Supporting Information

Thermoresponsive drug delivery to mitochondria in vivo

Lifo Ruan,‡ab Mengxue Zhou,‡ab Jun Chen,‡ab Hui Huang,‡b Jiayu Zhang,‡a Hongyan Sun,‡c Zhifang Chai‡ab and Yi Hu*‡ab

‡CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, Multidisciplinary Research Division, Institute of High Energy Physics, Chinese Academy of Sciences (CAS), Beijing 100049, China.
§University of Chinese Academy of Sciences, Beijing 100049, China.
*Department of Chemistry and COSDAF (Centre of Super-Diamond and Advanced Films), City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China.

‡ These authors contributed equally to this study.
*Corresponding Author, E-mail: huyi@ihep.ac.cn
Experimental Section

Materials. Paclitaxel (PTX), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), acrylamide (AAm), N-Succinimidyl 6-maleimidohexanoate (EMCS) and N,N′-Methylene-bis-acrylamide (MBA) were purchased from Inno-Chem Co., Ltd (Beijing, China). N-isopropylacrylamide (NIPAM), N-(3-Aminopropyl)methacrylamide hydrochloride (APM), 1, 6-hexanediol diacrylate (HDDA), 4-(Aminomethyl)piperidine (4-AMP), poly(ethylene glycol) methyl ether methacrylate (mPEG-MA, 950 g/mol) and (4-carboxybutyl)triphenylphosphonium bromide were obtained from Sigma-Aldrich (Shanghai, China). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Aladdin Chemical Co., Ltd. (Beijing, China). Mitotracker, Lysotracker and Hoechst 33342 were purchased from Life Technologies (Shanghai, China). Cy5 NHS ester was purchased from Xi’an Ruixi Biological Technology Co., Ltd. (Xi’an, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Invitrogen Inc. iRGD (sequence: c(CRGDKGPDC), purity > 98%) was obtained by GL Biochem (Shanghai, China).

Preparation of nanocarriers. poly(N-isopropylacrylamide) (PNIPAM) and polyacrylamide (PAM) nanoparticles were prepared as follows: NIPAM (226 mg, 2 mmol) or AAm (142 mg, 2 mmol), crosslinker (250 μL, 0.05 mmol), mPEG-MA (240 mg, 0.25 mmol), SDS (20 mg, 0.07 mmol), APMH (14.3 mg, 0.08 mmol) and MBA (3 mg, 0.02 mmol) were dissolved in 20 mL of H2O under vigorous stirring and argon purging. At 70 °C, the initiator APS (22.8 mg, 0.1 mmol) was added and allowed to react at 70 °C for 4 h under argon protection. Afterward, the synthesized nanoparticles were purified by dialysis (MWCO = 10000 Da). Afterward, the nanoparticles were mixed with (4-carboxybutyl)triphenylphosphonium bromide (a mass ratio of 25:1) activated by EDC/NHS and incubated at 30 °C for 24 h. After being dialyzed against ddH2O for 24 h (MWCO = 3500 Da), the nanoparticles were mixed with EMCS at a mass ratio of 35:1 and incubated at 30 °C for 30 min. Afterward, iRGD was mixed with the nanoparticles at a mass ratio of 1:10 overnight at 30 °C. Finally, the nanoparticles were dialyzed in ddH2O for 24 h (MWCO = 3500 Da) to remove free iRGD and collected by lyophilization. The chemical structures of the nanocarriers prepared in this study were analyzed by 1H NMR.

Formulation of PTX-loaded nanoparticles. PTX-loaded PNIPAM nanoparticles (PNIPAM@PTX) and PTX-loaded PAM nanoparticles (PAM@PTX) were prepared by using a solvent replacement method as previously described.1 The concentration of PTX in nanocarriers was determined by Waters Acquity Ultra Performance Liquid Chromatographic (UPLC) measurements at 227 nm with acetonitrile/water (50/50, v/v) as the mobile phase. Drug loading efficiency (DLE) and drug loading content (DLC) of PTX-loaded nanoparticles were measured according to the following formula:

\[
\text{DLE (%)} = \left( \frac{\text{weight of loaded PTX}}{\text{weight of feeding PTX}} \right) \times 100%
\]

\[
\text{DLC (%)} = \left( \frac{\text{weight of loaded PTX}}{\text{weight of nanoparticles}} \right) \times 100%
\]

Characterization of nanocarriers and PTX-loaded nanoparticles. The chemical structures of the nanocarriers prepared in this study were analyzed by Fourier-transform infrared spectroscopy (FTIR). The morphology of nanocarriers was investigated by using Transmission Electron Microscopy (TEM). The TEM samples were prepared by phosphotungstic acid staining method. Images of nanocarriers were recorded by a Joel 1010 Transmission Electron Microscope. The hydrodynamic radii and zeta potentials of the nanocarriers 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 as described previously.¹

**Stability of nanocarriers.** The nanocarriers were incubated in PBS at 48 °C for 72 h. The hydrodynamic diameter was measured at 12, 24, 48, and 72 h by DLS.

**Release of PTX from PTX-loaded nanoparticles.** Drug release profiles of PTX-loaded nanoparticles were analyzed as follows. PTX-loaded nanoparticles were dispersed in PBS at 25 °C, 37 °C and 48 °C. At predetermined time points, the solution was centrifuged at 10,000 rpm for 1 min, and the supernatant was collected and harvested by freeze-drying. Meanwhile, 1 mL of fresh PBS was added to redisperse the nanoparticles for further incubation. Subsequently, freeze-dried powder was dissolved in 1 mL of acetonitrile and the amounts of PTX released at different time points were determined by UPLC.

**Cell culture.** 4T1 murine breast carcinoma cells were used in this study. 4T1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (RPMI 1640) supplied with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U/mL). The cells were incubated at 37 °C with 5% CO₂.

**Lysosomal escape.** For cell imaging, the nanocarriers were labeled with Cy5 through the reaction between nanocarriers and Cy5-NHS with a mass ratio of 50:1. 4T1 cells were seeded in culture dishes at an initial density of 1 × 10⁴ cells. After 24 h, Cy5-labeled nanocarriers (50 μg/mL) were added and incubated with the cells for another 1 h or 4 h. After the incubation, the cells were washed with PBS, and a fresh medium was added. Green fluorescent Lysotracker was added into dishes and incubated with the cells for 30 min. The nuclei were stained with Hoechst 33342, and the cells were observed with confocal laser scanning microscopy. Pearson’s correlation coefficient for colocalization was determined by NIS-Elements Viewer.

**Cellular uptake.** 4T1 cells were seeded in culture dishes at an initial density of 1 × 10⁴ cells. After incubation of the cells with or without iRGD (100 μmol/L) for 1 h, the medium was discarded, followed by rinsing the cells with PBS twice and incubating the cells with Cy5-labeled nanocarriers (50 μg/mL) for 2 h. The cells were washed with PBS solution, and a fresh medium was added. The nuclei were stained with Hoechst 33342, and the cells were observed with confocal laser scanning microscopy. The intracellular fluorescence of Cy5-labeled nanocarriers was quantified with ImageJ.

**Cytotoxicity of nanocarriers.** The cytotoxicity of nanocarriers against 4T1 cells was evaluated by MTT assay. 4T1 cells were seeded in 96-well plates at a density of 8 × 10³ cells/well overnight. The samples were added at different concentrations and incubated for 24 h. Afterward, the medium was replaced with serum-free medium containing MTT solution (0.5 mg/mL) and it was incubated with the cells for 4 h. The medium was removed and 100 μL DMSO was added to each well. The samples were gently mixed on an orbital shaker (10 min) and then 570 nm absorbance was measured with a microplate reader (Spectra Max M5). Non-treated cells were used as a control. Cell viability (%) was calculated based on the following formula:

\[
\text{Cell Viability} \% = \left( \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \right) \times 100\%
\]
Where, \( A_{\text{sample}} \), \( A_{\text{blank}} \), and \( A_{\text{control}} \) were the absorbance of the sample, blank and control.

**Cytotoxicity of PTX-loaded nanoparticles.** 4T1 cells were seeded in 96-well plates at a density of \( 8 \times 10^3 \) cells/well overnight. Afterward, the samples were added at different concentrations and incubated for 24 h. The relative cell viability was determined by MTT assay.

**Intracellular drug distribution of PTX-loaded nanoparticles.** For cell imaging, PTX was labeled with Cy5 through the reaction between PTX and Cy5-NHS at a 1:1 molar ratio. 4T1 cells were seeded in culture dishes at an initial density of \( 1 \times 10^4 \) cells. After 24 h, Cy5-PTX-loaded nanoparticles (containing 0.5 μg/mL of PTX) were added and incubated with cells for another 4 h or 24 h. After the incubation, the cells were washed with PBS solution and a fresh medium was added. Green fluorescent Mitotracker 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.

**Establishment of a mouse model of subcutaneous breast cancer and cancer treatment.** All the animal experiments were carried out following the guidelines approved by the Animal Care and Use Committee of CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety. Female Balb/c mice (4-6 weeks, 13 ± 2 g) were obtained from Beijing WeiTongLiHua Animal Co. Ltd. The subcutaneous model of breast cancer was prepared as follows. Female Balb/c mice were anesthetized by intraperitoneal injection of 4% chloral hydrate at 10 mL/kg, followed by subcutaneous injection of \( 1 \times 10^7 \) 4T1 cells. When the tumor was palpable, the mice were subjected to the treatments.

The mice were randomly divided into 4 groups (5 mice per group) after the tumors reaching a size of ~50 mm\(^3\). PTX was dissolved in DMSO and diluted in PBS before administration. The treatments at PTX dose of 5 mg/kg were performed every two days for three weeks by intravenous injection through the tail vein. The body weight and tumor were monitored every other day. After the treatments, all the mice were sacrificed, and tumors were excised and weighed.

**Biodistributions of Cy5-PTX in tumor-bearing mice.** After the treatments, all groups of mice were sacrificed and hearts, livers, spleens, lungs, kidneys and tumors were sampled and then the fluorescent images were taken by PerkinElmer IVIS Spectrum system (Ex/Em = 640/680 nm).

**H&E staining.** Following drug treatments, the mice were sacrificed and mouse organs were fixed at 4 °C in 4% paraformaldehyde overnight. The samples were cut as 5 μm slices and stained with H&E. The images were recorded by inverted fluorescence microscope (Olympus IX73).

**Statistical analysis.** All data are shown as mean ± SD. Statistical significance between two groups was analyzed by two-tailed Student’s t test.

**Reference**

Fig. S1 FTIR characterization of TPP, iRGD, pristine PAM (without TPP and iRGD decoration), PAM-based nanocarriers (PAM), pristine PNIPAM (without TPP and iRGD decoration) and PNIPAM-based nanocarriers (PNIPAM).
Fig. S2 $^1$H NMR spectra recorded in DMSO-d6 for PNIPAM-based nanocarriers (A) and PAM-based nanocarriers (B).
**Fig. S3** Characterization of LCST of PNIPAM-based nanocarriers by pyrene fluorescence spectroscopy.

**Fig. S4** Characterization of PNIPAM-based nanocarriers (A) and PAM-based nanocarriers (B) before and after loading PTX. Scale bar is 200 nm.
**Fig. S5** Zeta potentials of different nanocarriers before and after loading PTX in ddH$_2$O.

**Fig. S6** Average sizes of PNIPAM-based and PAM-based nanocarriers in PBS for 3 days at 48 °C. Each value represents mean ± SD (n = 3).
**Fig. S7** (A) The UPLC analysis of PTX at various concentrations. (B) Calibration curve of PTX.

**Fig. S8** High resolution fluorescence images of Cy5-labeled PTX (red) and mitochondria (green) in 4T1 cells at 24 h. The nuclei were stained with Hoechst 33342 (blue). Scale bar is 5 μm.
Fig. S9 Images of Cy5-labeled nanocarriers (red) and lysosomes (green) in 4T1 cells at 1 h and 4 h. The nuclei were stained with Hoechst 33342 (blue). Scale bar is 50 μm.
Fig. S10 (A) Images of Cy5-labeled nanocarriers (red) in 4T1 cells with or without iRGD pretreatment. The nuclei were stained with Hoechst 33342 (blue). Scale bar is 50 μm. (B) Quantitative analysis of intracellular fluorescence intensity of Cy5-labeled nanocarriers with ImageJ. Each value represents mean ± SD (n = 5). ***, P < 0.001.
**Fig. S11** (A) Representative ex vivo fluorescence images of tumors and other tissues of mice after the treatments with free PTX, PAM@PTX or PNIPAM@PTX. (B) The average radiant efficiency of Cy5 in tumors. **, \( P < 0.01 \); ###, \( P < 0.001 \).

**Fig. S12** H&E-stained sections of heart, liver, spleen, lung and kidney of tumor-bearing mice after different treatments. Scale bar is 100 \( \mu m \).