Supporting Information

Stimuli-responsive perallyloxycucurbit[6]uril-based nanoparticles for selective drug delivery in melanoma cells

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Materials and Equipment

$AO_{12}CB[6]$ was purchased from CB Tech, Republic of Korea. Paclitaxel (PTX) was purchased from Xi'an Hao-Xuan Bio-Tech Co., Ltd (Xi'an, China). Dulbecco's Modified Eagle Medium (DMEM) medium and fetal bovine serum (FBS) were obtained from Gibco (USA). Cyanine Dye (Cy5) and 4', 6-Diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was supplied by Sigma Aldrich. Poly(vinyl alcohol) (PVA) (88 mol% hydrolyzed, Mw = 25 kDa) were obtained from Acros Organics. All the reagents and solvents employed were commercially available and used as supplied without further purification. The size and zeta potential of particles were determined by dynamic light scattering (DLS) at 25 °C with a Zetasizer (Malvern. Co., UK). Transmission Electron Microscopy (TEM) analysis was performed using a Tecnai G20 TEM (FEI, Co., USA) at operation voltage of 200 KV. Scanning Electron Microscope (SEM) analysis was performed using a Zeiss Zigma FESEM. The drug concentration of NPs was analyzed using HPLC with conditions as follows: flow rate 1.0 mL/min, column XDB C18 (4.6×250 mm, 5 mm), mobile phase acetonitrile/water (60/40, v/v). Cellular uptake and cell apoptosis were analyzed by using a FACS flow cytometer (Beckman coulter). A Confocal Laser Scanning Microscopy (CLSM, Zeiss LSM710) was used to directly visualize the intracellular location of micelles. Cell viability was measured by a multi-mode microplate reader (FlexStation 3).

The RAW 264.7 cell lines were obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The B16 cell lines were purchased from American Type Culture Collection (ATCC, Shanghai, China).

Preparation of Nanoparticles (NPs)

The GSH-responsive NPs were prepared according to oil-in-water (o/w) emulsion solvent evaporation method. 20 mg $AO_{12}CB[6]$ material was dissolved in 1.0 mL

dichloromethane (DCM) solution and then emulsified via probe sonication into 4.0 mL of 1.0 wt.% aqueous solution of PVA for 1 h. The NPs were collected by centrifugation and rinsed with deionized water for three times.

Stability study

The aqueous solution containing $AO_{12}CB[6]$ NPs was stored at ambient conditions. At selected time intervals (1, 7 and 14 d), 1.0 mL of the solution was taken out for DLS measurements. The results were shown in Table S1.

Table S1. Stability of $AO_{12}CB[6]$ NPs.

Time (days)	1	7	14
Diameter (nm)	220	226.2	348.6
PDI	0.108	0.256	0.467

Preparation of cargo-loaded NPs

PTX loaded NPs (PTX-NPs) were fabricated with similar process to that of blank NPs, where both PTX and $AO_{12}CB[6]$ were dissolved in DCM during the first step with the rest of the procedures remained the same. Cy5-NPs were prepared in the same process by replacing PTX with Cy5.

The content of PTX encapsulated by NPs was measured by dissolving NPs in DMSO and analyzing PTX quantitatively using HPLC-PDA detector. The drug encapsulation efficiency (DEE) and drug loading efficiency (DLE) were calculated using the following equations, respectively. The results were shown in Table S2.

$$DEE(\%) = \frac{amount\ of\ PTX\ loaded}{amount\ of\ PTX\ feeding} \times 100\%$$

$$DLE(\%) = \frac{amount\ of\ PTX\ loaded}{amount\ of\ PTX\ loaded + NPs} \times 100\%$$

Table S2. DEE and DLE results.

PTX feed ratio	Diameter(nm)	PDI	DEE	DLE
20%	220	0.108	91.0%	34.7%

GSH-responsive behaviors of the NPs and *in vitro* payload release

The release profile of GSH-responsive NPs was measured by DLS in buffer solutions with different GSH concentrations added under different UV conditions (λ = 365 nm). Briefly, NPs treated with 0 mM and 10 mM GSH were irradiated with or without h UVA (λ = 365 nm) for 2 h, and the diameter of the NPs was determined by DLS at specific time point.

As for the GSH triggered release profile study of PTX-NPs, three PTX-NPs solutions

(1 mL) treated with 0, 1 mM and 10 mM GSH, respectively, during the irritation of UV (λ = 365 nm), 100μL medium were taken out at set time points (0, 1 h, 1.5 h, 2 h and 2.5 h) for the determination of PTX content. The same volume of fresh medium was subsequently added to the original release media each time.

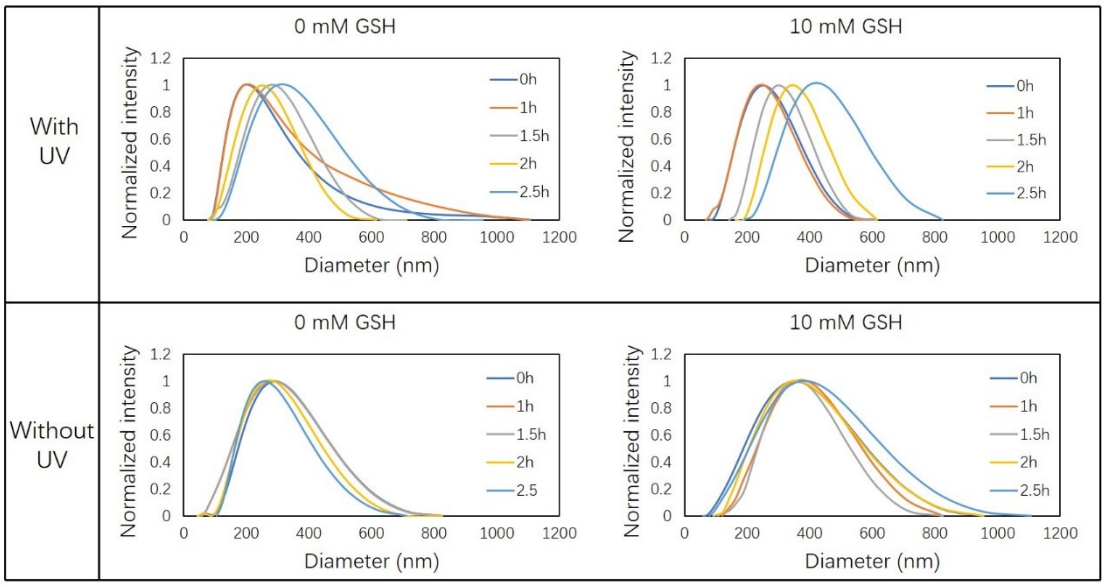

Fig. S1. The size distributions of $AO_{12}CB[6]$ NPs incubated in 0 and 10 mM GSH without and with UV irradiation at set time points.

Cell culture

The B16 and RAW 264.7 cell lines were incubated with DMEM and supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were sub-cultured regularly using trypsin/EDTA.

Biocompatibility study

RAW 264.7 and B16 cells were respectively seeded in a 96-well plate at a density of 8 × 10^3 cells per well in 100 μL of DMEM containing 10 % FBS and 1 % PS, respectively, and incubated in a humidified 5 % CO_2 atmosphere at 37°C for 24 h. The cell culture medium was replaced with 100 μL of a fresh one containing 12.5, 25, 50, 100 and 200 μg/mL of NPs and incubated for additional 36 h. After discarding the medium, the cells were incubated with 100 μL of a fresh medium containing 10 μL of MTT (5 mg/mL) for an additional 4 h at 37°C, and then the medium was gently removed. The purple, water insoluble crystals formed by live cells remaining at the bottom of the wells were dissolved with 100 μL of DMSO and the solution was gently shaken for 10 min. UV absorption of the solution at 590 nm was measured by a multi-well plate reader. The control experiment without the treatment of NPs was executed. The experiment was performed 3 times to obtain a standard deviation. The results were shown in Figure S2.

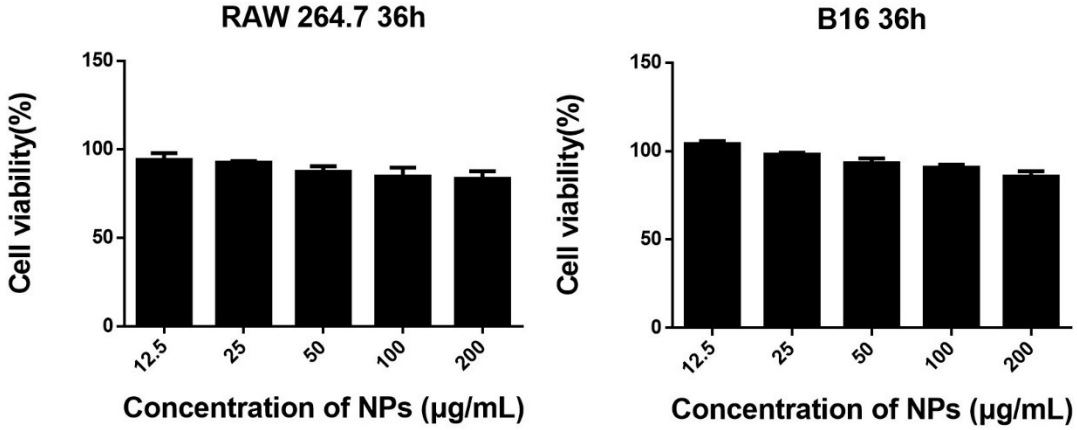

Fig. S2. Cytotoxicity of $AO_{12}CB[6]$ NPs against RAW 264.7 and B16 cells after incubation for 36 h.

Cytotoxicity assay of PTX-NPs

B16 and RAW 264.7 cells were seeded in a 96-well plate at a density of 8×10^3 cells per well in 100 μL of DMEM containing 10 % FBS and 1 % PS and incubated in a humidified 5 % CO_2 atmosphere at 37°C for 24 h. The cell culture medium was replaced with 100 μL of a fresh one containing PTX or PTX-NPs in PBS. After incubation at 37°C for 12 h, the cells were exposed to UV light (λ = 365 nm) for 2 h. The cells were further incubated for additional 24 h, then the medium was replaced with 100 μL of a fresh medium containing 10 μL of MTT (5 mg/mL) for an additional 4 h at 37°C. After gentle removal of the medium, the purple water insoluble crystals formed by live cells remaining at the bottom of the wells were dissolved with 100 μL of DMSO and the solution was gently shaken for 10 min. UV absorption of the resulting solution at 590 nm was measured by a multi-well plate reader. The same experiments without the treatment of PTX or PTX-NPs were performed as a control. These experiments were performed for 3 times to obtain a standard deviation.

Cellular uptake study

The fluorescence probe Cy5 was encapsulated in NPs. The intracellular uptake behaviors of NPs were investigated after B16 cells were seeded in confocal dishes (20 mm) with a density of 10^5 cells per well and incubated for 24 h. The cell culture medium was replaced with 300 μL fresh medium containing Cy5-NPs in PBS. After incubation at 37°C for 2 h, 4 h, 8 h and 12 h, the cells were washed three times with PBS and fixed in 300 μL of paraformaldehyde for 15 min. The cells were subsequently washed for another three times with PBS and the cell nuclei were counterstained by 300 μL of DAPI aqueous solution for 15 min. Finally, the cells were washed again with PBS three times and confocal laser scanning microscopy (TCS SP8, Leica) was used to qualitatively observe the intracellular uptake of NPs.

Cell Apoptosis Assays

The apoptosis effects of PTX-NPs and free PTX at an equivalent PTX dose of 30 nM were determined by the Annexin V/PI staining assay. B16 and RAW 264.7 cells were seeded in a 12-well plate at a density of 10^5 cells per well in 1 mL of DMEM containing 10 % FBS and 1 % PS and incubated in a humidified 5 % CO_2 atmosphere at 37°C for 24 h. The cell culture medium was replaced with 1 mL of a fresh one containing PTX or PTX-NPs in PBS. After incubation at 37°C for 12 h, the cells were exposed to UV light (λ = 365 nm) for 2 h. The cells were further incubated for additional 24 h, both non-adherent and adherent cells were collected, washed with cold PBS, and re-suspended in 200 μL binding buffer containing 5 μL Annexin V-FITC. Cells were gently mixed and incubated in dark at room temperature for 10 min. Cells were centrifuged and resuspended in 200 μL binding buffer containing 10 μL propidium iodide (PI) solution. Cell apoptosis was analyzed immediately by a FACS flow cytometer (Beckman coulter). The data were summarized in Figure S3.

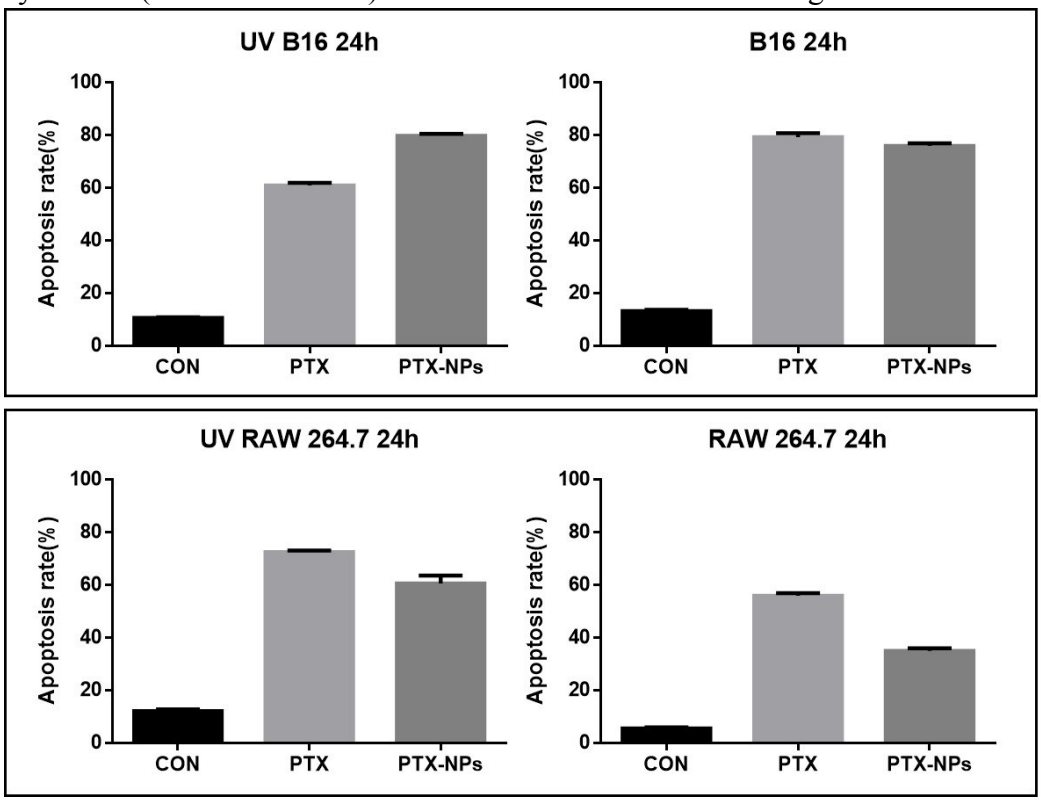

Fig. S3. Apoptosis rates of B16 (up) and RAW 264.7 cells (down) when incubated with free PTX or PTX-NPs 24h with and without UV irradiation, respectively. The PTX concentration was 30 nM.