Supporting Information

A H₂O₂-Responsive Nanocarrier for Dual-Release of Platinum Anticancer Drugs and O₂: Controlled Release and Enhanced Cytotoxicity against Cisplatin Resistant Cancer Cells

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I.1. Materials. Poly(D,L-lactic-*co*-glycolic acid) (PLGA, with a lactide/glycolide molar ratio of 75:25 and an inherent viscosity of 0.17 dL/g) was obtained from Daigang BIO Engineer Ltd. Co. (Shandong, China). Cisplatin, catalase, H_2O_2 , Black Hole Quencher-2 (BHQ-2), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Poly (vinyl alcohol) (PVA, M_W = 57-66 kDa), phorbol 12-myristate 13-acetate (PMA), Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride complex [Ru(dpp)₃]Cl₂ were all obtained from Sigma-Aldrich (St. Louis, MO, USA). [PtLCl]Cl was synthesized in our laboratory.¹ Ultrapure water was prepared using a Millipore Simplicity System (Millipore, Bedford, USA).

I.2. Fabrication and characterization of PLGA NPs. PLGA NPs encapsulating [PtLCl]Cl were prepared using a water-inoil-in-water (W/O/W) double-emulsion, solvent-diffusion-evaporation approach method.² A 2.5 mg catalase powder and 10 mg [PtLCI]Cl were added to 1 mL PVA aqueous (10 mg/mL) and then mixed thoroughly for 15 min. Subsequently, the mixture was emulsified with 2 mL of PLGA solution (5 mg/mL in CH₂Cl₂) containing BHQ-2 (10 mg/mL) to obtain the primary W/O emulsion. The primary emulsification was carried out by using an ultrasonicator for 1 min in an ice bath. The primary emulsion was then added to 6 mL of PVA aqueous (20 mg/mL) and emulsified by using an ultrasonicator for 1 min in an ice bath to form the W/O/W double-emulsion. To evaporate CH₂Cl₂ and solidify the particles, the resultant double-emulsion was transferred into 20 mL of ultrapure water and stirred overnight at room temperature. After washing three times with ultrapure water, the PLGA NPs were collected by centrifugation and finally resuspended in 10 mL phosphate buffered saline (PBS) solution. The encapsulation efficiency of [PtLCl]Cl is 15.8%. The similar procedures were used to prepare the PLGA NPs encapsulating cispaltin. The encapsulation efficiency of cisplatin is 14.2%. The surface morphology and size of the as-prepared PLGA NPs were investigated by scanning electron microscopy (Hitachi s-4800 high resolution SEM) on the conductive adhesive tapes. The NPs for transmission electron microscopy (TEM, JEM-2100) measurement was prepared by dropping the solution onto a carbon-coated copper grid following negative staining with 2.0% (w/v) phosphotungstic acid. The particle size and size distribution of PLGA NPs were measured by dynamic light scattering (DLS) (a Mastersizer 2000 particle size analyzer) with a fixed scattering angle of 90°. The measurement was performed. Zeta potential measurement was performed at 25 °C on a Malvern Zeta sizer-Nano Z instrument. The glass transition temperature (T_g) was measured with differential scanning calorimetry (DSC, Mettler-Toledo, Switzerland).

I.3. Discussion on the mean diameter, polydispersity index (PDI) and zeta potential of NPs (containing catalase and cisplatin) at different preparation conditions. The influence of some preparation conditions (PVA concentration in outer aqueous phase and amount of PLGA) on the particle size, PDI and zeta potential was investigated and summarized in Table S1. The PLGA NPs were spherical in shape with a core-shell structure under these technical conditions.

PLGA (mg)	PVA ^a (%, w/w)	Mean		Zeta
		diameter	PDI	potential
		(nm)		(mV)
5	1	239	0.17	-19
	2	223	0.19	-20
	4	216	0.13	-25
10	1	245	0.18	-15
	2	230	0.11	-18
	4	224	0.14	-20
20	1	269	0.21	-11
	2	257	0.17	-13
	4	246	0.19	-15

Table S1. Mean diameter, PDI and zeta potential values of NPs prepared with different amount of PLGA and PVA.

^aPVA concentration in outer aqueous phase.

I.4. Long-term-stability study of PLGA NPs in various media. A significant challenge in application of NPs is to retain their stability in physiological environments. Aiming at biomedical applications, we evaluated the stability of PLGA NPs (containing catalase/cisplatin) by suspending the NPs in two commonly used biological media: PBS and Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 10% fetal bovine serum (FBS). Nanoparticle size was measured by DLS. No significant size change was observed after incubation of the NPs in these media for at least 7 days (Fig. S3), demonstrating good colloidal stability of the NPs under various culture media.

I.5. Study on in vitro release of payload from the PLGA NPs. The profiles for the in vitro release of payload from NPs were established by dialysis of the PLGA NPs suspensions in PBS. Briefly, 3 mL the NPs suspensions (500 μ g/mL) were dialyzed against 15 mL PBS buffer (molecular-weight cutoff: 12000) and gently shaken in a thermostatic rotary shaker at 100 rpm and 37 °C. Samples were removed at different intervals, and an equal amount of the same medium was added to maintain a constant volume. The amount of cisplatin released from the NPs was analyzed by ICP-OES. The cisplatin concentration corresponding to 100% release is 61.94 mg/L.

I.6. Investigation on the catalase activity after encapsulation and catalase release behavior. The activity of catalase after encapsulation was assessed by an indirect method³: by determining the total amount of catalase added in the formulation and subtracting what was not encapsulated. Free catalase in the supernatant and washings collected during the preparation of NPs were assayed for enzyme activity. Catalase activity was determined with Catalase Assay Kit (KeyGen Biotec Co. Ltd., China).

The reaction of H_2O_2 decomposition by catalase was terminated by adding ammonium molybdate, with the remaining H_2O_2 and ammonium molybdate producing a pale yellow complex which was measured at 405 nm. All assays were carried out in triplicate. The catalase activity after encapsulation was measured to be 47 U in each mg of PLGA NPs, which corresponding to 13.4% of encapsulated catalase. In vitro release was performed by suspending catalase-loaded NPs in 20 mL PBS with 100 μ M H_2O_2 equilibrated at 37 °C. At fixed time intervals, 1 mL suspension was transferred to a 2 mL microtube and the supernatant was taken for enzyme quantization after centrifugation.

I.7. Cell culture and confocal imaging. Human cervical carcinoma HeLa cells, human breast cancer MCF-7 cells, human alveolar adenocarcinoma A549 cells, drug resistance A549/CDDP, human gastric cancer SGC-7901, drug resistance SGC-7901/DDP cells were cultured in glass bottom dishes. Cells were seeded at a density of 1×10^6 cells/mL in RPMI 1640 supplemented with 10% FBS, NaHCO₃ (2 g/L) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). The cells were maintained in a humidified incubator at 37 °C, in 5% CO₂/95% air. One day before imaging, cells were passed and plated on 18-mm glass bottom dishes. Cell imaging was carried out after washing cells with PBS for three times. Confocal fluorescence imaging studies were performed with a ZEISS Laser Scanning Microscope (Zeiss LSM 710).

I.8. In vitro cytotoxicity assay. To investigate cytotoxicity of the samples: free [PtLC1]Cl, [PtLC1]Cl-loaded NPs, free cisplatin, cisplatin-loaded NPs, and NPs (no catalase), MTT assay were carried out on HeLa and MCF-7 cells. Cells (10⁶ cells/mL) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μ L/well. Plates were maintained at 37 °C in a 5% CO₂/95% air incubator for 24 h. The samples were diluted to different concentrations of solution and added to each well after the original medium has been removed. Cells were incubated with different concentrations of the samples for 48 h. Then, 20 μ L of 5 mg/mL MTT solution in pH 7.4 PBS was added to each well. After 4-h incubation, the medium containing unreacted MTT was removed carefully, and 150 μ L of DMSO was added to each well to dissolve the formazan crystals. After 1 h the absorbance (Abs.) was measured at 490 nm in a TRITURUS microplate reader. The cell viability was then determined by the following equation: Cell viability (%) = (mean of Abs. value of treatment group/mean Abs. value of control) × 100%. Calculation of the half lethal dose (IC₅₀) values was done according to Huber and Koella.⁴

I.9. Flow Cytometry Analysis. To study the cell apoptosis induced by free cisplatin and NPs on SGC-7901/DDP cells, a flow cytometric assay of annexin V-FITC and propidium iodide (PI) costaining was employed.⁵ The cells were harvested, rinsed in PBS, resuspended, and determined by flow cytometry using Cytomics FC500 Flow Cytometry (Beckman Coulter Ltd.). All experiments detected at least 10,000 cells and the data were analyzed with FCS Express V3.

I.10. Study of the intracellular release of O₂ from the PLGA NPs. The intracellular release of O₂ was investigated by confocal fluorescence imaging. The intracellular O₂ levels were assessed with an O₂ sensing probe $[(Ru(dpp)_3)]Cl_2$, the fluorescence of which is strongly reduced by molecular oxygen)⁶ by comparing the $[Ru(dpp)_3]Cl_2$ fluorescence intensity of NPs (containing catalase only)-treated cells with untreated cells. PMA-stimulated HeLa cells were incubated with 5 μ M $[Ru(dpp)_3]Cl_2$ for 4 h and further incubated with 50 μ g/mL NPs for 24 h. The cells were excited at 488 nm and the emission was collected between 600 nm and 700 nm.

I.11. JC-1 assay. The membrane-permeant JC-1 dye is widely used to determine the early stage of apoptosis.⁷ In healthy cells with high mitochondrial membrane potential, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low mitochondrial membrane potential, JC-1 remains in the monomeric form, which shows only green fluorescence. In JC-1 assay, cells were treated with free cisplatin or NPs for 24 h, and then loaded with the 5 μ M JC-1 dye for 30 min at 37 °C. Prior to imaging, the medium was removed and cells were washed with PBS for three times. The fluorescence of green channel was excited at 488 nm and the emission was collected

between 510-545 nm. The fluorescence of red channel was excited at 543 nm and the emission was collected between 575-630 nm.

II.1.



Fig. S1. Zeta potential distribution of the PLGA NPs.

II.2.



Fig. S2. Stability study on PLGA NPs at physiological temperature in the absence of H_2O_2 : the in vitro release profiles of cisplatin from PLGA NPs incubated in PBS at 37 °C. Insets: the SEM micrographs of PLGA NPs incubated in PBS at 37 °C at 0 h and 24 h. Scale bar: 200 nm.



Fig. S3. Long-term-stability study of PLGA NPs in PBS and in RPMI 1640 with 10% FBS.

II.4.



Fig. S4. The size stability of PLGA NPs (without catalase) in the presence of 100 μ M H₂O₂. Insets: the SEM micrographs of PLGA NPs (without catalase) incubated with 100 μ M H₂O₂ at 0 h and 24 h. Scale bar: 200 nm.

II.3.



Fig. S5. Normalized fluorescence of [PtLCl]Cl upon release from the PLGA NPs in the presence of $100 \ \mu M \ H_2O_2$.

II.6.



Fig. S6. Absorption spectrum of BHQ-2.

II.7.



Fig. S7. Confocal fluorescence images of PMA-stimulated HeLa cells incubated with the NPs for 16 h and then incubated with (a) LysoTracker Red FM and (b) Hoechst 33342. Scale bars: 20 µm.

II.8.



Fig. S8. Catalase release behavior from the NPs incubated with 100 μ M H₂O₂ quantified by measuring the enzyme activity within 24 h.

II.9.



Fig. S9. MTT assay of HeLa cells in the presence of different concentrations of free [PtLCl]Cl.



Fig. S10. MTT assay of HeLa cells in the presence of different concentrations of [PtLCl]Cl-loaded NPs and [PtLCl]Cl-loaded NPs (without catalase).





Fig. S11. MTT assay of HeLa cells in the presence of 20 μ M free [PtLCl]Cl, NPs, NPs (no catalase) and NPs (no [PtLCl]Cl). The concentration of free [PtLCl]Cl used was equivalent to what was incorporated into the NPs.

II.12.



Fig. S12. Confocal fluorescence images showing increased intracellular O_2 level after treated with catalase-loaded NPs. PMAstimulated HeLa cells incubated with 5 μ M [Ru(dpp)₃]Cl₂ for 4 h, followed by incubation with the NPs for a) 0 h, b) 8 h, c) 16 h and d) 24 h. e)-h) represent the bright field images of a)-d) respectively. Scale bars: 10 μ m. i) Normalized average intracellular fluorescence intensity of PMA-stimulated and [Ru(dpp)₃]Cl₂-loaded HeLa cells incubated with NPs for different time.



Fig. S13. Confocal fluorescence images of apoptosis by the JC-1 assay in drug-sensitive SGC 7901 and drug resistance SGC 7901/DDP cells treated with free cisplatin, NPs or NPs (no catalase) for 24 h. The amount of the cisplatin incorporated in PLGA NPs was equivalent to that used in free cisplatin. Scale bars: 25 µm.

II.14.



Fig. S14. Confocal fluorescence images of apoptosis by the JC-1 assay in a) drug-sensitive A549 and b) drug resistance A549/CDDP cells treated with free cisplatin or cisplatin-loaded NPs for 24 h. Scale bars: 25 µm.



Fig. S15. Flow cytometry analysis of drug resistance SGC-7901/DDP cells after free cisplatin or cisplatin-loaded NPs treatment. a) Control. b) SGC-7901/DDP cells treated with free cisplatin for 24 h. c) SGC-7901/DDP cells treated with cisplatin-loaded NPs for 24 h.

II.16.



Fig. S16. MTT assay of (a) SGC 7901cells and (b) SGC 7901/DDP cells in the presence of different concentrations of free cisplatin and cisplatin inside the NPs (with/without catalase).

Supplementary references

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II.15.