Supporting Information

Dimeric Drug Polymeric Micelles with Acid-Active Tumor Targeting and FRET-Traceable Drug Release

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Experimental Section

Materials. TAT (CYGRKKRRQRRR-NH_2) was purchased from GenScript. Maleimide-PEG-OH (M_n 5000), ε-caprolactone, 2,3-dimethylmaleic anhydride, campothecin, 2,3-dibromomaleimide, and tris(2-carboxyethyl)phosphine hydrochloride were purchased from Sigma-Aldrich. 2-Hydroxyethyl disulfide was purchased from Santa Cruz Biotechnology. AlamarBlue® and propidium iodide were purchased from ThermoFisher. Calcein AM and Annexin V-FITC apoptosis detection kit was purchased from eBioscience. All chemicals were purchased from commercial supplier and used as received.

Synthesis of Polymer and Drug Dimer

Amidization of TAT. TAT (0.01 mmol, 16.6 mg) was dissolved in HEPES buffer (pH 8.5) and cooled to 0 °C, followed by adding 2,3-dimethylmaleic anhydride (DA) (0.03 mmol, 3.8 mg) and stirred for 24 h. The mixture was then dialyzed to remove the unreacted DA and lyophilize to get DA-TAT.

Synthesis of Maleimide-PEG-PCL (Mal-PECL). Mal-PECL was synthesized through ring-opening polymerization (ROP) of ε-caprolactone initiated by Maleimide-PEG-OH in the presence of stannous octoate ($Sn(Oct)_2$) as the catalyst. Briefly, Maleimide-PEG-OH (0.02 mmol, 100 mg), ε-caprolactone (1.1 mmol, 125 mg) and $Sn(Oct)_2$ (1 wt% of ε-caprolactone) were degassed under vacuum for 5 h with continuous stirring, then heated at 150 °C for 4 h. After that, the mixture was allowed to cool down, dissolved in dichloromethane, precipitated

in cold ethanol, and collected by centrifugation. The Mal-PEG-PCL polymer was obtained by drying overnight under vacuum.

Synthesis of TAT-PEG-PCL (TAT-PECL). TAT-PECL was synthesized according to the literature.[24] Mal-PECL (0.005 mmol, 56.3 mg) was dissolved in acetonitrile, followed by rotary evaporation to form thin film at 37 °C and hydrated with phosphate buffer solution (PBS, pH 7.4). Then TAT (0.005 mmol, 8.3 mg) was added and stirred for 24 h. The unreacted TAT was removed by dialysis against PBS. The final product TAT-PEG-PCL was obtained by freeze drying.

Synthesis of DA-TAT-PECL. For the conjugation of DA-TAT, Mal-PECL (0.005 mmol, 56.3 mg) was dissolved in acetonitrile, followed by rotary evaporation to form thin film at 37 °C and hydrated with PBS buffer (pH 7.4). Then DA-TAT (0.005 mmol, 9.9 mg) was added and stirred for 24 h. The unreacted DA-TAT was removed by dialysis against PBS. The final product DA-TAT-PECL was obtained by freeze drying.

Synthesis of $(CPT)_2$-ss.[20] $(CPT)_2$-ss with a disulfide linker was synthesized through activating the hydroxyl group of CPT by triphosgene and then reacting with 2-hydroxyethyl disulfide. First, the dichloromethane solution of triphosgene (0.4 mmol, 118.7 mg) and 4-(dimethylamino)pyridine (DMAP, 1.0 mmol, 122.2 mg) was added dropwise into CPT (1.0 mmol, 348.4 mg) suspended in dichloromethane. Then, the mixture was stirred at room temperature for 6 h until the solution became clear, and reacted for another 24 h after addition of 2-hydroxyethyl disulfide (0.4 mmol, 61.7 mg). The product was obtained by removal of solvent through rotary evaporation and purified by silica gel column chromatography.

Synthesis of $(CPT)_2$-ss-Mal. The dithiomaleimide-based $(CPT)_2$-ss-Mal was synthesized by conjugating $(CPT)_2$-ss to 2,3-dibromomaleimide. Briefly, triethylamine (0.75 mmol, 75.9 mg) and TCEP.HCl (0.25 mmol, 71.6 mg) were dissolved in THF/methanol (1/3, v/v) and stired at room temperature for 1 h to remove the hydrochloric acid of TCEP.HCl. Then, the mixture was added dropwise into the solution of $(CPT)_2$-ss (0.25 mmol, 225.5 mg) and 2,3-

dibromomaleimide (0.25 mmol, 63.7 mg), and stirred for another 24 h. The product was obtained by removal of solvent through rotary evaporation and purified by silica gel column chromatography.

^{1}H NMR Determination. ^{1}H Nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Bruker Avance Ⅲ 500 apparatus. $CDCl_3$ or DMSO-d_6 was used as a solvent. Chemical shifts are expressed in parts per million, ppm (δ).

Preparation of Micelles. Blank micelles were prepared by solvent evaporation method. Briefly, polymer was dissolved in 5 mL of tetrahydrofuran amd added dropwise into 10 mL of deionized water under high-speed stirring. Then the mixture was stired under a moderate speed to completely remove tetrahydrofuran. Drug-loaded micelles were prepared by dialysis method. Polymer and $(CPT)_2$-ss-Mal with a weight ratio of 1 ∶ 1 were dissolved in 1 mL of DMSO and dropped into 9 mL of deionized water. After that, the mixture was transferred into a dialysis flask (MWCO 3500) against deionized water to remove the unloaded drug.

Characterization of Micelles and Drug Dimer

DLS Determination. Dynamic light scattering (DLS) (Malvern Zetasizer) was employed to detect the average size distribution of micelles. Each measurement was carried out in triplicate at 25 °C and an average value was reported.

TEM Observation. For transmission electron microscopy (TEM) observation, sample was prepared by negative staining. Briefly, the DA-TAT-PECL micellar solution was dropped onto a 300-mesh formvar film-supported copper grid for 1 min and soaked up the excess solution by filter paper. Afterwards, a drop of phosphotungstic acid (2%, pH 7.0) was added onto the grid for another 1 min and wicked off with filter paper. The grid was air dried and observed by JEOL JEM 1010 TEM operated at a voltage of 100 kV.

Stability Against Protein Adsorption. The stability of cationic charge-unshielded TAT-PECL micelles and shielded DA-TAT-PECL micelles were evaluated by size change against protein

incubation. DLS was used to determine the size of the two micelles in the presence of 0.5 mg mL^{-1} bovine serum albumin (BSA) at different time points. Each measurement was carried out in triplicate at 25 °C and an average value was reported.

Charge-Reversal Behavior. TAT-PECL and DA-TAT-PECL polymers were dissolved in PBS at pH 7.4 or pH 6.8, and DLS was performed to measure their ζ-potentials at different time points. Each measurement was carried out in triplicate at 25 °C and an average value was reported.

GSH-Induced FRET Inactivation. A fluorescence spectrometer (FluoroMax-3, JY-Horiba) was used to record the emission spectra of $(CPT)_2$-ss-Mal solution in the presence of 10 mM GSH at different time points. The excitation wavelength was set at 370 nm. In the meantime, the pictures of $(CPT)_2$-ss-Mal solution after GSH incubation were taken under UV irradiation (365 nm).

Drug Loading. To detect the drug-loading content (LC) and encapsulation efficiency (EE), the $(CPT)_2$-ss-Mal-loaded micelles were lyophilized, redissolved in DMSO and measured by a Cary 50 UV-vis spectrophotometer (Agilent Technologies). The CPT concentration was quantified by absorpbance at 365 nm through standard curve. LC was calculated as LC(%) = weight of CPT in micelles/weight of CPT-loaded miclles, and EE was calculated as EE(%) = weight of CPT in micelles/weight of CPT in feed.

***In Vitro* Drug Release.** The lyophilized powder of CPT-loaded DA-TAT-PECL micelles were redissolved in 1 mL DMSO and dropped into 9 mL PBS (pH 7.4) with different concentrations of GSH (0, 10 μM and 10 mM). Then the micelles were dialyzed against the corresponding PBS solutions in a shaking incubator at 37 °C. The CPT concentrations in the media at selected time intervals from 0.5 to 48 h were measured by a FluoroMax-3 spectrofluorimeter upon the emission wavelength at 438 nm. The excitation wavelength was set at 370 nm.

Cytocompatibility Assay. Cytocompatibility of blank micelles was evaluated by alamarBlue

assay and live/dead staining. For alamarBlue assay, MDA-MB-231 cells were seeded onto 48-well plates at a density of 1×10^4 cells/well for 24 h prior to treatment. Then PECL, TAT-PECL and DA-TAT-PECL blank micelles with concentrations in the range of 10 μg mL^{-1} to 500 μg mL^{-1} were added to the corresponding well. After 24 h incubation, cells were washed by PBS and treated with 300 μL of alamarBlue solution (10% alamarBlue, 80% media 199 (Gibcos), and 10% FBS, v/v) for further 3 h incubation. The plate was read on a Synergy HT fluorescent plate reader (BioTek Instruments Inc., Winooski, VT, USA) at an adsorption wavelength of 570 nm. Each experiment was performed in quintuplicate. Cell viability was calculated as cell viability(%) = OD_{570}(sample) - OD_{570}(blank)/OD_{570}(control) - OD_{570}(blank).

For live/dead staining, MDA-MB-231 cells were seeded onto 12-well plates at a density of 2×10^4 cells/well. After 24 h incubation with different concentrations of blank micelles, cells were treated with 10 μM Calcein-AM for 10 min and 1 μM propidium iodide (PI) for 10 min. The images were taken using an Olympus BX 50 Fluorescence Microscope. The Calcein-AM fluorescence (green) indicated live cells and the PI fluorescence (red) indicated dead cells.

Intracellular FRET Inactivation. To observe the GSH-triggered intracellular FRET inactivation, MDA-MB-231 breast cancer cells (10×10^4 cells/well) grown in 6-well plates were treated with $(CPT)_2$-ss-Mal at a CPT dose of 1 mM. The images were taken at different time points under the exitation wavelength of 365 nm using an Olympus BX 50 Fluorescence Microscope. The Mal fluorescence (green) indicated FRET activation and the CPT fluorescence (blue) indicated FRET inactivation.

Cellular Uptake Observed by Fluorescence Microscopy. MDA-MB-231 cells were seeded onto 6-well plates at a density of 10×10^4 cells/well and allowed to grow for 24 h. Then the culture medium was replaced with $(CPT)_2$-ss-Mal-loaded PECL, TAT-PECL and DA-TAT-PECL micelles suspended in neutral medium (pH 7.4) and acidic medium (pH 6.8) (at a CPT dose of 1 mM). After 2 h of incubation, cells were washed with PBS, fixed by paraformaldehyde, stained with propidium iodide (PI), and imaged by fluorescence

microscopy. CPT (blue) and PI (red) were excited at 365 nm and 535 nm, respectively.

Cellular Uptake Measured by Flow Cytometry. MDA-MB-231 cells were seeded onto 6-well plates at a density of 10×10^4 cells/well and allowed to grow for 24 h. The culture medium were replaced with $(CPT)_2$-ss-Mal-loaded PECL, TAT-PECL and DA-TAT-PECL micelles suspended in neutral medium (pH 7.4) and acidic medium (pH 6.8) (at a CPT dose of 1 mM). After 2 h incubation, cells were washed with PBS, harvested by trypsin, collected in tubes and centrifuged at 1500 rpm for 5 min, and then resuspended in PBS. Miltenyi Biotec 8-color MACSQuant VYB flow cytometer was used to analyze the intracellular fluorescence intensity.

Cell Cytotoxicity. The cytotoxicity of various CPT formulations against MDA-MB-231 cells at different pH values was evaluated by alamarBlue assay and live/dead staining. For alamarBlue assay, 1×10^4 cells were seeded in 48-well plates for 24 h, and the culture medium were replaced with free $(CPT)_2$-ss-Mal and $(CPT)_2$-ss-Mal-loaded PECL, TAT-PECL and DA-TAT-PECL micelles suspended in neutral medium (pH 7.4) and acidic medium (pH 6.8). CPT doses were ranging from 10^{-3} μM to 10^3 μM. 24 h later, cells were washed with PBS, incubated with 300 μL of alamarBlue solution for further 3 h, and read on a Synergy HT fluorescent plate reader.

For live/dead staining, MDA-MB-231 cells were seeded onto 12-well plates at a density of 2×10^4 cells/well, and incubated with various CPT formulations at different pH conditons for 24 h (at a CPT dose of 1 mM). Then cells were treated with 10 μM Calcein-AM for 10 min and 1 μM propidium iodide (PI) for 10 min. The images were taken using an Olympus BX 50 Fluorescence Microscope. The Calcein-AM fluorescence (green) indicated live cells and the PI fluorescence (red) indicated dead cells.

Cell Apoptosis and Necrosis. Cell apoptosis and necrosis induced by various CPT formulations at different pH values were analyzed by flow cytometry using the Annexin V-FITC/PI apoptosis detection kit. MDA-MB-231 cells seeded at 10×10^4 cells/well were

incubated with free $(CPT)_2$-ss-Mal and $(CPT)_2$-ss-Mal-loaded PECL, TAT-PECL and DA-TAT-PECL micelles suspended in neutral medium (pH 7.4) and acidic medium (pH 6.8) (at a CPT dose of 1 mM). After 24 h of incubation, cells were trypsinized, collected, and labeled with Annexin V-FITC and PI according to the manufacturer's protocols prior to fluorescence analysis on a flow cytometer.

Animals and Tumor Models. Female BALB/c mice and nude mice (20 ± 2 g, 5 weeks of age) were feed at the condition of 25 °C and 55% of humidity and allowed to access food and water freely. All animal experiments were approved by the Institutional Animal Care and Use Committee at Yale University and carried out in compliance with guidelines.

To set up the tumor xenograft model, mice were subcutaneously inoculated in the right lower leg with 1×10^6 murine breast cancer 4T1 cells. Tumor volume (V) was determined by the following equation: $V = L \times W^2/2$, where L and W are length and width of the tumor, respectively. 4T1 tumor bearing mice were used to carry out experiments when the tumor volumes reached around 50 mm^3.

In Vivo and ex Vivo Fluorescence Imaging. To evaluate the biodistribution of various micelles, PECL, TAT-PECL and DA-TAT-PECL micelles loading with a near-infrared dye, IR-780 iodide, were intravenously injected into 4T1 tumor bearing nude mice. Then mice were anesthetized and imaged by in vivo imaging system (IVIS) (Xenogen) with exitation wavelength of 745 nm and emission wavelength of 820 nm. In vivo images were taken at 1 h, 6 h, and 24 h, then mice were sacrificed to separate the organs and tumors for ex vivo imaging.

Intratumoral FRET Inactivation. To observe the GSH-triggered intratumoral FRET inactivation, 4T1 tumor bearing BALB/c mice were intratumorally injected with $(CPT)_2$-ss-Mal at a CPT dosage of 5 mg kg^{-1}. At 30 min, 60 min, 90 min and 120 min post-injection, mice were sacrificed to seperate the tumor tissues, which were embedded in optimal cutting temperature (OCT) compound and cut into 25 μm slices. The sections were stained with PI and observed using a fluoresence microscopy.

In Vivo Tumor Inhibition. 4T1 tumor bearing BALB/c mice were randomly divided into five groups (seven mice per group). Saline, free $(CPT)_2$-ss-Mal, and $(CPT)_2$-ss-Mal-loaded PECL, TAT-PECL and DA-TAT-PECL micelles were intravenously injected into mice every 3 days for 4 times. CPT dosage was 5 mg Kg^{-1} body weight. The first day of treatment was defined as day 0. The body weight and tumor size were recorded every 2 days, and the survival of mice were monitored throughout the experiment.

Immunohistochemical Analyses. At day 15, one mouse in each group was sacrificed to separate the tumors, which were then fixed in 4% formaldehyde solution in PBS. For immunohistochemical staining, the fixed tumors were dehydrated by gradient ethanol and embedded in paraffin block. Haematoxylin and eosin (H&E), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Ki-67 staining were performed by the Pathology core (Yale Universtiy, New Haven, CT).

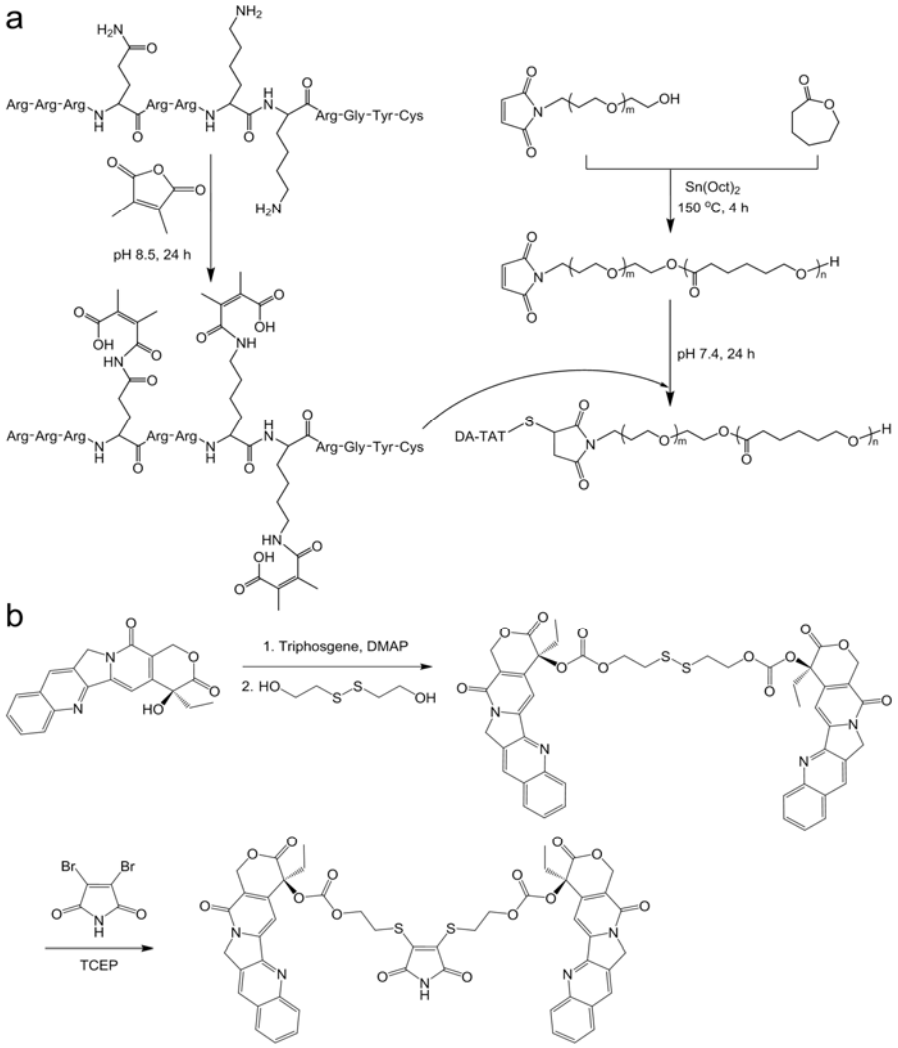

Figure S1. Synthesis of a) DA-TAT-PECL polymer and b) $(CPT)_2$-ss-Mal drug dimer.

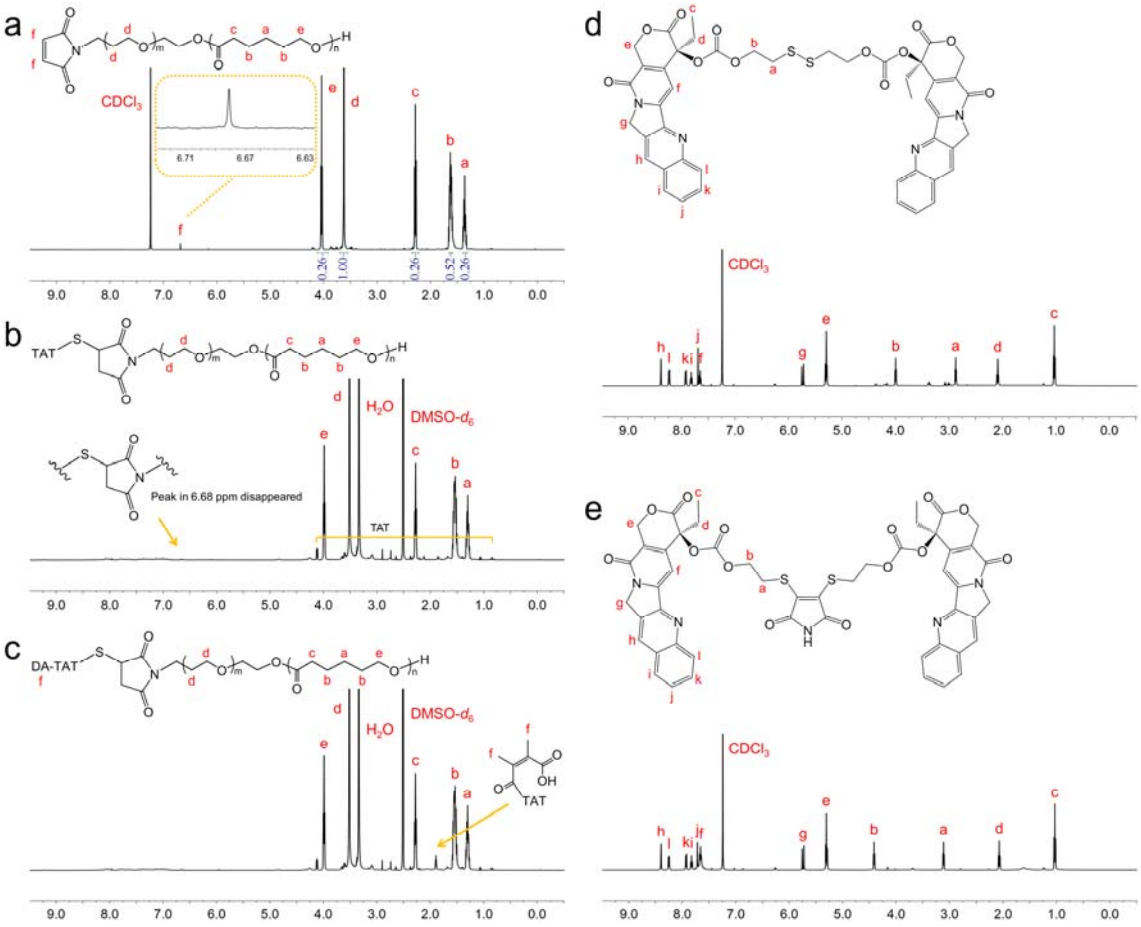

Figure S2. ^{1}H NMR spectra of a) Mal-PECL, b) TAT-PECL, c) DA-TAT-PECL, d) $(CPT)_2$-ss and e) $(CPT)_2$-ss-Mal in $CDCl_3$ or in DMSO-d_6.

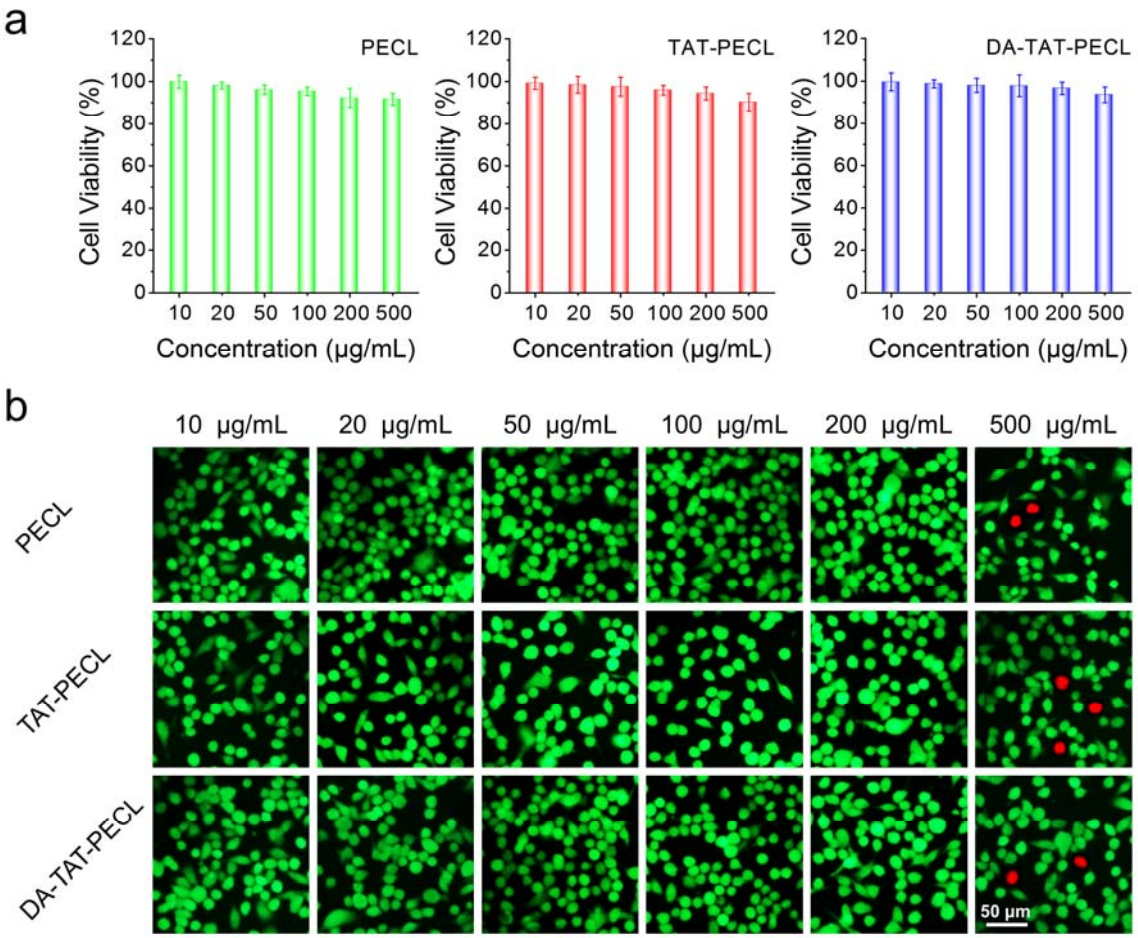

Figure S3. a) Viability of MDA-MB-231 cells by alamarBlue assay after treating with different concentrations of PECL, TAT-PECL and DA-TAT-PECL micelles for 48 h (n = 5). b) Fluorescence images showing the viability of MDA-MB-231 cells by calcein AM and propidium iodide double staining after treating with different concentrations of PECL, TAT-PECL and DA-TAT-PECL for 48 h. The live cells were stained green while the dead cells were stained red.