Supporting Information

Dual stimuli-responsive bispillar[5]arene-based nanoparticles for precisely selective drug delivery in cancer cells

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Materials and Equipment

The disulfide-bridged bispillar[5]arene material was synthesized by following a previously published method.[1]

Paclitaxel (PTX) was purchased from Xi'an haoxuan Biological Co., Ltd (Xi'an, China). Spermine (SPM) and glutathione (GSH) was purchased from Sigma-Aldrich (Hong kong). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (USA). Cyanine Dyes (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 Amresco. Poly (vinyl alcohol) (PVA) (88 mol% hydrolyzed, Mw=25 kDa) were obtained from Acro Organics. All the reagents and solvents employed were commercially available and used as supplied without further purification. The size and zeta potential of micelles were determined by doing 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 200KV. The drug concentration was detected using HPLC, and the chromatographic conditions were as follows: the column used was an XDB C18 (4.6×250 mm, 5 mm), and the mobile phase consisted of acetonitrile and water (60/40, v/v). Cellular uptake and apoptosis were analyzed by a FACS flow cytometer (Beckman coulter). A confocal laser scanning microscopy (CLSM, Zeiss LSM710) was used to directly visualize the intracellular location of micelles. Cellular viability was measured by a multi-mode microplate reader (FlexStation 3).

The L02 cell lines were obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The A549 cell lines were purchased from American Type Culture Collection (ATCC, Shanghai, China).

Preparation of Nanoparticles (NPs)

The GSH and SPM responsive NPs were prepared according to oil-in-water (o/w) emulsion solvent evaporation method. 20 mg bisP5A material was dissolved in 1.0 mL trichloromethane (TCM) 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.

Stability study

The aqueous solution containing bisP5A 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 bisP5A NPs.

Time (days)	1	7	14
Diameter (nm)	245.9	256.2	298.6
PDI	0.093	0.147	0.367

Preparation of cargo-loaded NPs

PTX loaded NPs (PTX-NPs) were fabricated with similar process to that of blank NPs, where both PTX and bisP5A were dissolved in TCM during the first step with the rest of the procedures remained the same. Cy5 loaded NPs (Cy5-NPs) and Nile red loaded NPs (NR-NPs) were prepared in the same process by replacing PTX with Cy5/Nile red.

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.

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

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

Table S2. DEE and DLE results.

PTX feed ratio	Diameter(nm)	PDI	DEE	DLE
10%	245.9	0.093	84.5%	11.5%

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

The GSH and SPM responsive behaviors of the NPs were followed by DLS in buffer solution under different stimuli. Firstly, the diameters of four bisP5A NPs samples containing 0, 0.01 mM, 1 mM and 10 mM concentrations of GSH, respectively, were evaluated by DLS at various time points, the selected time was 0 h, 1 h, 6 h, 18 h, 24 h, 42 h, 49 h, 66 h and 86 h, respectively. Figure S1 shows the size evolution of the NPs under different GSH conditions over time. Then the diameters of four bisP5A NPs samples contain not only GSH but also SPM (1 mM) were evaluated by DLS at same time intervals. Figure S2 shows the size evolution of the NPs under different GSH conditions and 1 mM SPM over time.

As for the GSH and SPM triggered payload release of PTX-NPs, similarly, a certain amount of PTX-NPs solution was placed with different concentration of GSH stimuli and SPM environment (0, 1 mM SPM, 10 mM GSH, 1 mM GSH+0.1 mM SPM, 10 mM GSH+1 mM SPM). At selected time intervals (0 h, 1 h, 6 h, 18 h, 24 h, 42 h, 49 h, 66 h and 86 h), 100 μL of the release media was taken out for measuring the released

PTX concentrations by HPLC. The same volume of fresh medium was subsequently added to the original release media.

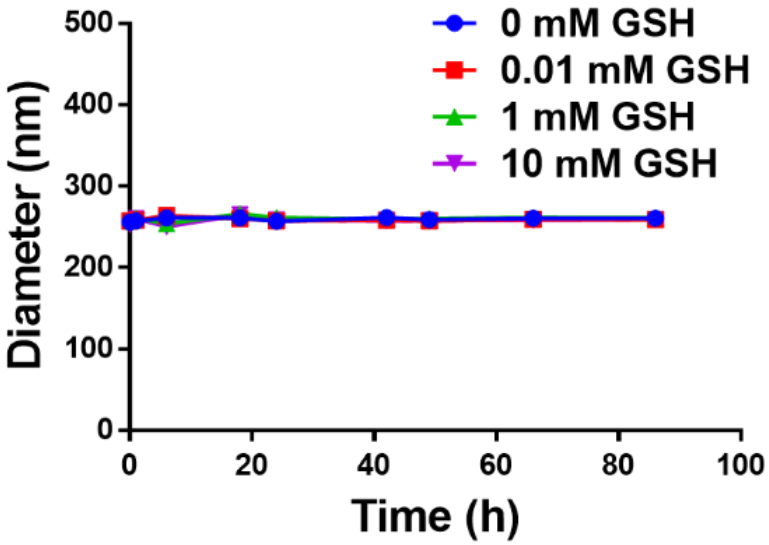

Fig. S1. The size and size (diameter) distributions of bisP5A NPs at various time points, when incubated in various concentrations of GSH. (n = 3)

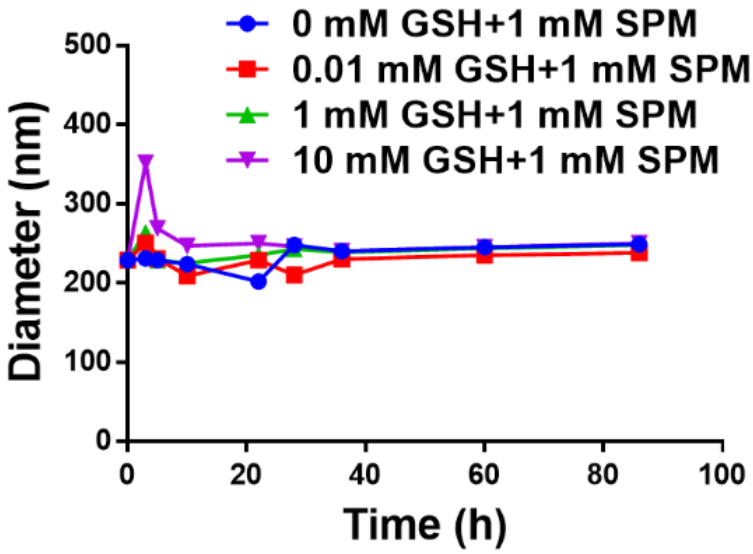

Fig. S2. The size and size (diameter) distributions of bisP5A NPs at various time points, when incubated in various concentrations of GSH and SPM (1 mM). (n = 3)

Cell culture

The L02 and A549 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

L02 and A549 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 25, 50, 100 and 200 μg/mL of NPs and incubated for additional 48 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 S3.

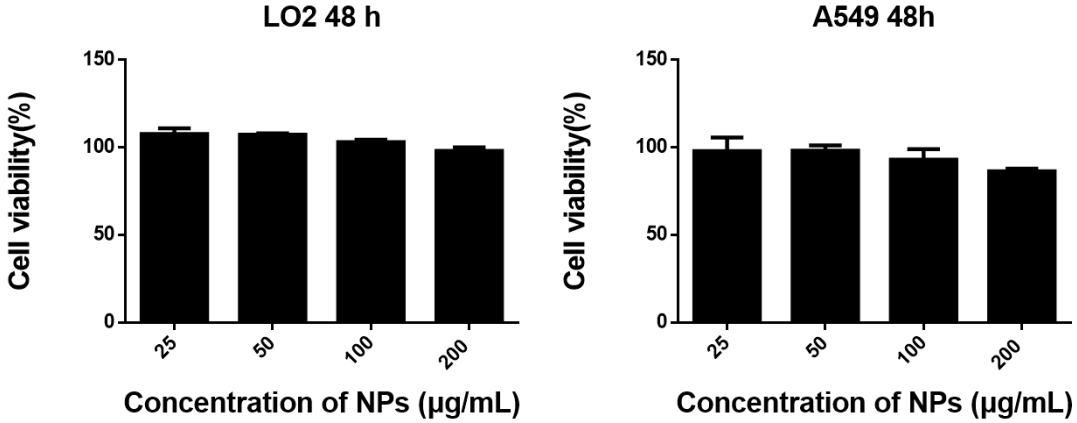

Fig. S3. Cytotoxicity of bisP5A NPs against L02 and A549 cells after incubation for 48 h.

Cytotoxicity assay of PTX-NPs

L02 and A549 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 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. The cells were further incubated for additional 48 h, subsequently the medium was replaced with 100 μL of a fresh medium containing 10 μL of methylthiazolyldiphenyl-tetrazolium bromide (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 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 cellular uptake behaviors of NPs were investigated after Cy5-NPs were incubated for 2, 4, 8 and 12 h with L02 and A549 cells. L02 and A549 cells were respectively seeded in confocal dishes (20 mm) with a density of 10^5 cells per well and incubated for 24 h. The culture medium was replaced with fresh medium containing Cy5-NPs. After incubation for additional 2, 4, 8 and 12 h, the culture medium was removed. The cells were washed for three times with PBS and were subsequently fixed in 300 mL of paraformaldehyde for 15 min. The cells were subsequently washed for another three times with PBS and the cell nuclei were counterstained by 300 mL of DAPI aqueous solution for 15 min. Finally, the cells were washed again with PBS for three times and confocal laser scanning microscopy (TCS SP8, Leica) was used to observe the intracellular uptake behavior of NPs.

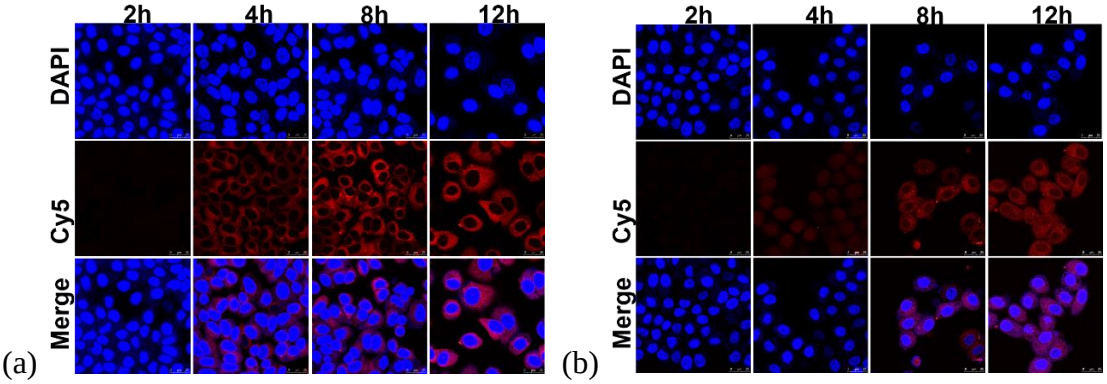

Fig. S4. CLSM images showing cellular uptake of Cy5-NPs: A549(a) and L02 cells (b) incubated with Cy5-NPs for up to 12 h. The cell nucleuses were stained by DAPI (blue fluorescence).

L-O2 and A549 cells were incubated into 24 well plates at a density of 2×10^5 cells per well in DMEM medium for 24 h. The final concentration of 50 μg/mL concentration Cy5-NPs was added. Then, the cells were cultured for 48 h and washed three times with PBS. Cells were treated with trypsin and centrifuged for 5 min at 1000 rpm. Then the cells were suspended in 0.5 mL of PBS and analyzed using a FACScan flow cytometer.

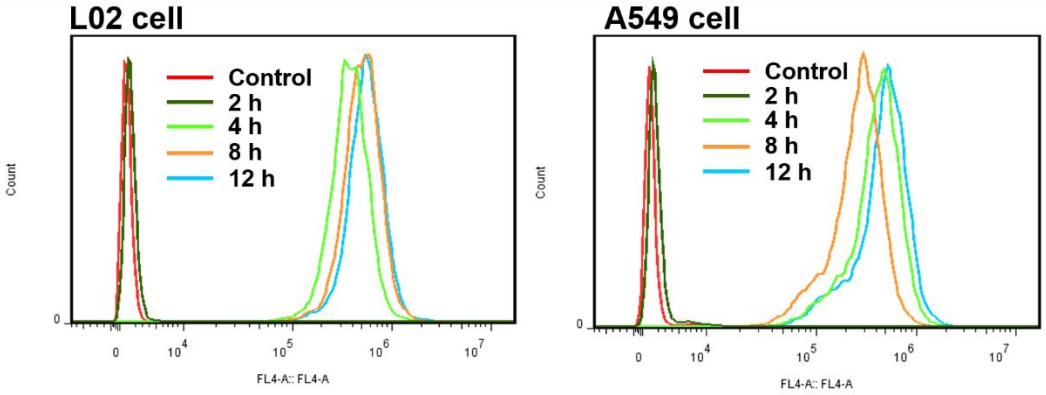

Fig. S5. Cellular uptake of flow cytometry analysis upon incubation with of 50 μg/mL concentration Cy5-NPs for 2 h, 4 h, 8 h, and 12 h in L02 (left) and A549 (right) cells, respectively.

Intracellular release study

The intracellular release behaviors of GSH and SPM responsive NPs were investigated after they had been incubated for 2 h and 12 h in L02 and A549 cells, respectively. The fluorescence probe Nile red (NR) was encapsulated in GSH and SPM responsive NPs as previous described. L02 and A549 cells were seeded in confocal dishes (20 mm) with a density of 10^5 cells per well and incubated for 24 h. The culture medium was replaced with fresh medium containing NR loaded NPs. After incubation for additional 4 h the culture medium was replaced with fresh medium and continued to incubation for 2 h and 12 h. The cells were washed three times with PBS and fixed in 300 mL of paraformaldehyde for 15 min. The cells were subsequently washed for another three times with PBS and the cell nuclei were counterstained by 300 mL 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 observe the intracellular release behaviour of NPs.

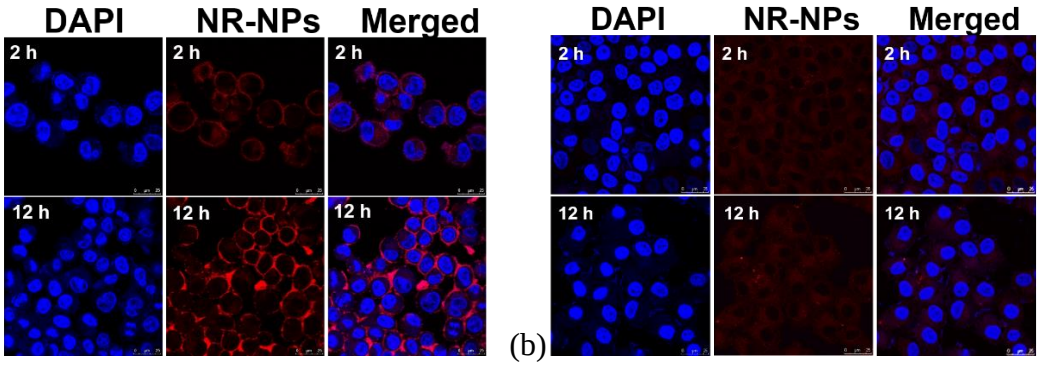

Fig. S6. CLSM images showing intracellular release of NR-NPs: A549(a) and L02 cells (b) incubated with NR-NPs for up to 12 h. The cell nucleuses were stained by DAPI (blue fluorescence).

Cell Apoptosis Assays

Cellular apoptosis effects of PTX and PTX-NPs at a concentration equivalent to 20 nM PTX were determined by the Annexin V/PI staining assay. L02 and A549 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, respectively, 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 DMEM. 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 re-suspended in 200 μL binding buffer containing 5 μL propidium iodide (PI) solution. Cell apoptosis rates were analyzed immediately by a FACS flow cytometer (Beckman coulter), and the data were summarized in Fig. S7.

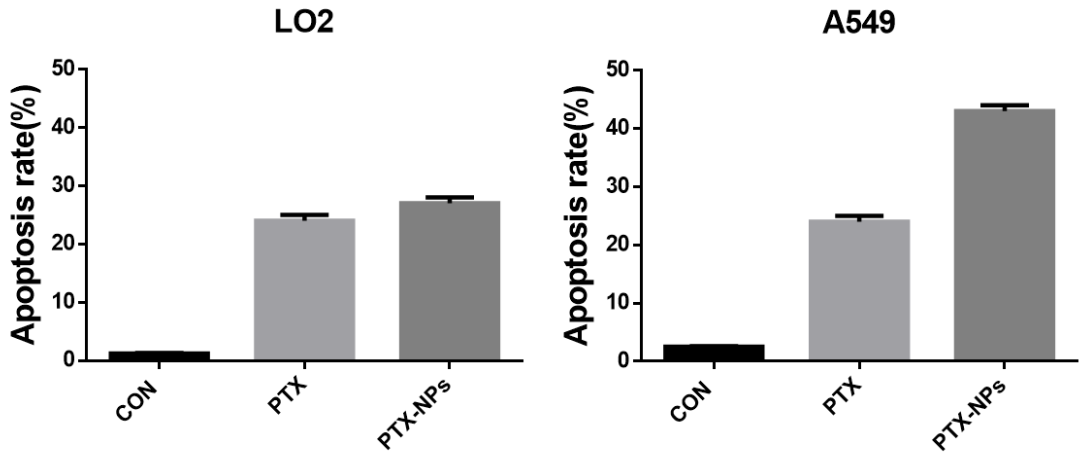

Fig. S7. Apoptosis rates of L02 and A549 cells incubated with free PTX and PTX-NPs for 24h. The PTX concentration was 20 nM.

Reference:

1. C.-L. Sun, H.-Q. Peng, L.-Y. Niu, Y.-Z. Chen, L.-Z. Wu, C.-H. Tung and Q.-Z. Yang, *Chem. Commun.*, 2018, **54**, 1117.