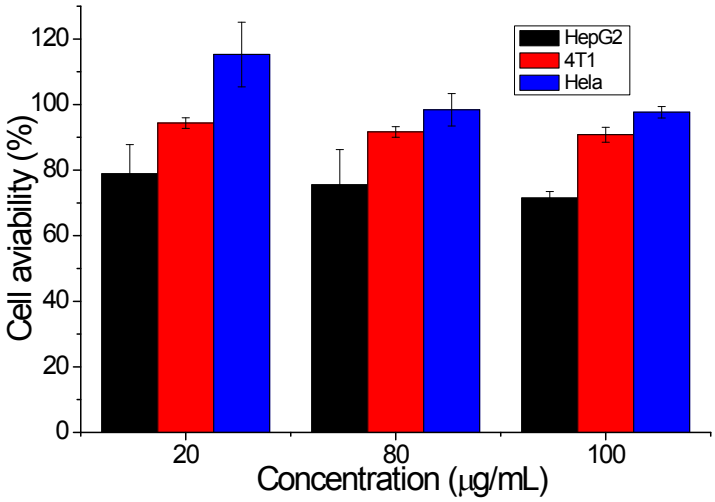

Figure S8. The cytotoxicity of blank nanoparticles.

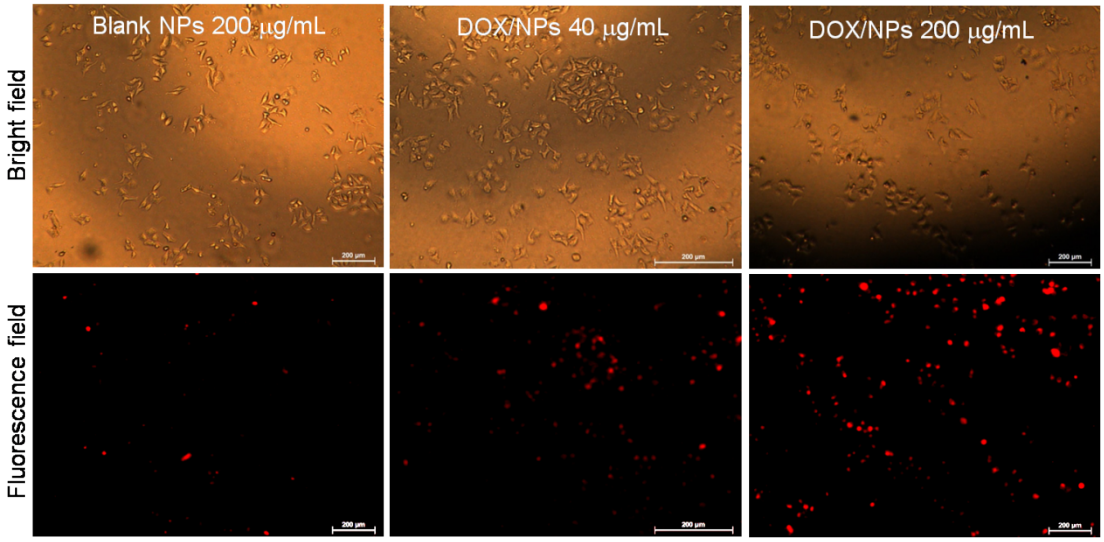

Figure S9. The morphologies of 4T1 cells incubated with DOX/NPs for 24 h.

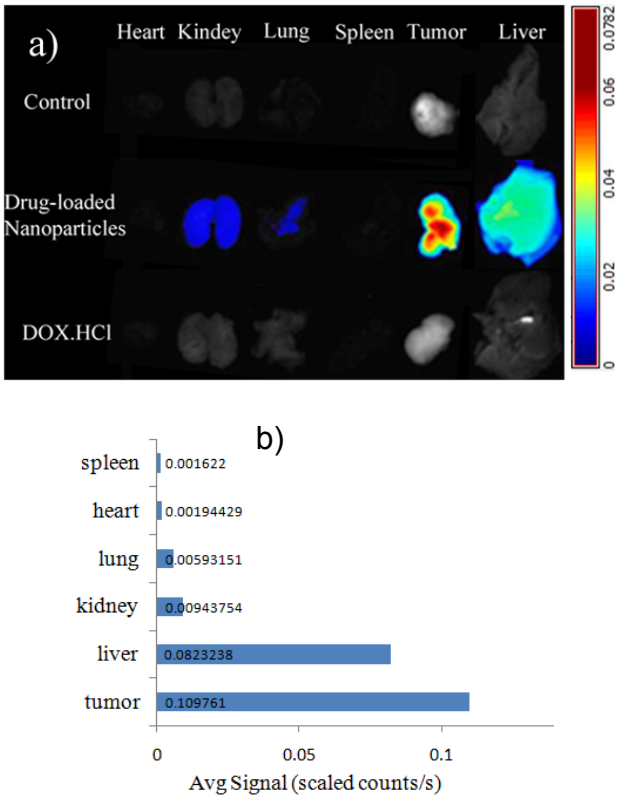

Figure S10. The ex vivo fluorescence of the organs separated from tumor-bearing mice administrated with DOX/NPs (a) and the fluorescence intensity of the organs (b), the DOX dose was 5 mg/kg, the time was 10 hours.

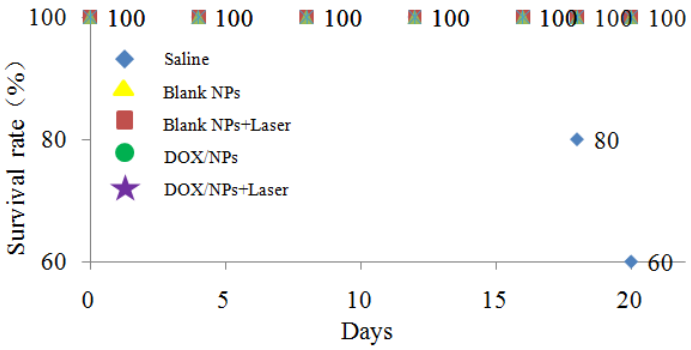

Figure S11. The survival rates of mice bearing tumor after treatments.