Supporting Information

Acid Active Cell-Penetrating Peptide for In Vivo Tumor-Targeted Drug Delivery

Erlei Jin[†,¶], Bo Zhang[†,¶], Xuanrong Sun[‡], Zhuxian Zhou[†], Xinpeng Ma[†], Qihang Sun[†], Jianbin Tang[‡], Youqing Shen[‡]* Edward Van Kirk[±], William J. Murdoch[±], Maciej Radosz[†],

[‡]Center for Bionanoengineering and the State Key Laboratory for Chemical Engineering, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou, China 310027, [†]Department of Chemical and Petroleum Engineering and [±]Department of Animal Science, University of Wyoming, Laramie, Wyoming 82071

1. Materials and methods

1.1. Materials

Cysteamine, poly(ethylene glycol) (Mn: 3,300) and tin(II) 2-ethylhexanoate, ε-caprolactone (CL) were purchased from Fluka (St. Louis, MO). *N*-Maleoyl-beta-alanine, ^{1}H-pyrazole-1-carboxamidine hydrochloride, thionyl chloride, succinyl chloride, doxorubicin (DOX), 2,2'-azobisisobutyronitrile (AIBN), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich (St. Louis, MO). TAT (YGRKKRRQRRRC[NH2]) was purchased from Invitogen (Carlsbad, CA). *N, N*-Dimethylformamide (DMF), triethylamine (TEA) and dichloromethene (DCM) were dried over 4 Å molecular sieves. SKOV-3 ovarian cancer cells and MCF-7 breast cancer cells were purchased from American Type Culture Collection (ATCC) and maintained in our laboratory.

1.2. Synthesis of ^{a}TAT-PEG-PCL (Figure S1)

Amidization of TAT: TAT (5 mg) was dissolved in 5 mL of pH 8.5 PBS buffer and cooled to 0 ℃. Several drops of succinyl chloride were added to the solution and

stirred for 24 h. The mixture was loaded into a dialysis bag (Spectra Por-7, MWCO 1000) and dialyzed against pH 7.4 PBS buffer followed by lyophilization (4.2 mg, yield 81%). The product amidized TAT (^{a}TAT) was analyzed using HPLC and MALDI-TOF MS spectra.

Synthesis of ω-allyl-α-hydroxy PEG (allyl-PEG-OH): allyl alcohol (0.1 g, 1.72 mmol) in 30 mL of anhydrous THF solution and potassium naphthalide solution (1.6 mmol in 5 mL of anhydrous THF) synthesized according to the literature[1] were charged to a 250 mL round-bottom flask under nitrogen atmosphere. The solution was stirred at room temperatures for 10 min to form potassium allyl alcoholate as an initiator. The mixture was cooled to 0 ℃ and ethylene oxide (4.9 g, 110 mmol) was added to the flask using a cold glass syringe. The reaction system was stirred at 0 ℃ for several hours and then room temperature for another 40 h. The solution was poured into an excess of diethyl ether. The solid was isolated and purified by reprecipitation, and dried under vacuum at room temperature to give the final product (4.55 g, yield 91%). ^{1}H-NMR (400 MHz, CDl_3): δ (ppm): 5.95-5.83 (m), 5.28 (d), 5.18 (d), 4.09-4.03 (m), 3.68 (br). The molecular weight of the allyl-PEG was 3.3 KDa determined by ^{1}H-NMR. The PDI of the polymer was 1.36 determined by gel permeation chromatograph (GPC, Waters, Milford, MA)

Synthesis of ω-allyl-PEG-*block*-poly(ε-caprolactone) *(allyl-PEG-PCL)*: Allyl-PEG-OH (0.99 g, 0.3 mmol) and CL (0.61 g, 5.3 mmol) were dissolved in 15 ml of anhydrous toluene at room temperature. The solution was bubbled with nitrogen for 20 minutes and tin (II) 2-ethylhexanoate (0.12 g, 0.3 mmol) was added as catalyst. The mixture was stirred at 110 ℃ for 4 h. After cooled to room temperature, the mixture was poured into 100 ml cold ether. The solid was isolated and purified by reprecipitation, and dried in vacuum at room temperature to give the product allyl-PEG-PCL (1.33 g, yield 83%). ^{1}H-NMR (400 MHz, CDl_3): δ (ppm): 6.00-5.89 (m), 5.28 (d), 5.18 (d), 4.25 (t), 4.09-4.03 (m), 3.72 (br), 2.38-2.26 (m), 2.07 (br), 1.70-1.58 (m), 1.57-1.36 (m). The molecular weight of the PCL block was 2.1 KDa determined by ^{1}H-NMR. The PDI of the block copolymer was 1.43 determined by GPC.

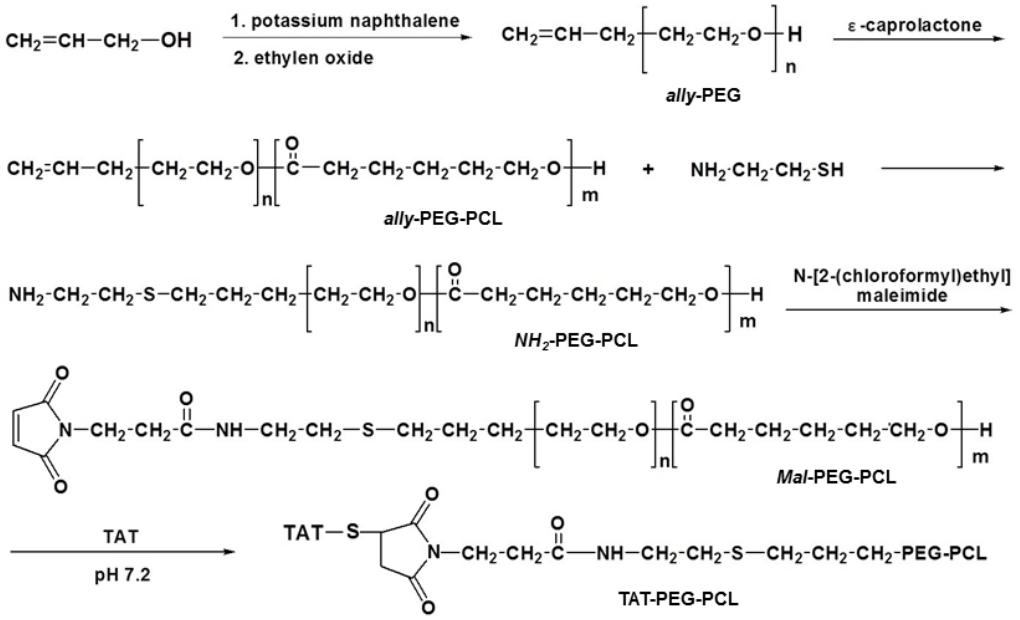

Figure S1 | Synthesis of the end-functionalized PEG-PCL block copolymers.

Synthesis of ω-amino-PEG-*block*-poly(ε-caprolactone) *(NH_2-PEG-PCL)*: Allyl-PEG-PCL (0.54 g, 0.1 mmol) and cysteamine (115.5 mg, 15 mmol) were dissolved in 5 ml of anhydrous DMF. AIBN (3.2 mg, 0.02 mmol) and one drop of TEA were added as catalyst. The mixture was bubbled with nitrogen for 20 minutes at room temperature and then reacted at 70 ℃ for 24 h. The reaction was tracked by ^{1}H-NMR spectra until the double bonds disappeared. The solvent was removed under vacuum and the excess of cysteamine was sublimated in the meanwhile. The final product NH_2-PEG-PCL (0.40 g, yield 72.7%) was obtained by reprecipitation in anhydrous ether. ^{1}H-NMR (400 MHz, CDl3): δ (ppm): 4.25 (t), 4.09-4.03 (m), 3.72 (br), 2.75-2.63 (br), 2.62-2.45 (br), 2.38-2.26 (m), 1.70-1.58 (m), 1.57-1.36 (m).

Synthesis of Mal-PEG-PCL: *N*-Maleoyl-β-alanine (1.69 g, 10 mmol) and 2 mL of thionyl chloride were dissolved in 15 mL of anhydrous DCM and one drop of DMF was added as catalyst. The mixture was stirred at room temperature overnight. The solvent and excess of thionyl chloride were removed under vacuum at room temperature to obtain the *N*-[2-(Chloroformyl)ethyl] maleimide. NH_2-PEG-PCL (0.28 g, 0.05 mmol) and TEA (5 mg, 0.05 mmol) were dissolved in 10 mL of anhydrous DCM. At 0 °C, the solution of the *N*-[2-(Chloroformyl) ethyl] maleimide (47 mg, 0.25

mmol) in 5 mL of anhydrous DCM was added dropwise into the polymer solution. The mixture was stirred at 0 °C for several hours and then room temperature overnight. DCM was removed under vacuum. The solid was dispersed in 5 mL of water, and the mixture was loaded into a dialysis bag (MWCO 3500) and dialyzed against 2 liter of deionized water. The pure *Mal*-PEG-PCL (0.17 g, yield 53%) was obtained after lyophilization. ^{1}H-NMR (400 MHz, CDl_3): δ (ppm): 6.72 (s), 4.25 (t), 4.09-4.03 (m), 3.72 (br), 3.48-3.58 (br), 2.75-2.63 (br), 2.62-2.43 (br), 2.38-2.26 (m), 1.70-1.58 (m), 1.57-1.36 (m).

Synthesis of TAT or ^{a}TAT-functionalized PEG-PCL (TAT-PEG-PCL or ^{a}TAT-PEG-PCL): Maleimide-end capped PEG-PCL(*Mal*-PEG-PCL, 56 mg, 0.01 mmol) was dispersed in 5 mL of pH 7.4 PBS buffer. TAT or ^{a}TAT (20 mg, 0.012 mmol) was added into the solution. The mixture was stirred in the dark at room temperature for 24 h. The mixture was loaded into a dialysis bag (Spectra Por-7, MWCO 3500) and dialyzed against 2 liter of pH 7.4 PBS buffer to remove the excess free peptides. The TAT-PEG-PCL (yield 66%) was obtained after lyophilization. ^{1}H-NMR spectra showed that the double bonds of maleimide group (δ: 6.72 ppm) disappeared. Similarly, ^{a}TAT-PEG-PCL were obtained (yield 92%).

1.3. Fabrication of micelles

PEG-PCL, ^{a}TAT-PEG-PCL and FA-PEG-PCL (5 mg, mixed at the needed molar ratio) were dissolved in 1 mL of DMSO. The DMSO solution was dropped into 5 mL of pH 8.5 PBS buffer with stirring. The solution was loaded into a dialysis bag (Spectra Por-7, MWCO 3500) and dialyzed against pH 8.5 PBS buffer.

DOX or DiR loaded micelles: DOX hydrochloride salt (1 mg) was dissolved in 1 mL of DMSO and stirred for 10 min. Triethylamine (20 μL) was added to the solution and stirred for another 1 h. PEG-PCL, ^{a}TAT-PEG-PCL and FA-PEG-PCL (5 mg, mixed at the needed molar ratio) were added into the above DMSO solution and stirred for 1 h. The solution was dropped into 5 mL of pH 7.4 PBS buffer and stirred. The mixture was then loaded into a dialysis bag (Spectra Por-7, MWCO 3,500) and dialyzed against with pH 8.5 PBS. The solution was filtered through a 0.45 μm filter.

The DOX loading efficiency and content were analyzed by measuring DOX's UV absorbance at 486 nm in DMSO. The encapsulation efficiency was 83%, and the loading content was 13.6%. DiR loaded nanoparticles were fabricated similarly except for not adding TEA. The DiR loading was analyzed by measuring its fluorescence absorbance at excitation 748 nm and emission 780 nm. The loading content was 14.3%.

1.4. Reverse-phase HPLC

Ion-pairing reverse-phase HPLC (Agilent 1100, Santa Clara, CA) was performed with a RP-C18 HPLC column (4.6 × 250 mm^2, 5 μm particle size) and UV detector. The mobile phase was a gradient of 10-50% of acetonitrile aqueous solution containing 0.5% TFA at a total flow rate of 1 mL/min. An aliquot of 200 μL sample at approximately 0.1 mg/mL was injected, of which 20 μL went into the column at 25 ℃. The UV absorption peaked at 210 of the elution was recorded for analysis.

1.5. Transmission electron microscope (TEM) observation

The micelles were prepared as described above. The micelle solution (two drops) was applied onto a 300-mesh carbon-coated copper grid for a while and the excess solution was wicked off with a piece of filter paper. A drop of phosphotungstic acid (PTA, 2%) was added onto the grid and dried for a while. Images were recorded using a transmission electron microscope (HITACHI H-7000 TEM) operated at a voltage of 75 kV.

1.6 Cellular uptake observation under confocal microscopy

SKOV-3 ovarian cancer cells were plated onto glass-bottom petri dishes (MatTek, Ashland, MA, no.P35G-1.0-14-C) at a density of 80,000 cells per plate in 2 mL of medium (RPMI-1640, Sigma-Aldrich, supplemented with 10 % FBS). The cells were incubated at 37 °C and 5 % CO_2 for 24 h prior to the treatments. Treatments were prepared in 10 mM pH 7.4 HEPES solution. Each well was added with 20 μL of the nile red-loaded micelles at an nile red concentration of 1 μg/mL. After 60 min, LysoTracker (Molecular Probes, Carlsbad, CA) was added to each well at a concentration of 200 nM. The images were taken at different time points using a

confocal laser scanning microscope (Leica TCS SP2 microscope). DRAQ5^{TM} (Biostatus Limited, Leicestershire, UK) was added into each well at a concentration of 5 μM before observation. The nuclear staining was observed using a 633-nm laser, and the emission wavelength was from 660 to 760 nm and expressed as blue. LysoTracker was observed using a 488-nm laser, and the emission wavelength was from 510 to 540 nm and expressed as green. Nile red loaded micelles were observed using a 543-nm laser, and the emission wavelength was from 560 to 610 nm and expressed as red. Images were taken by using the lasers sequentially with a 63× objective lens.

1.7 Cellular uptake measured by flow cytometry

SKOV-3 ovarian cancer cells were seeded onto six-well plates (2.5 mL of cell suspension per well) at a density of 2×10^5 cells/mL and allowed to grow for 24 h. The DOX-loaded micelles (at a DOX dose of 1 μg/mL) were added into each well and incubated for 1 or 5 h. The cells were then washed with cold PBS twice, harvested by 0.25% (w/v) trypsin/0.03% (w/v) ethylenediaminetetraacetic acid (EDTA), pelleted in eppendorf tubes and centrifuged at 1,000 g for 4 min at 4 ^{o}C, and resuspended in PBS. Each sample was quickly analyzed on a Millipore's Guava flow cytometer (Billerica, MA) using the 488 nm argon laser for excitation and the emitted 525 nm fluorescence for detection. Data were collected of 5000 gated events and analyzed with the Millipore's Guava software program.

1.8 In vitro MTT assay

The cytotoxicity assay was carried out using the (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) cell proliferation kit (ATCC, Manassas, VA) according to the manufacturer's protocol. Cells were seeded in 96-well plates at an initial density of 5,000 cells/well in 200 μL of RPMI medium and grown for 24h. The original medium in each well was replaced with 100 μl of fresh medium. Free DOX, TAT-PEG-PCL/DOX, ^{a}TAT-PEG-PCL/DOX or (FA/^{a}TAT)-PEG-PCL/DOX solutions were added to the medium at DOX doses ranging from 0.05 to 10 μg/mL. Each dosage was replicated in 3 wells. Treated cells were incubated at 37 °C under a

humidified air with 5% CO_2 for 24 h. The medium in each well was then replaced with fresh culture medium and the cells were cultured for another 24 h. The MTT solution was then added to the plates for an additional 3 h incubation, allowing viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Finally, DMSO was added into each well to dissolve the formazan crystals. The absorbance in each well was determined at 562 nm using a microplate reader. The cytotoxicity was expressed as a percentage of the control.

1.9 Animals

All animal studies were approved by the Animal Care and Use Committee of University of Wyoming or Zhejiang University in accordance with the guidelines for the care and use of laboratory animals. Eight-week-old athymic nude mice (BALB/c nu/nu, Charles River Laboratories, Wilmington, MA, USA or Zhejiang University Animal Center) were used in this study.

2.0 Blood circulation in mice

Each group had four mice. 100 μL of DiR-loaded micelles (at a DiR dose of 10 μg/mL) saline solution was intravenously (*i.v.*) injected into the tail vein of each mice. At timed intervals, about 100 μL of blood was collected from the tail vein and stored in heparin containing eppendorf tubes at 4 ℃ for further analysis. The plasma was separated from the blood by centrifuging at a speed of 2,000 RPM for 5 min. The plasma was diluted with methanol and centrifuged to remove the insoluble solid. At the excitation wavelength of 748 nm, the fluorescence intensity at 780 nm was measured using a microplate reader (SpectraMax M2e) and the corresponding DiR concentration was calculated according to an established standard curve. The percent injected dose per milliliter (%ID/mL) blood was calculated accordingly.

2.1 In vivo antitumor study

Subcutaneous tumors were established in athymic female BALB/c mice by subcutaneous inoculation of 1×10^6 cancer cells in the flank region. One week after tumor implantation, the tumor bearing nude mice were randomly divided into five groups with five animals per group. Mice were *i.v.* administrated with PBS, DOX,

PEG-PCL/DOX, TAT-PEG-PCL/DOX or [a]TAT-PEG-PCL/DOX at the DOX or DOX-equivalent dose of 4 mg/kg every two or three days, depending on the healthy status of mice. Each mouse was earmarked and followed individually throughout the whole experiments. The width and length of the tumors and the body weight of mice were measured at the time of each injection until the animals were terminated. Tumor volume (V) were calculated using the following formula: $V\ (mm^3) = \pi \times \text{width (mm)} \times \text{width (mm)} \times \text{length (mm)}/6$ [2]. The therapeutic efficacy of the treatments was evaluated by comparing the experimental group with control group. Mice were terminated after the 7th injections on day 18. Hearts and tumors were excised, and the tumors were weighed. The inhibition rate (TIR) of tumor growth was calculated using the following equation: TIR = 100% × (mean tumor weight of control group - mean tumor weight of experimental group)/mean tumor weight of control group.

2.2 DOX accumulation in tumor tissues

Quantitation: A fraction of the removed tumors (0.2 g) in each group was homogenized in 1 mL of lysis buffer (0.25 M sucrose, 10 mM phosphate buffer) with a Homogenizer (T 10B, IKA, Germany). For DOX measurements, tissue lysate (200 μL) was mixed with Triton X-100 (10%, 1 mL). After vigorous vortexing, 1.5 mL of the extraction solution (0.75 M HCl in isopropanol) was added, and the samples were incubated at -20 °C for overnight. The solution was centrifuged at 14,800 rpm for 30 min. The fluorescence of the supernatant was measured at an emission of 590 nm with an excitation wavelength of 488 nm. The DOX concentration in the tumor was calculated accordingly.

Confocal microscopy observation: The removed tumors were fixed with neutral paraformaldehyde and embedded into paraffin. Tumor sections of 7 μm thickness were mounted onto glass slides. The fluorescence images were acquired on a confocal microscope (Nikon-A1 system, Japan) from the DOX emission fluorescence between 550 and 600 nm excited with a 488 nm laser. Background subtraction of the images was performed using ImageJ to reduce artifacts resulting from the laser beam profile. The imaging parameters were kept constant for different groups.

2.3 TdT-mediated dUTP nick end labeling assay

Apoptotic events after the treatments were determined by TdT-mediated dUTP nick end labeling (TUNEL) assay in xenograft tumor sections as reported [3]. Briefly, 7 μm thick paraffin-embedded tissue sections were prepared from the dissected tumors. The slides were then subjected to TUNEL staining using an In Situ Cell Death Detection Kit according to the manufacturer's protocol (Roche, Penzberg, Germany). Apoptotic cells were identified by positive TUNEL staining under light microscopy. Three randomly chosen microscopic fields in each group were used to calculate the percentages of apoptotic cells.

2.4 Statistics

Assignments to treatments and selections of fields of microscopic inspection were made at random. Within-animal/subsample data were averaged. Mean comparisons were made by analysis of variance and protected least significant difference or Student's *t*-test. Contrasts were considered significant at $p<0.05$. Data are plotted as means ± standard errors (SE).

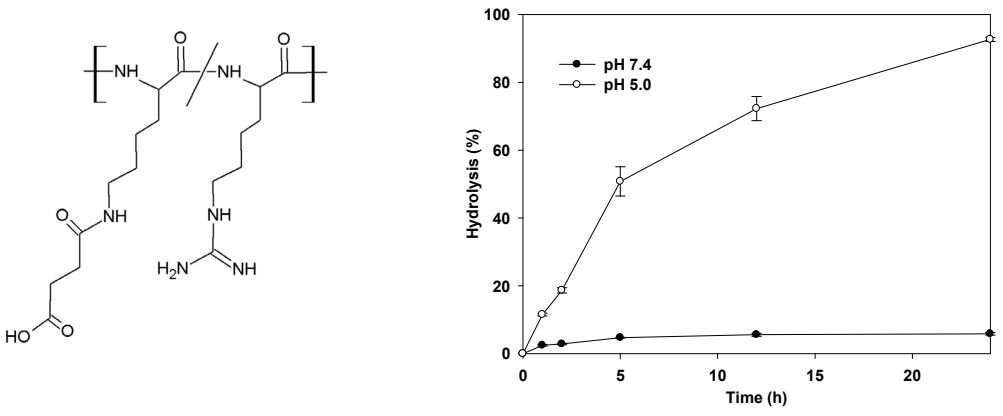

Figure S2 | Hydrolysis of succinylamide in guanidinated PLL at pH 7.4 and pH 5.0 detected by ^{1}H-NMR spectra. Data were presented as mean ± SE (n=3). A polylysine (number average molecular weight of 2 KDa) was reacted with 1H-pyrazole-1-carboxamidine hydrochloride to convert 50 % of the lysine residues' primary amines into guanidinyl groups. The polymer was isolated and reacted with

succinic chloride to amidize the remaining primary amines to succinylamides (structure shown in the Figure) as described in amidization of TAT. The polymer was purified and dissolved D_2O. The solution pH was adjusted to 7.4 or 5.0 using a D_2O solution of NaOD or DCl. The solution was warmed in a water bath at 37 °C with shaking. At timed intervals, samples were withdrawn and their ^{1}H-NMR spectra were recorded on a Bruker Avance DRX-400 spectrometer. The hydrolysis of the amide was calculated as we reported.[4] At pH 7.4, only 5% of amides hydrolyzed within 5 h and less than 8% hydrolyzed after 24 hours. While at pH 5.0, about 90% of these amides hydrolyzed after 24h. In contrast, the succinylamides of pure PLL was not hydrolysable at pH 5. [4]

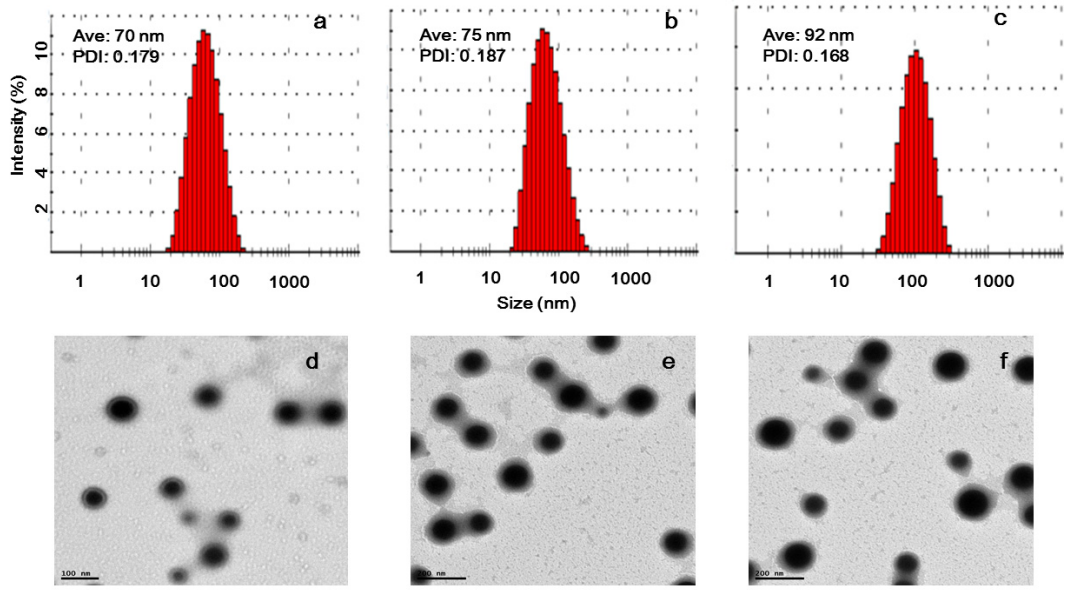

Figure S3 | Sizes and morphologies of the micelles of [a]TAT-PEG-PCL (**a** and **d**), [a]TAT-PEG-PCL/DOX (13.6 wt% DOX) (**b** and **e**) and [a]TAT-PEG-PCL/DiR (14.3 wt% DiR) (**c** and **f**). The sizes were determined by dynamic light scattering, and the morphologies were observed by transmission electron microscopy (nanoparticles were stained by 2% phosphotungstic acid).

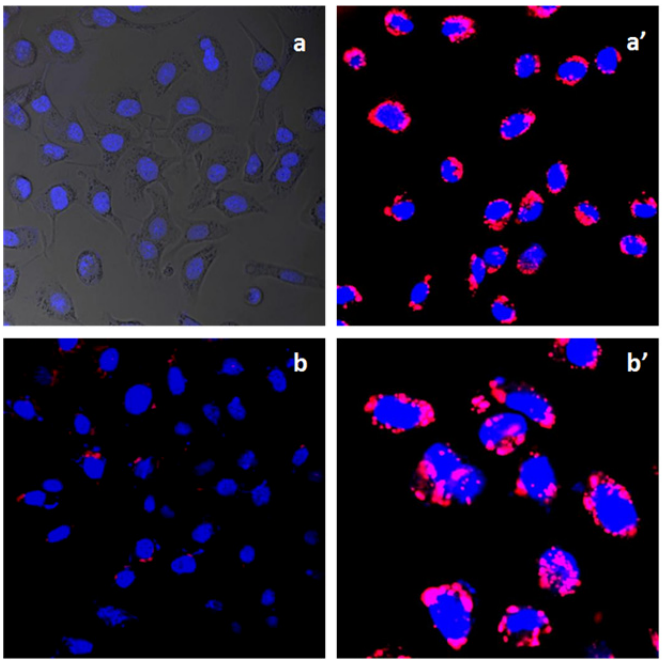

Figure S4 | Cellular uptake of PEG-PCL/nile red (a, b) or TAT-PEG-PCL/nile red micelles (a', b') observed by confocal laser scanning microscopy. SKOV-3 ovarian cancer cells were cultured with the nile red loaded micelles for 1 h (a, a') or 5 h (b, b') at a nile red dose of 1 μg/mL. The nuclei were stained with DRAQ5 (blue). The nanoparticles/nile red were assigned as red. Original magnification was 63 ×.

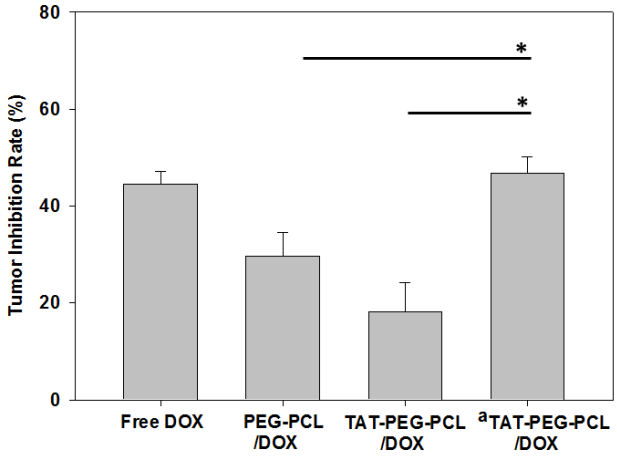

Figure S5 | Tumor inhibition rates. At day 18, mice were terminated and tumors were dissected and weighed. TIR = 100% × (mean tumor weight of control group - mean tumor weight of experimental group)/mean tumor weight of control group. A 47% tumor inhibition rate was achieved by [a]TAT-PEG-PCL/DOX, compared to 29% and 18% for PEG-PCL/DOX and TAT-PEG-PCL/DOX respectively. Data were presented as mean ± SE, n=5. * $p<0.05$.

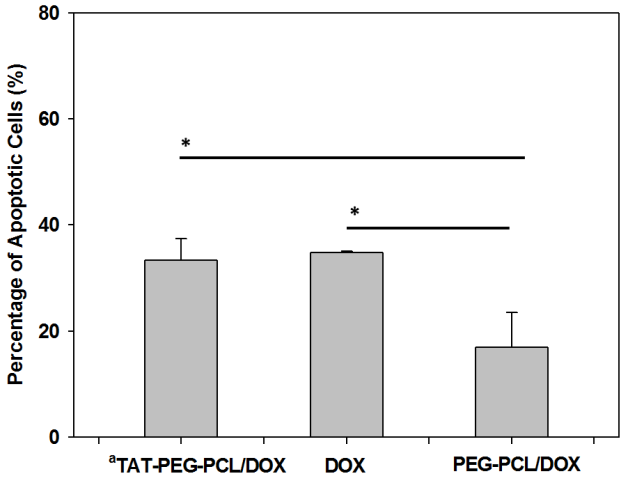

Figure S6 | Statistical analysis of apoptotic cells in tumor tissues by TUNEL assay. The apoptotic cancer cells in each slide were statistically analyzed by randomly taking three areas in a large view. The number of brown cells in each view was counted, and the percentage of the number of positive cells to the total number of cells was then calculated. Data were presented as mean ± SE (n=3). * p<0.05.

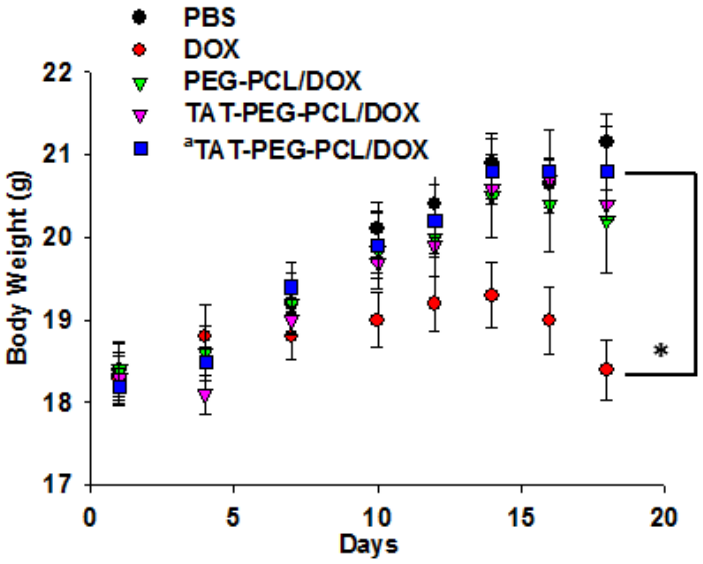

Figure S7 | The change of mice body weights during the treatment. Data were expressed as mean ± SE, n=5. * p<0.05.

References:

1. Scott, N. D.; Walker, J. F.; Hansley, V. L., Sodium Naphthalene. I. A New Method for the Preparation of Addition Compounds of Alkali Metals and Polycyclic Aromatic Hydrocarbons. *J. Am. Chem. Soc.* **1936,** 58, (12), 2442-2444.

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3. Gavrieli, Y.; Sherman, Y.; Ben-Sasson, S. A., Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **1992,** 119, (3), 493-501.
4. Zhou, Z. X.; Shen, Y. Q.; Tang, J. B.; Fan, M. H.; Van Kirk, E. A.; Murdoch, W. J.; Radosz, M., Charge-reversal drug conjugate for targeted cancer cell nuclear drug delivery. *Adv. Funct. Mater.* **2009,** 19, (22), 3580-3589.