Supplemental materials for:

Solid Tumor Therapy Using a Cannon and Pawn Combination Strategy

Wantong Song[1], Zhaohui Tang[1,*], Dawei Zhang[1], Xue Wen[2], Shixian Lv[1,3], Zhilin Liu[1,3], Mingxiao Deng[2], Xuesi Chen[1,*]

[1]*Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, 130022, PR China*

[2]*Department of Chemistry, Northeast Normal University, Changchun, 130021, PR China*

[3]*University of Chinese Academy of Sciences, Beijing, 100039, PR China*

Supplemental Materials and Methods

Materials

mPEG5k was purchased from Sigma-Aldrich. γ-Benzyl-L-glutamate-N-carboxyanhydride (BLG-NCA) was purchased from Shanghai Yeexin Biochem & Tech Co. Ltd. Combretastatin A4 (CA4) was purchased from Hangzhou Great Forest Biomedical Ltd., China. Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng United Technology Corporation. Diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) and N-hydroxysuccinimide (NHS) were supplied by Aladdin Reagent Co. Ltd. Rhodamine B-NH2 (RhoB-NH_2) was a gift from Dr. Chunsheng Xiao, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. Anti-CD31 antibody (ab28364) was purchased from Abcam. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Sigma-Aldrich. All other reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. and used as received.

Synthesis of mPEG-PLG-*g*-CA4

Methoxy poly(ethylene glycol)-*block*-poly(L-glutamic acid) copolymer (mPEG-PLG) was prepared as previously described [1]. Herein, mPEG5k (average 113 ethylene glycol repeating units) was applied to initiate the ring-opening polymerization of BLG-NCA, and the final copolymer had average 25 L-glutamic

acid repeating units. CA4 was grafted to the prepared mPEG-PLG by Steglich esterification. Briefly, mPEG-PLG (0.100 mmol, 850 mg) and CA4 (1.00 mmol, 286 mg) were dissolved in 30 mL anhydrous DMF in a glass reactor, then DIC (1.20 mmol, 186 μL) and DMAP (0.750 mmol, 91.6 mg) were added. The reaction proceeded at room temperature for 24 h. Then the reaction mixture was precipitated into excess diethyl ether, re-dissolved in DMF, and dialyzed in distilled water. The final product was obtained after lyophilization with a yield of 86%.

Chemical structures of the synthesized mPEG-PLG and mPEG-PLG-*g*-CA4 were confirmed by ^{1}H NMR (D_2O, Bruker AV 400 NMR spectrometer) and Fourier transform infrared spectroscopy (FT-IR, Bio-Rad Win-IR). Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC, Waters 515 pump, 2414 detector, DMF containing 0.01 M LiBr as the eluent, polystyrene as standard samples).

Preparation and characterization of PLG-CA4/DOX

mPEG-PLG-*g*-CA4 (500 mg) and DOX·HCl (13.0 mg) were dissolved in 50 mL DMF, the mixture was then added to 100 mL distilled water in a dropwise manner under vigorous stirring. The mixture was dialyzed in distilled water and the micelles were obtained after lyophilization.

The hydrodynamic radius (R_h) of the prepared micelles in phosphate buffered saline (PBS, pH 7.4) was determined by dynamic laser scattering (DLS) on a WyattQELS instrument with a vertically polarized He-Ne laser with 90^{o} collecting

optics. Zeta-potentials were measured with a Zeta Potential/BI-90 Plus particle size analyzer (Brookhaven, USA) at room temperature. Transmission electron microscopy (TEM) images were taken from JEOL JEM-1011 transmission electron microscope at an accelerating voltage of 100 kV. Critical micelle concentration (CMC) of the prepared micelles was determined by a fluorescence spectrometer (Perkin-Elmer LS50B) using pyrene as the probe, with excitation from 280 nm to 360 nm and emission at 390 nm. CMC was estimated as the inflexion-point in the curve of I_{339}/I_{335} *vs*. micelle concentration. Exact CA4 and DOX loading contents were calculated by measuring the UV-Vis absorbance at 290 nm and 480 nm in DMF/H_2O (v/v = 1/1), respectively. The drug loading content (DLC%) was calculated with the following formulation:

DLC% = (weight of loaded drug/weight of micelles) × 100%.

***In vitro* release**

In vitro release of DOX and CA4 from PLG-CA4/DOX micelles was conducted using a dialysis method. Briefly, 3 mg of PLG-CA4/DOX was dissolved in 5 mL phosphate buffered saline (PBS) solution at pH 7.4 or 5.5. The samples were sealed in a dialysis bag (MWCO 3500 Da) and incubated in 45 mL of the respective release media at 37°C with a shaking rate of 100 rpm. At predetermined time points (1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 36 h, and 48 h), 3 mL of the incubated solution was taken out and replaced with fresh media. The concentration of CA4 in the released media was determined by comparing absorbance at 290 nm using the UV-Vis spectrometer, while

DOX concentrations were determined by measuring the emission fluorescent intensity at 590 nm with excitation wavelength at 490 nm.

The time-dependent size changes of PLG-CA4/DOX in pH 7.4 and 5.5 PBS were measured by DLS. Briefly, PLG-CA4/DOX was dissolved in pH 7.4 and 5.5 PBS at a concentration of 0.2 mg/mL, and placed at 37°C with a shaking rate of 100 rpm. At predetermined time points (0 h, 4 h, 12 h, 24 h, and 48 h), R_h was determined by DLS.

Cell culture

Murine colon carcinoma C26 cells and murine mammary carcinoma 4T1 cells were purchased from Shanghai Bogoo Biotechnology Co. Ltd. Cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, supplemented with 50 U/mL penicillin and 50 U/mL streptomycin, and incubated at 37°C in 5% CO_2 atmosphere.

***In vitro* tumor cell inhibition test**

C26 cells were seeded in 96-well plates at a density of 7, 000 cells per well in 200 μL DMEM. After 24 h, the culture medium was removed and replaced with DOX, CA4, mPEG-PLG-*g*-CA4, and PLG-CA4/DOX at gradient concentrations. After another 48 h, the cells were subjected to MTT assay, and the absorbance was measured with a Bio-Rad 680 microplate reader at 492 nm. Cell viability was calculated according to the following equation:

viability (%) = $(A_{sample}/A_{control}) \times 100\%$

where A_{sample} and $A_{control}$ are the absorbances of the sample and control wells, respectively.

Animal use

All the animal experiments were conducted in accordance with the guidelines of the Laboratory Protocol of Animal Care and Use Committee, Jilin University. Balb/C mice were bought from Beijing Huafukang Biological Technology Co. Ltd. (HFK Bioscience, Beijing). The C26 xenograft tumor model was prepared by inoculating the right flank of Balb/C mice with a 0.10 mL C26 cell suspension, obtained by homogenation of a freshly dissected C26 solid tumor. The 4T1 orthotopic tumor model was prepared by injecting 4T1 cells (1.0×10^6) into the mammary fat pad of Balb/C mice [2].

Immunohistochemical staining

Initially, we prepared the RhoB-labeled mPEG-PLG-*g*-CA4 by reacting mPEG-PLG-*g*-CA4 with RhoB-NH_2 with the help of EDC/NHS (a polymer/RhoB-NH_2 molar ratio of 1:2). Then Balb/C mice bearing C26 tumors were injected with RhoB-labeled mPEG-PLG-*g*-CA4 or PLG-CA4/DOX with a relative DOX dose of 5.0 mg/kg and CA4 dose of 50.0 mg/kg. 4 h later, the mice were sacrificed and tumors were collected and embedded in Tissue-Tek OCT embedding medium. Cryogenic slides (5 μm thickness) were prepared with a freezing microtome (Leica CM 1900) and placed on polylysine-coated glass slides (Wuhan Boster

AR1065). Immunohistochemical staining was carried out following the immunocytochemistry (ICC) protocol developed by Abcam, similar to our previous work [3]. Sample photos were taken under a confocal laser-scanning microscope (CLSM, Carl Zeiss LSM 780).

H & E analysis

For comparison purposes, mPEG-PLG/DOX was prepared as previously described [4]. mPEG-PLG formed an amphiphile complex with DOX in the aqueous phase, with an R_h of 111 ± 26.3 nm, zeta potential of -18.1 ± 2.3 mV and DOX loading content of 3.0%.

Balb/C mice bearing C26 tumors were divided into three groups and injected with mPEG-PLG/DOX, mPEG-PLG-*g*-CA4, or PLG-CA4/DOX at a relative DOX dose of 5.0 mg/kg and CA4 dose of 50.0 mg/kg. The mice were sacrificed at different time points (24 h and 72 h). Tumors were collected, embedded with paraffin, and stained with H & E. Histological photos were taken and analyzed under a microscope (Nikon TI-S/L100).

Intra-tumor drug concentration measurements

Balb/C mice bearing C26 tumors were divided into three groups, injected with free DOX, free CA4 or PLG-CA4/DOX, at a relative DOX dose of 5.0 mg/kg and CA4 dose of 50.0 mg/kg. At predetermined time points (4, 24, 48, and 72 h), the mice were sacrificed and the tumors were collected, homogenized in H_2O/MeOH, and

centrifuged. The extracts were passed through a 0.22 μm membrane and the drug concentrations were determined using high-performance liquid chromatography (HPLC); for CA4, a reverse-phase C-18 column (Symmetry), with a mobile phase of acetonitrile and water (80/20, v/v) and UV detector set at 305 nm was used. For DOX, a reverse-phase C-18 column (Symmetry), with a mobile phase of acetonitrile and water (30/70, v/v, the water phase containing 30 mM phosphate buffer pH adjusted to 3-4) and fluorescent detector set at 480 nm Ex and 590 nm Em was used.

***In vivo* tumor therapy studies**

The antitumor efficacy was first evaluated in C26 tumor bearing mice. After tumor growth reached approximately 100 mm^3, the mice were randomly divided into seven groups and treated with saline, DOX, mPEG-PLG/DOX, CA4, mPEG-PLG-*g*-CA4, DOX+CA4, and PLG-CA4/DOX at a relative DOX dose of 5.0 mg/kg and CA4 dose of 50.0 mg/kg. Injections were carried out on days 1 and 8, with especially additional injections of DOX and mPEG-PLG/DOX on days 3 and 10. Measurements of tumor volume and body weight were used to evaluate the treatment efficacy and safety, respectively. Photos of mice bearing tumor were taken on day 15. Tumor volume (V_t) and tumor suppression rate (TSR%) were calculated based on the following equations:

$$V_t = a \times b^2/2$$

$$\text{TSR\%} = [(V_c - V_x)/V_c] \times 100\%$$

where a and b are the major and minor axes of the tumors measured by caliper. V_c

represents tumor volume of the control group; V_x represents tumor volume of the treatment group.

Therapy for large C26 tumors was conducted similar to the above, except that treatment started when tumor volume grew to about 250 mm^3. Saline and PLG-CA4/DOX were injected on days 1 and 5 at a relative DOX dose of 5.0 mg/kg and CA4 dose of 50.0 mg/kg. Tumor volumes and body weights were recorded.

Therapy for large 4T1 tumors was conducted similar to the above. Treatment started when tumor volume grew to about 250 mm^3. Saline, mPEG-PLG/DOX, mPEG-PLG-*g*-CA4 and PLG-CA4/DOX were injected on days 1 and 14 at a relative DOX dose of 5.0 mg/kg and CA4 dose of 50.0 mg/kg. Tumor volumes and body weights were recorded. Photos of mice bearing tumor were taken on day 21.

Supplemental figures

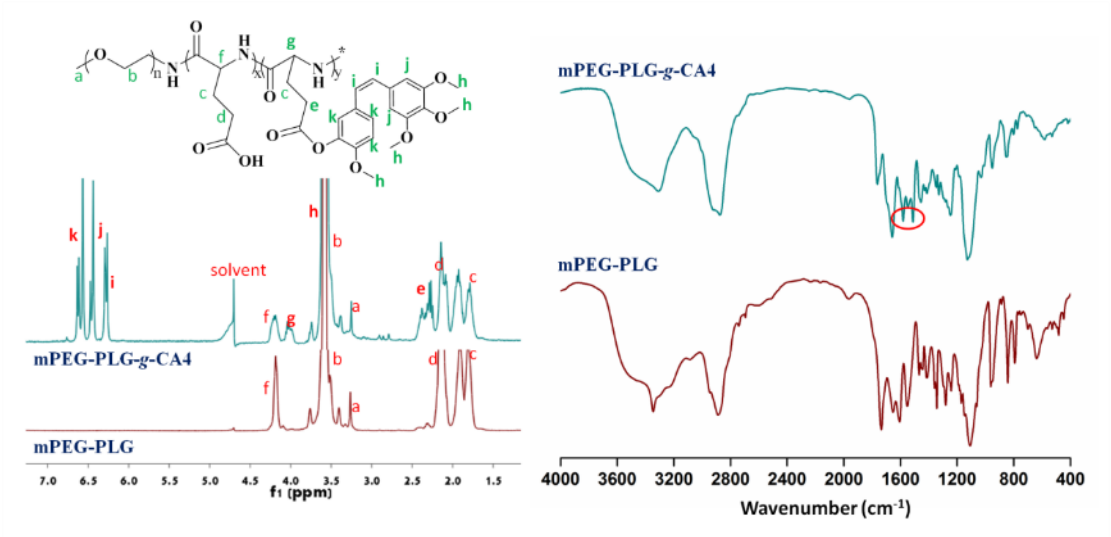

Figure S1. ^{1}H NMR (A) and FT-IR (B) characterization of mPEG-PLG-*g*-CA4. Bold numbers indicate the newly appeared resonance peaks in mPEG-PLG-*g*-CA4 compared to mPEG-PLG. Red circles point to characteristic peaks (1600 and 1500 cm^{-1}) of benzene in the CA4 of mPEG-PLG-*g*-CA4.

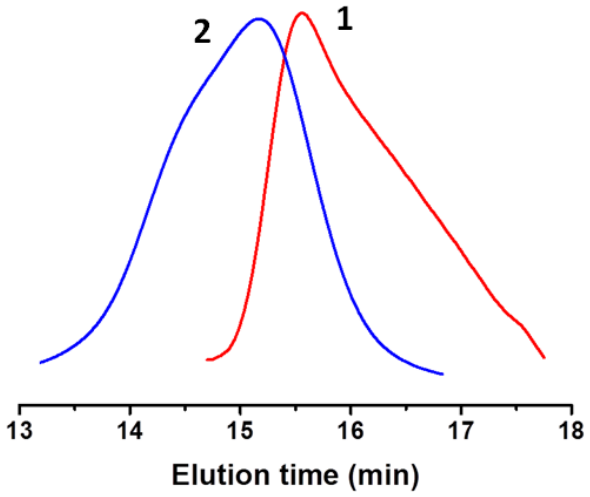

Figure S2. GPC elution curves of mPEG-PLG (1) and mPEG-PLG-*g*-CA4 (2) in DMF.

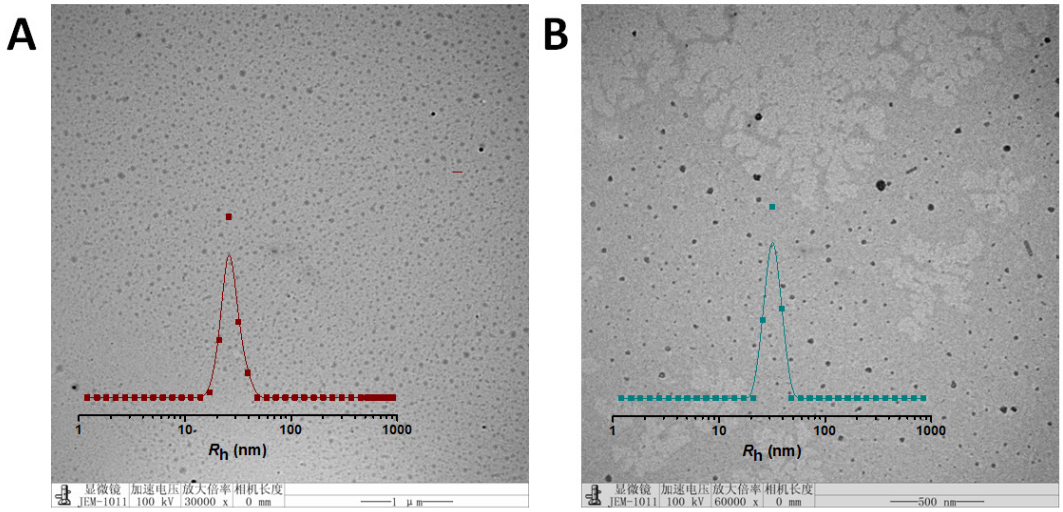

Figure S3. TEM and DLS results of (A) mPEG-PLG-*g*-CA4 and (B) PLG-CA4/DOX in pH 7.4 phosphate buffered saline.

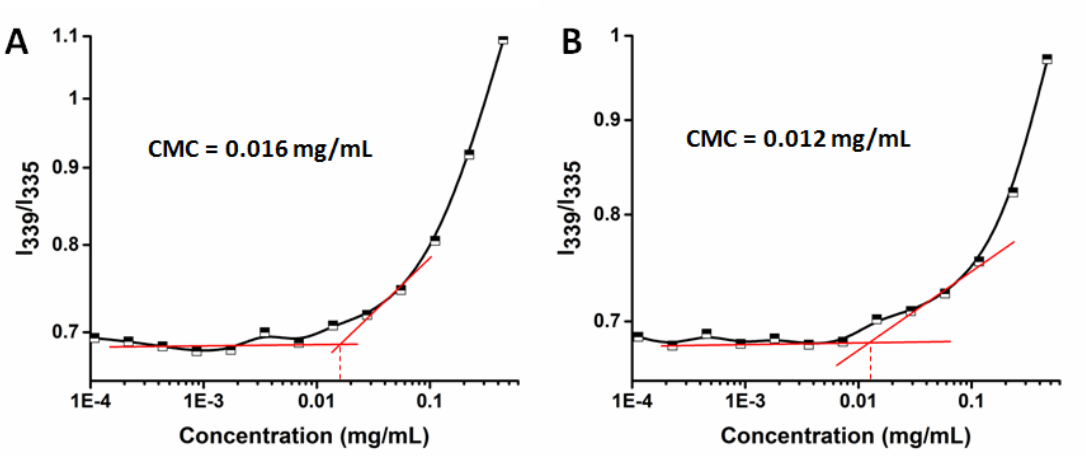

Figure S4. Correlation curves of fluorescence intensity ratios (I_{339}/I_{335}) *vs.* micelle concentrations. (A) mPEG-PLG-*g*-CA4, (B) PLG-CA4/DOX. CMC was determined as the inflexion-point of the curve.

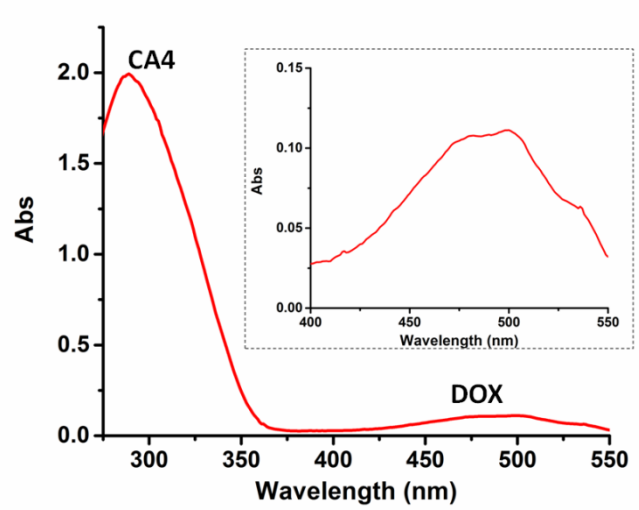

Figure S5. UV-Vis spectrum of 0.25 mg/mL PLG-CA4/DOX in DMF/H_2O (v/v = 1/1). The absorbance peaks of CA4 and DOX are at 290 nm and 480 nm. Insert is the enlarged spectrum from 400 nm to 550 nm.

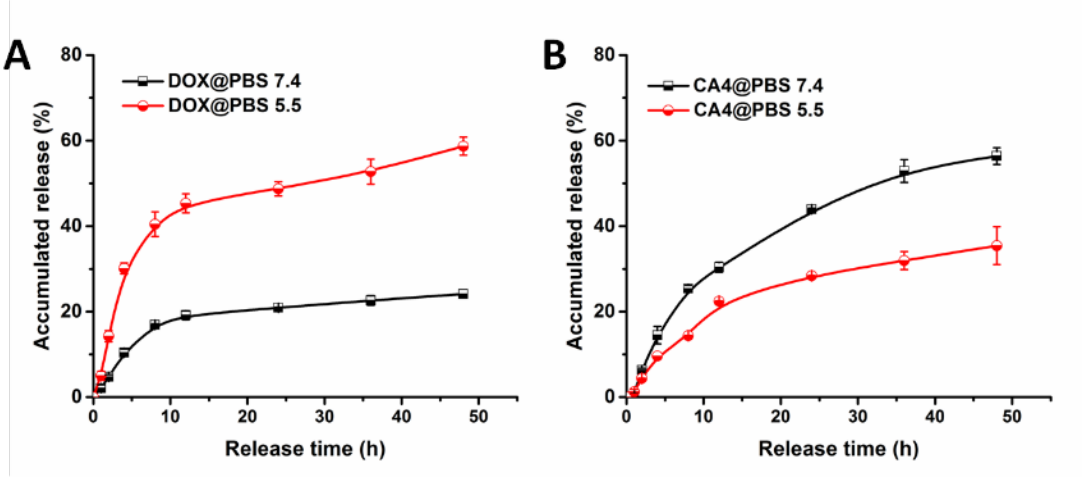

Figure S6. *In vitro* release of DOX and CA4 from PLG-CA4/DOX in pH 7.4 and 5.5 phosphate buffered saline (n = 3).

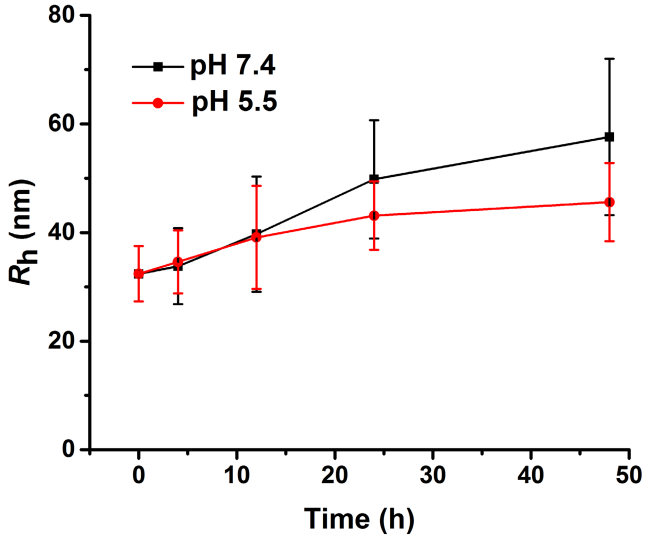

Figure S7. Time-dependent size changes of PLG-CA4/DOX micelles in pH 7.4 and 5.5 phosphate buffered saline, determined by DLS (n = 3).

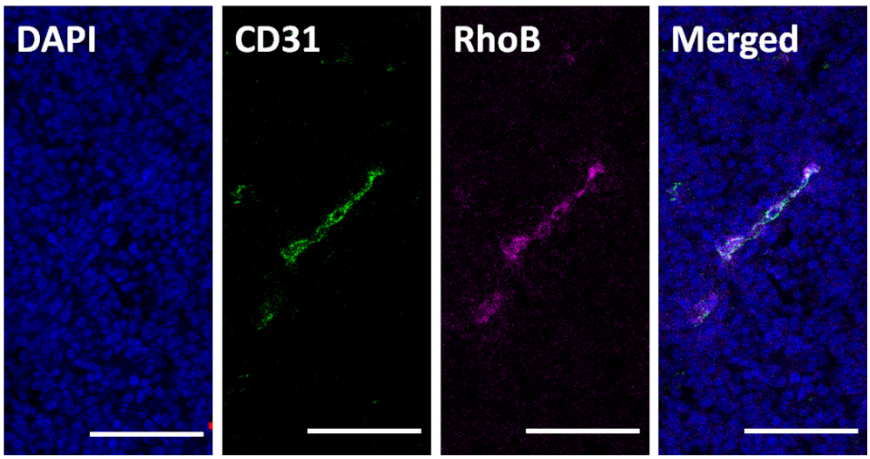

Figure S8. Histopathochemical analysis of tumor tissues 4 h after injection of RhoB-labeled mPEG-PLG-*g*-CA4. Scale bar = 50 μm.

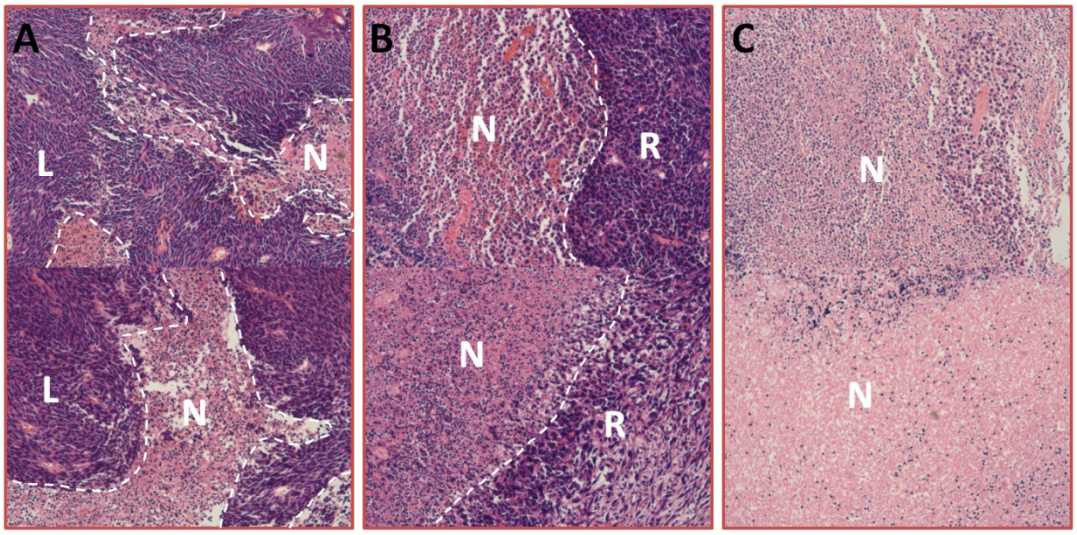

Figure S9. H & E analysis of tumor tissues 72 h after injection of mPEG-PLG/DOX (A), mPEG-PLG-*g*-CA4 (B), and PLG-CA4/DOX (C) at a relative DOX dose of 5.0 mg/kg and CA4 dose of 50.0 mg/kg. **L**, **N** and **R** indicate the live, necrotic and relapsed regions.

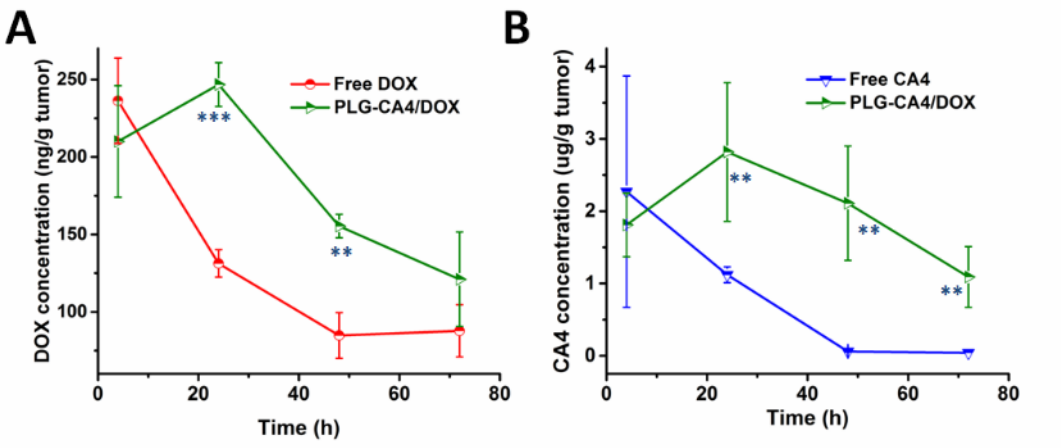

Figure S10. Intra-tumor DOX (A) and CA4 (B) concentrations after injection of free DOX, free CA4 or PLG-CA4/DOX at a relative DOX dose of 5.0 mg/kg and CA4 dose of 50.0 mg/kg, $n = 3$, ** $p < 0.01$, *** $p < 0.001$.

References

[1] Song W, Li M, Tang Z, Li Q, Yang Y, Liu H, et al. Methoxypoly(ethylene glycol)-block-poly(L-glutamic acid)-loaded cisplatin and a combination with iRGD for the treatment of non-small-cell lung cancers. Macromol Biosci. 2012; 12: 1514-23.

[2] Li M, Tang Z, Zhang D, Sun H, Liu H, Zhang Y, et al. Doxorubicin-loaded polysaccharide nanoparticles suppress the growth of murine colorectal carcinoma and inhibit the metastasis of murine mammary carcinoma in rodent models. Biomaterials. 2015; 51: 161-72.

[3] Song W, Tang Z, Zhang D, Yu H, Chen X. Coadministration of vascular disrupting agents and nanomedicines to eradicate tumors from peripheral and central regions.

Small. 2015; 11: 3755-61.

[4] Li M, Song W, Tang Z, Lv S, Lin L, Sun H, et al. Nanoscaled poly(L-glutamic acid)/doxorubicin-amphiphile complex as pH-responsive drug delivery dystem for effective treatment of non-small cell lung cancer, Acs Appl Mater Inter. 2013; 5: 1781-92.