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Supporting Information

Glutathione-responsive homodithiacalix[4]arene-based nanoparticles for selective intracellular drug delivery

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Materials and Equipment

The GSH-responsive homodithiacalix[4]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). 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) was 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 was 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 RAW 264.7 cell lines were obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The MCF-7 cell lines were purchased from American Type Culture Collection (ATCC, Shanghai, China).

Preparation of NPs

The GSH-responsive NPs were prepared by oil-in-water (o/w) emulsion solvent evaporation method. In a typical process, 20 mg homodithiacalix[4]arene material was dissolved in 1 mL dichloromethane (DCM) solution, followed by emulsification via probe sonication into 4 mL of 1.0 wt.% aqueous solution of PVA. The prepared emulsion was stirred in a round bottom flask and heated with blower to remove the dichloromethane solvent. The NPs were collected by centrifugation at 14,500 rpm for 6 min, rinsed with deionized water for three times.

Stability study

The aqueous solution containing HDT-C4A NPs was stored at ambient conditions. At selected time intervals (1, 7 and 14 d), 1 mL of the solution was taken out for DLS measurements. The results were shown in Table S1.

Table S1. Stability of HDT-C4A NPs.

Time (days)	1	7	14
Diameter (nm)	214	275	345.2
PDI	0.136	0.181	0.388

Preparation of cargo-loaded NPs

PTX loaded NPs (PTX-NPs) were fabricated in the similar process, where both PTX and homodithiacalix[4]arene 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 with the replacement of PTX with Cy5.

The content of PTX encapsulated by nanoparticles was measured by dissolving it in DMSO and analyzed 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.

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

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

Table S2. DEE and DLE results.

PTX feed ratio	Diameter(nm)	PDI	DEE	DLE
20%	214	0.136	89.50%	51%

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

The GSH-responsive behaviors of the NPs were followed by DLS in buffer solution with different GSH concentrations. Briefly, the diameters of four GSH-responsive NPs samples containing 0, 10 μM, 1 mM and 10 mM concentrations of GSH, respectively, were evaluated by DLS at various time points, the selected time was 0 h, 18 h, 24 h, 30 h and 40 h, respectively. Figure S1 shows the size evolution of the NPs under different GSH conditions over time.

As for the GSH triggered payload release of PTX-NPs, similarly, a certain amount of PTX-NPs solution was placed with 0, 10 μM, 1 mM and 10 mM GSH concentrations, respectively. At selected time intervals (0, 4 h, 8 h, 20 h, 26 h and 32 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.

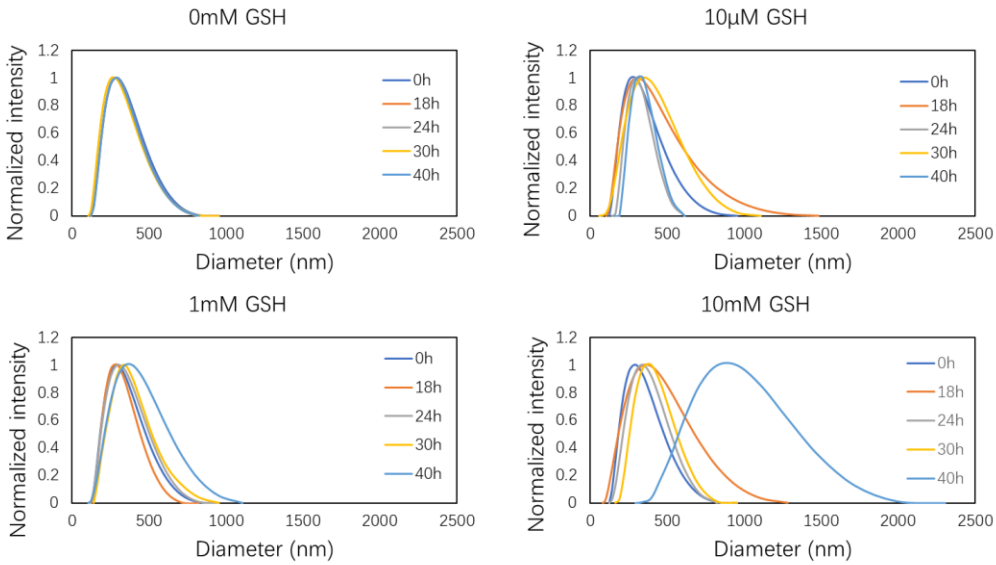

Figure S1. The size and size (diameter) distributions of HDT-C4A NPs at various time points, when incubated in various concentrations of GSH.

Cell culture

The MCF-7 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 MCF-7 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 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 subsequently 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 same experiment without treatment of NPs was executed for control. The experiment was performed 3 times to obtain a standard deviation. The results were shown in Figure S2.

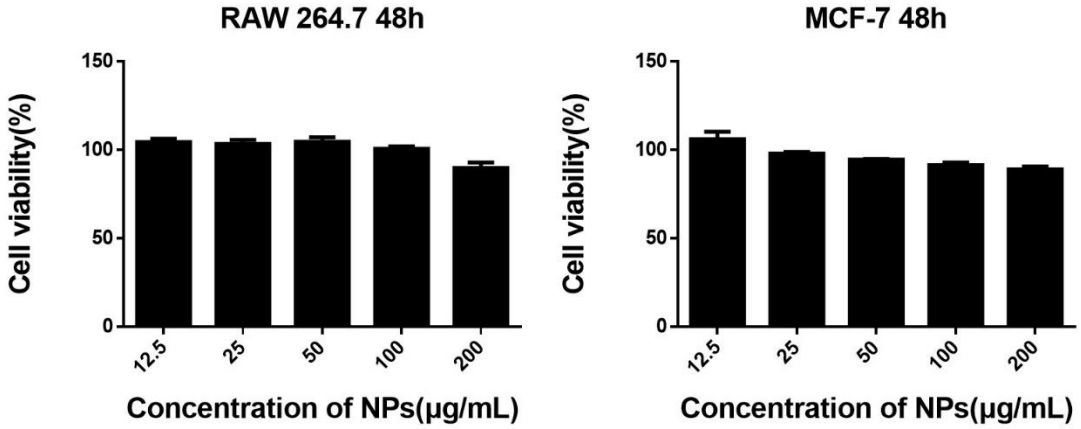

Figure S2. Cytotoxicity of HDT-C4A NPs against RAW 264.7 and MCF-7 cells after incubation for 48 h.

Cytotoxicity assay of PTX-NPs

MCF-7 and RAW 264.7 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 36 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 MCF-7 cells. MCF-7 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 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 aqueous solution of DAPI (0.1μg/mL) 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.

Cell Apoptosis Assays

Cellular apoptosis effects of PTX and PTX-NPs at a concentration equivalent to 30 nM PTX were determined by the Annexin V/PI staining assay. MCF-7 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, 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 PBS. 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 10 μL propidium iodide (PI) solution. Cell apoptosis rates were analyzed immediately by a FACS flow cytometer (Beckman coulter), and the data were summarized in Figure S3.

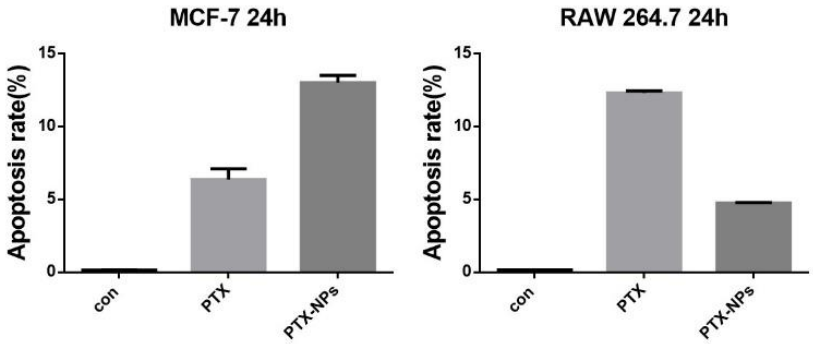

Figure S3. Apoptosis rates of MCF-7 and RAW 264.7 cells incubated with free PTX and PTX-NPs for 24h. The PTX concentration was 30 nM.

1. J. Thomas, G. Reekmans, P. Adriaensens, L. Van Meervelt, M. Smet, W. Maes, W. Dehaen, and L. Dobrzanska, *Angew. Chem. Int. Ed.* 2013, **52** (39), 10237-10240.