Supporting Information

A thermoresponsive nanocarrier for mitochondria-targeted drug delivery

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Experimental Section

Materials

N-isopropylacrylamide, acrylamide, paclitaxel (PTX) were purchased from Inno-Chem Co., Ltd (Beijing, China). 1, 6-hexanediol diacrylate, 4-(Aminomethyl)piperidine, poly(ethylene glycol) methyl ether methacrylate and ammonium persulfate were purchased from Sigma-Aldrich (Shanghai, China). All chemicals were used without further purification unless otherwise noted. CCK-8 kit was purchased from Dojindo Molecular Technologies (Kumamoto Techno, Japan). Mitotracker, Lysotracker and Hoechst 33342 were obtained from Life Technologies (Shanghai, China).

Preparation of nanocarriers and PTX-loaded nanoparticles

The macromolecular crosslinker was synthesized via a Michael addition polymerization. Briefly, 1,6-hexanediol diacrylate (452.5 mg, 2 mmol) and 4-(Aminomethyl)piperidine (114.2 mg, 1 mmol) were mixed in 5 mL DMSO. The solution was stirred for 3 d at 60 °C under the protection of argon. The poly(β-aminoester)s containing acrylates as end groups were obtained and stored in dark at -20 °C.

The poly(*N*-isopropylacrylamide) (PNIPAM) and polyacrylamide (PAM) nanoparticles were prepared as follows: *N*-isopropylacrylamide (240 mg, 2.12 mmol) or acrylamide (151 mg, 2.12 mmol), crosslinker (30 mg, 0.005 mmol), poly(ethylene glycol) methyl ether methacrylate (240 mg, 0.25 mmol), sodium dodecyl sulfate (20 mg, 0.069 mmol) were dissolved in 20 mL DMSO/H_2O mixed solvent (volume ratio of 1/4) under vigorous stirring and argon purging. After the temperature of the solution reached 70 °C, the initiator ammonium persulfate (22.4 mg, 0.1 mmol) was rapidly added to the solution and then the reaction was allowed to last for 4 h at 70 °C under argon protection. Afterward, the solution was cooled to room temperature and dialyzed against distilled water for two days (molecular weight cut-off = 10000 Da).

The nanoparticles were then collected by lyophilization.

The fluorescently labeled nanocarriers Cy5-PNIPAM and Cy5-PAM were prepared as follows: *N*-isopropylacrylamide (240 mg, 2.12 mmol) or acrylamide (151 mg, 2.12 mmol), crosslinker (30 mg, 0.005 mmol), poly(ethylene glycol) methyl ether methacrylate (240 mg, 0.25 mmol), sodium dodecyl sulfate (20 mg, 0.069 mmol), *N*-(3-Aminopropyl)methacrylamide hydrochloride (3.78 mg, 0.0212 mmol) were dissolved in 20 mL DMSO/H_2O mixed solvent (volume ratio of 1/4) under vigorous stirring and argon purging. After the temperature of the solution reached 70 °C, the initiator ammonium persulfate (22.4 mg, 0.1 mmol) was rapidly added to the solution, and the reaction was allowed to last for 4 h at 70 °C under argon protection. Afterward, the solution was cooled to room temperature and dialyzed against distilled water for two days (molecular weight cut-off = 10000 Da). The nanoparticles were then collected by lyophilization. 11.3 mg of the nanoparticles and Cy5-NHS (1 mg, 0.0015 mmol) were incubated in 2 mL DMSO for 24 h, and the mixture was purified by dialysis until no fluorescence was detected in supernatant.

PTX-loaded PNIPAM nanoparticles (PNIPAM-PTX) and PTX-loaded PAM nanoparticles (PAM-PTX) were prepared by using a solvent replacement method. Typically, 20 mg of PNIPAM nanoparticles in ethanol and 20 mg of PTX in DMSO were mixed, followed by stirring for 1 h. The solution was then transferred into a dialysis bag and dialyzed against distilled water for 48 h (molecular weight cut-off = 3500 Da). The drug-loaded nanoparticles were obtained by centrifugation at 3500 rpm for 10 min.

Encapsulation efficiency and drug-loading capacity of PTX-loaded NPs were calculated according to the following equations: PTX encapsulation efficiency as a percentage = (weight of loaded PTX/weight of feeding PTX) × 100%; drug-loading capacity as a percentage = (weight of loaded PTX/weight of nanoparticles) × 100%.

Characterization of nanocarriers and PTX-loaded nanoparticles

The morphology of nanoparticles was investigated by using Transmission Electron Microscopy (TEM). The TEM samples were prepared by dropping the solution onto a carbon-coated copper grid followed by rinsing with deionized water. The counterstaining of the nanoparticles was performed by dropping phosphotungstic acid (weight ratio of 2.5%) onto the grid. After 2 min, the solution was absorbed and washed by distilled water for three times. After complete drying in air, TEM images of nanoparticles were recorded by a Joel 1010 Transmission Electron Microscope. The hydrodynamic radii and zeta potentials of the nanoparticles were examined by dynamic light scattering (DLS). Measurements were performed at 25 °C by using Zetasizer Nano-S (Malvern Instruments Ltd, Worcestershire, United Kingdom).

The LCST of PNIPAM was examined with pyrene fluorescence. 20 μL of pyrene acetone solution (20 μg/mL) was added to a 20-mL brown bottle and air-dried. A total of 4 mL of aqueous solution containing 2 mg/mL PNIPAM was added to the bottle. After being equilibrated for 1 h at different bath temperatures, the excitation spectra (300~360 nm) of the solutions were recorded at an emission wavelength of 395 nm with excitation and emission bandwidths set at 5 nm.

The in vitro drug release profile of PTX-loaded nanoparticles was analyzed as follows. Typically, Cy5-PTX loaded nanoparticles were dispersed in Roswell Park Memorial Institute 1640 (RPMI 1640) with 10% FBS, and the temperatures of solutions were fixed at 25 °C, 37 °C, 50 °C. At predetermined time points, the solutions were centrifuged at 10,000 rpm for 1 min, and the supernatant was collected. 1-mL fresh medium was added to redisperse the nanoparticles for further incubation. The amounts of Cy5-PTX released at different time points were determined by fluorescence spectrophotometer.

Cell culture

Murine bladder cancer MB49 cells were cultured in RPMI 1640 medium supplying with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U mL^{-1}). Murine

macrophage RAW 264.7 cells were cultured in DMEM/F-12 supplying with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U mL^{-1}). Human umbilical vein endothelial cells (HUVEC) were cultured in DMEM/F-12 supplying with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U mL^{-1}). All cells were incubated at 37 °C with 5% CO_2.

Intracellular distribution of nanocarriers and mitochondria-targeted drug release

To investigate the intracellular distribution of nanocarriers, MB49 cells were seeded on culture dishes at an initial density of 1×10^4 cells. After 24 h, Cy5-PNIPAM or Cy5-PAM was incubated with cells for another 2 h or 24 h. After the incubation, the cells were washed with PBS solution and a fresh medium was added. Mitotracker or Lysotracker (Life Technologies) was added into dishes and incubated for 30 min. The nuclei were stained with Hoechst 33342, and the cells were observed with confocal laser scanning microscopy.

For cell imaging of PTX-loaded nanoparticles, PTX was labeled with Cy5 through the esterification reaction between PTX and Cy5-NHS at a mass ratio of 200:1. Confocal laser scanning microscopy was used to investigate the intracellular distribution of PTX-loaded nanoparticles in MB49 cells, RAW 264.7 cells and HUVEC cells. Typically, the cells were seeded in culture dishes at an initial density of 1×10^4 cells. Cy5-PTX loaded nanoparticles were incubated with the cells. After predetermined time, the medium was replaced by fresh medium. The nuclei were stained with Hoechst 33342, and the cells were observed with confocal laser scanning microscopy.

To investigate the mitochondria-targeted drug release, MB49 cells, RAW 264.7 cells and HUVEC cells were seeded on culture dishes at an initial density of 1×10^4 cells. After 24 h, Cy5-PTX-loaded nanoparticles were added and incubated with cells for another 2 h or 24 h. After the incubation, the cells were washed with PBS solution

and a fresh medium was added. Green fluorescent Mitotracker (Life Technologies) was added into dishes and incubated for 30 min. The nuclei were stained with Hoechst 33342, and the cells were observed with confocal laser scanning microscopy.

To verify mitochondrial temperature in different cell lines, we measured the mitochondrial temperature of MB49, RAW264.7, HUVEC cells by using Mito thermo yellow probe. Briefly, MB49 cells, RAW 264.7 cells and HUVEC cells were seeded on culture dishes at an initial density of 1×10^4 cells and cultured for 24 h. Mito thermo yellow probe was added in a pre-warmed culture medium. This medium was used to replace the cell culture medium and then incubated at 37 °C with 5% CO_2 for 15 min. The nuclei were stained by Hoechst 33342. The confocal microscopic images of cells were obtained by visualizing the fluorescent probe through excitation at 561 nm.

Flow cytometry

MB49 cells were cultured at a density of 20,000 cells/well in 6-well dishes. After 24-h incubation, free PTX, PNIPAM-PTX or PAM-PTX was incubated with the cells for another 24 h, followed by flow cytometry analysis.

***In Vitro* cytotoxicity assay**

The cytotoxicity of PTX-loaded nanoparticles against MB49, RAW264.7, HUVEC cells was examined by using CCK8 assays. The cells were seeded in 96-well plates at the density of 6,000 cells per well and allowed to culture for 24 h at 37 °C with 5% CO_2. Afterward, the samples were added at different concentrations and incubated for 48 h. After the incubation, the culture medium was substituted by 200 μL fresh medium. CCK8 (10%) solution was added to each well and incubated for about 30 min. The absorbance of each well was measured by multifunctional microplate reader at 450 nm. The relative cell viability was calculated as the following equation: Cell Viabililty (%) = $(OD_{samples}-OD_{blank})/(OD_{control}-OD_{blank}) \times 100$.

Analysis of mitochondrial membrane potentials

The MB49 cells were seeded at a density of 2×10^4 cells/well on culture dishes, and cultured at 37 °C with 5% CO_2 for 24 h before the addition of the nanoparticles. 1-mL prewarmed medium containing rhodamine 123 (5 μg/mL) was added and incubated at 37 °C for 15 min. The nuclei were stained with Hoechst 33342 (5 μg/mL) for 10 min. The cells were washed three times with PBS buffer. The confocal microscopic images of cells were obtained by visualizing the fluorescent probe through excitation at 488 nm. The fluorescent intensity was quantified by ImageJ software.

Statistics

All data were expressed as the mean value ± standard deviation. Statistical significance was determined by two-tailed student's *t* test with $P < 0.05$.

Supporting figures

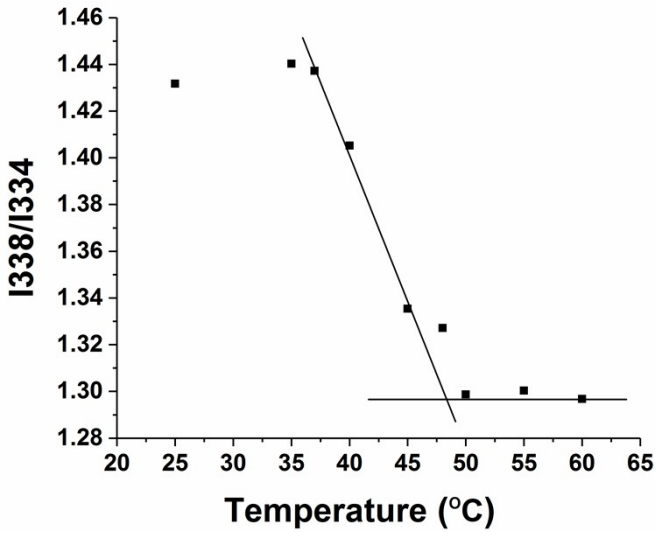

Fig. S1 Characterization of LCST of PNIPAM-based nanoparticles by pyrene fluorescence spectroscopy.

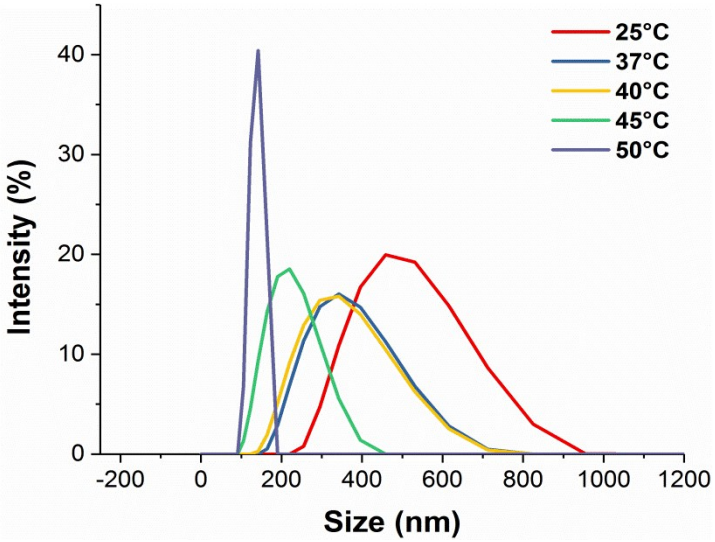

Fig. S2 Characterization of particle size changes at different temperatures for PNIPAM-PTX.

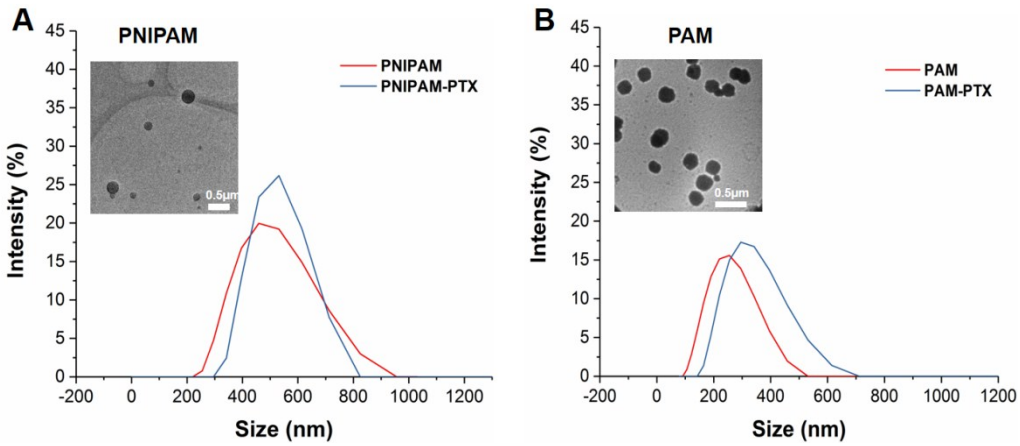

Fig. S3 Characterization of PNIPAM (A) and PAM (B) before and after loading PTX.

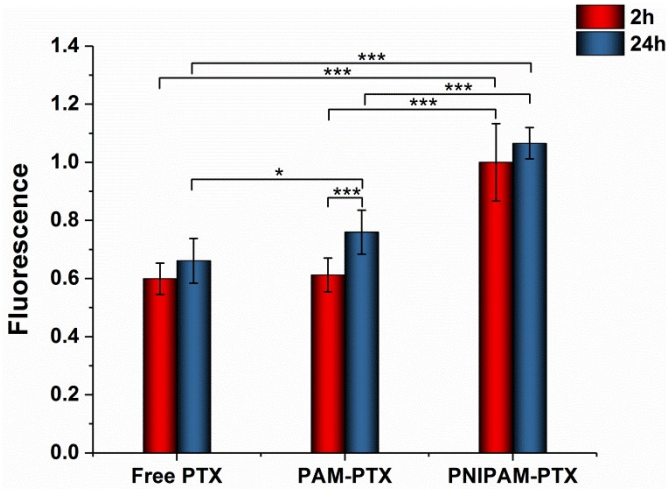

Fig. S4 Average fluorescence intensity of Cy5-PTX in MB49 cells using ImageJ (mean ± SD, n = 7). *, $P < 0.05$; ***, $P < 0.001$.

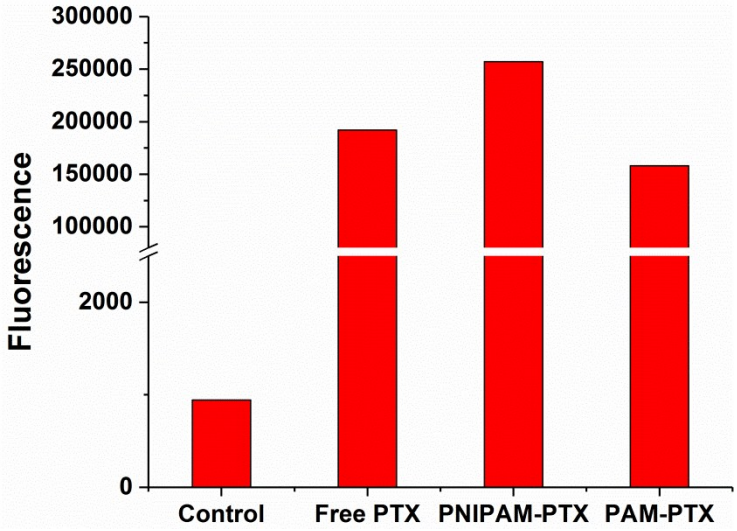

Fig. S5 Flow cytometry analysis of Cy5-PTX fluorescence in MB49 cells.

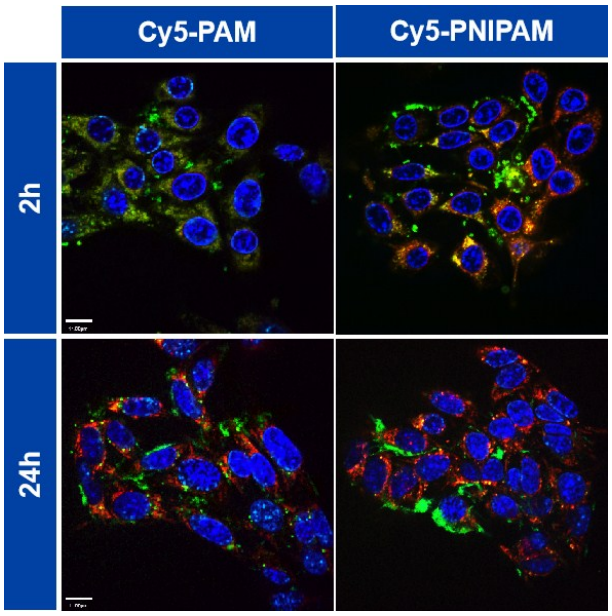

Fig. S6 Images of Cy5-labeled nanocarriers (red) and lysosomes (green) in MB49 cells at 2 h and 24 h. The nuclei were stained with Hoechst 33342 (blue). Scale bar is 11 μm.

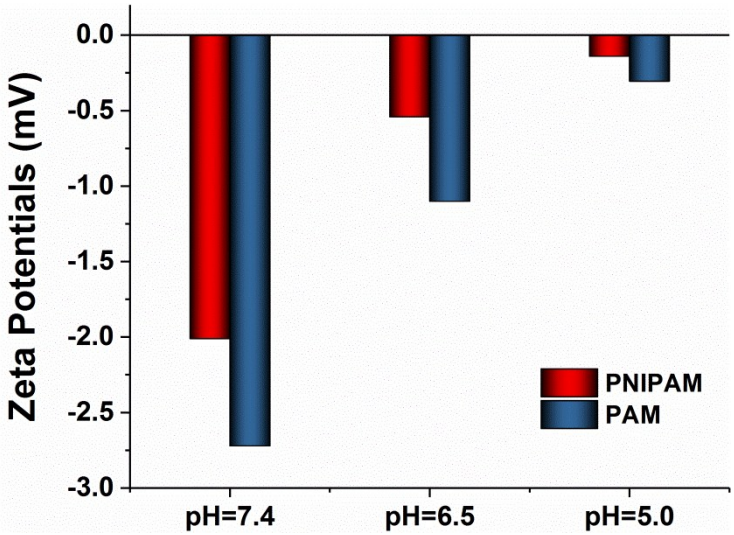

Fig. S7 Zeta potentials of nanocarriers at different pH.

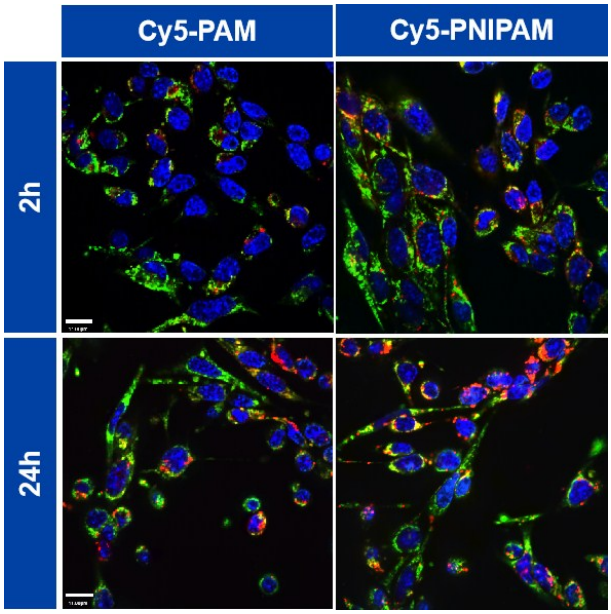

Fig. S8 Images of Cy5-labeled nanocarriers (red) and mitochondria (green) in MB49 cells at 2 h and 24 h. The nuclei were stained with Hoechst 33342 (blue). Scale bar is 11 μm.

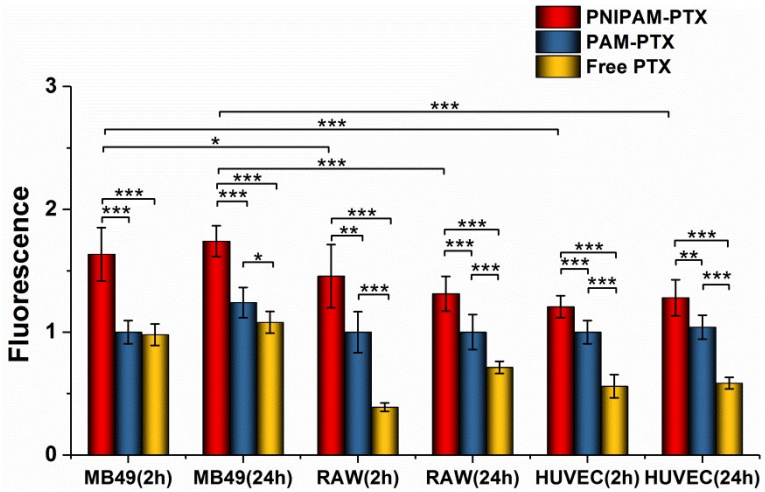

Fig. S9 Normalized fluorescence intensity of Cy5-PTX in MB49 cells, RAW 264.7 (RAW) cells, and HUVEC cells (mean ± SD, n = 7). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

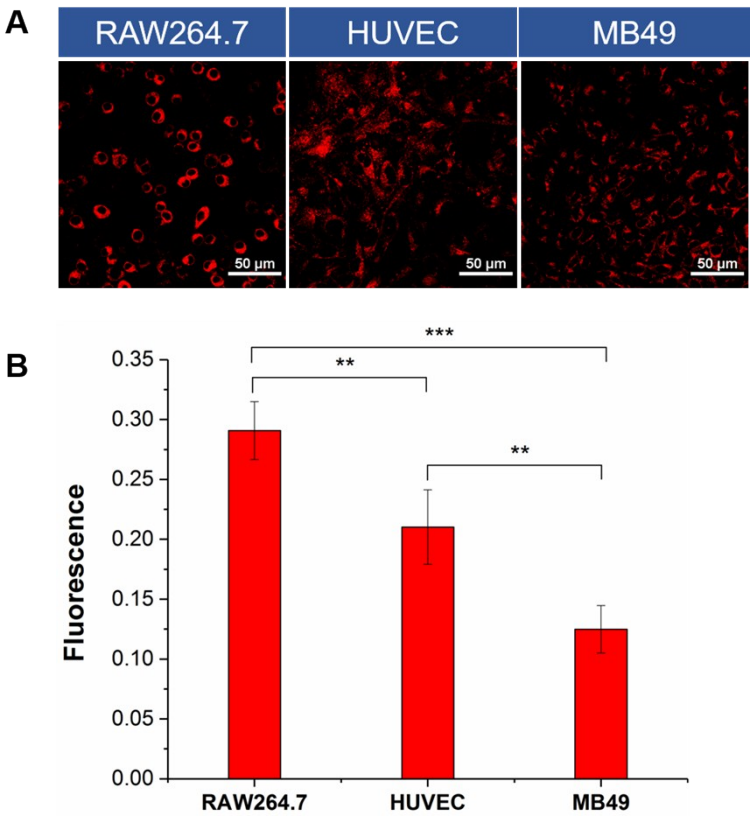

Fig. S10 (A) The images of Mito thermo yellow probe in RAW264.7 cells, HUVEC cells and MB49 cells. (B) Fluorescence intensity was determined by ImageJ (mean ± SD, n = 4). **, $P < 0.01$; ***, $P < 0.001$.

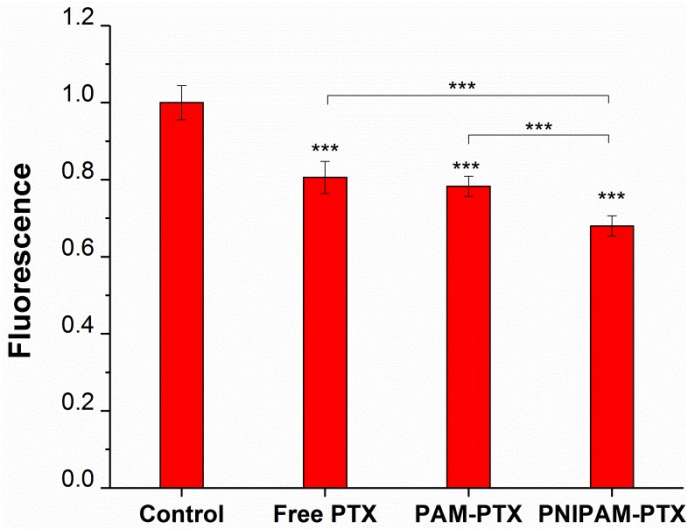

Fig. S11 Relative fluorescence intensity of mitochondrial membrane potentials in MB49 cells treated with free PTX, PAM-PTX and PNIPAM-PTX (mean ± SD, n = 5). The concentration of free PTX was 2 μg/mL, and PTX-loaded nanoparticles contained 2 μg/mL of PTX. ***, $P < 0.001$.

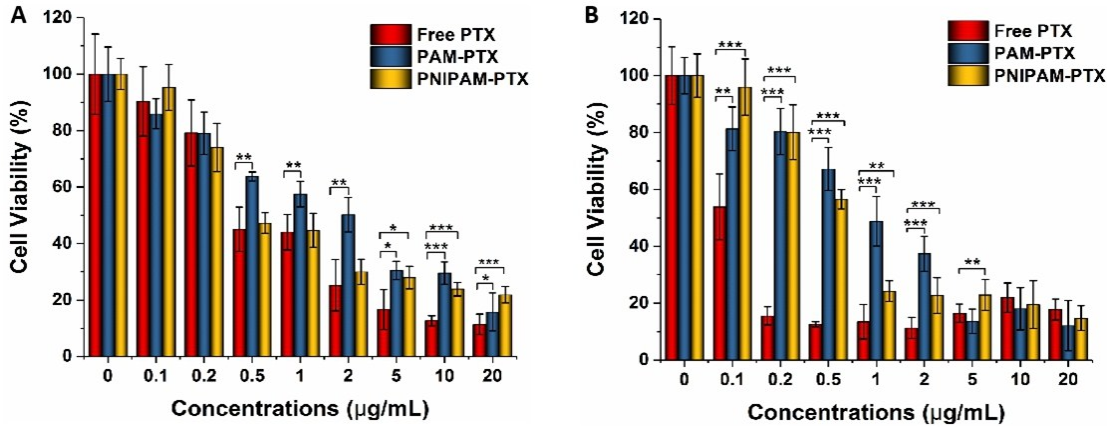

Fig. S12 The cytotoxicity of free PTX, PAM-PTX and PNIPAM-PTX in HUVEC cells (A) at 48 h and RAW 264.7 cells (B) at 12 h (mean ± SD, n = 6). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.