Supporting Information

Self-Assembly of Enzymes and Prodrugs with Clickable Amino Acid for Nucleus-Targeted Cancer Therapy

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1. General

1.1 Materials and Reagents

All chemical reagents were used as supplied without further purification unless otherwise specified. DNase I was purchased from Sigma (Shanghai, China). Cisplatin was purchased from (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Singlet oxygen sensor green (SOSG) were purchased from Thermo Fishcer Scientific (Waltham, MA, USA). Sodium azide (NaN_3), methyl-β-cyclodextrin (M-β-CD), Ivermectin, genistein was purchased from Bide Pharmatech Ltd.

1.2 Instruments

The morphology of nanoparticles was observed by transmission electron microscope (TEM, Talos F200S, Holland). Hydrodynamic sizes and zeta potentials were measured by multi angle particle size and high sensitive zeta potential analyzer (Omni, USA). The assembly behavior of Pt(IV) and nanomaterials was investigated using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, PerkinElmer).

2 Experimental Section

Preparation of DP-LDA nanoparticles

To prepare the nanoassembly, Lys-D and 4-A were first synthesized according to literature,[1] and c,t,c-[$Pt(NH_3)$ 2-($O_2CCH_2CH_2COOH$)$(OH)Cl_2$] (Pt (IV)), a prodrug of cis-platinum, was utilized. The amino acid derivative Lys-D solution (19.5 μL, 20 μg/μL) was mixed with the DNase I solution (2.5 μL, 20 μg/μL) to prepare D-LD

complexes. According to the molar ratio of Lys-D to Pt (IV) 1:28, Pt (IV) solution (3.4 μL, 5 μg/μL) was added and mixed for 15 min. Then, the cross-linking agent 4-A (5.5 μL, 20 μg/μL) was added to the solution and mixed evenly to obtained DP-LDA nanoparticles (18.3 μg/μL).

Drug loading and encapsulation efficiency of DNase I and Pt(IV)

For study the loading efficiency of DNase I, the different samples including free DNase I and the mass ratios of DNase I: Lys-D and 4-A was 1:2, 1:4, 1:6, 1:8, 1:10, and 1:12, independently. The prepared complex was loaded onto the agarose gel. Then the protein was stained with Coomassie brilliant blue to calculate the proteins encapsulation. Protein loading ratio was calculated as: Loading Ratio = (weight of protein in the nanoformulations/weight of nanoformulations) × 100%

To assess the binding capacity of Pt(IV) to nanoparticles, DP-LDA nanoparticle samples were incubated in water, transferred to ultrafiltration tubes, and subjected to centrifugation at 15,000 rpm for 30 minutes in a high-speed centrifuge. The filtrate was collected, and the Pt content in the filtrate was determined using inductively coupled plasma mass spectrometry (ICP-MS). The drug loading (DL) and encapsulation efficiency (EE) of Pt in the nanoparticles were calculated using the following formulas.

Evaluation of Pt(IV) release from DP-LDA

To determine the release content of Pt(IV) through dialysis, 30 mg of DP-LDA nanoparticles (with Pt content of 2.95 wt%) were dissolved in a 5 mL ultrapure water solution, sealed in a dialysis bag (Sigma, 1500 Da), and immersed in phosphate-buffered saline (PBS) solutions with different concentrations (0 mM PBS, 50 mM PBS, 100 mM PBS). Dialysis was conducted at 37°C under agitation at 100 rpm in a water

bath. Samples were withdrawn at predetermined time intervals, and the released medium was replenished with an equal volume of fresh release medium. The released amount of Pt was analyzed using inductively coupled plasma mass spectrometry (ICP-MS). All experiments were performed in triplicate.

Cell culture

Mouse breast cancer 4T1 cells were obtained from Shanghai Ek-Bioscience and cultured in complete RPMI-1640 medium (10% FBS, 1% penicillin/streptomycin) at 37 °C in the presence of 5% CO2.

Cellular uptake

4T1 cells (density of 1×104 cells/well) were seeded in 96-well plates and incubated for 12 h at 37 °C under 5% CO2. 100 μL fresh medium containing DNase I, D-LDA, DP-LDA (20 μg/mL for DNase I and 10 μg/mL for Pt(IV)) was then added, The above-mentioned DNase I was labeled using isothiocyanate (FITC).After 2 h incubation, the cells were fixed with 4% paraformaldehyde, stained with DAPI and imaged with fluorescent microscopy (ix51, Japan).

Endocytosis mechanism

4T1 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated 12 h at 37 °C under 5% CO_2. The cells were incubated with different endocytosis inhibitor (10 mM NaN_3, 10 mM M-β-CD or 200 μM genistein) or placed at 4°C for 1 h, and then DP-LDA nanoparticle was added and incubated at 37 °C or 4 °C for another 2 h. After that cells were trypsinsized and collected for flow cytometry analysis.

Nucleus transport mechanism

To explore the nuclear transport mechanism of DP-LDA nanoparticles, 4T1 cells were

preincubated with ivermectin (25 μM) for 1 h and then Lys-DDPN ((20 μg/mL for DNase I and 10 μg/mL for Pt(IV)) was added for further incubating another 2 h. After that, the cells were fixed with 4% paraformaldehyde, stained with DAPI and imaged with fluorescent microscopy.

Detection of cisplatin in the cells by using ICP-MS

4T1 cells were seeded in 6-well culture plates and incubated overnight at 37 °C. Then, the cells were incubated with free DNase I, Cisplatin, D-LD, D-LDA and DP-LDA group for 2 h. After that, cells were washed with PBS three times, and then lysed by using cell lysis buffer. Thereafter, it was subjected to centrifugation at 2×10^4 rpm for 15 min. The supernatant was collected for ICP-MS testing of cisplatin contents.

In vitro cytotoxicity

4T1 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated for 12 h at 37 °C under 5% CO_2. Then the medium was replaced with fresh medium containing free DNase I, Cisplatin, D-LD, D-LDA, P-LDA and DP-LDA (5 μg/mL for DNase I, 2 μg/mL for Pt(IV)). After 24 h incubation, the cell viability was measured by performing MTT assays. For cytotoxicity study of LDA nanoparticles, overnight incubated 4T1 cells were treated with different concentrations of LDA (0, 5,10, 20, 30, 40, 50 μg/mL) for 24 h, and MTT assay was used to determine the cell viabilities.

Animal model

All animal experiments were carried out in compliance with the requirements of the National Act on the Use of Experimental Animals (People's Republic of China) and were approved by the Experimental Animal Ethical Committee of Chongqing University Cancer Hospital. Female mice (6-8 weeks) were supplied by the Animal

Center of Chongqing Medical University (Chongqing, China).

Biodistribution

4T1 tumor-bearing mice were injected intravenously with free DNase I, DP-LDA (1 mg/kg Cy7-DNase I, 4 mg/kg Pt). The mice were imaged at different time points (2, 8, 12, 24 h) postinjection by small animal imaging system (IVIS Lumina III, USA). 24 h after administration, the mice were sacrificed, major organs (heart, liver, spleen, lung and kidney) and tumors were imaged and analyzed by an IVIS lumina imaging system.

Pharmacokinetic Study

Mice were randomly divided into two groups, each comprising 5 individuals: the Cisplatin group and the DP-LDA group (4 mg/kg Pt). The administration was carried out via tail vein injection. At specific time points post-administration (15 min, 30 min, 1 h, 2 h, 8 h, 12 h, 24 h), 0.5 mL of blood was collected from the mouse retro-orbital plexus and transferred to 1.5 mL centrifuge tubes coated with heparin sodium. To ensure thorough mixing, the tubes were inverted, and then, using a low-temperature centrifuge at 3000 rpm for 5 min at 4°C, plasma was obtained. The drug content in mouse plasma was extracted, and Pt levels were determined using ICP-MS. Graphical analysis was conducted to assess the pharmacokinetics of DP-LDA nanoparticles in the mouse body.

In vivo antitumor activity

For anti-tumor therapy studies, breast cancer 4T1 cells (2×10^6) were injected subcutaneously into female BALB/c-nude mice. When the tumor volume grew to approximately 60 mm3, the mice were randomly divided into 5 groups (n=5): (1) PBS control group, (2) DNase I group, (3) Cisplatin group, (4) D-LDA group, (5) DP-LDA

group. 150 μL of different samples with equal amount of DNase I (3 mg/kg) and Cisplatin (2 mg/kg) were intravenously injected into the mice every three days for a total of three times. The tumor volumes and weight were measured every two days for 20 days. The tumor volumes value was calculated as the following equation: tumor volumes = length × width^2 × 0.5. At the end of experiment, the tumor was excised and weighed. For histological examination, tumor tissues and major organs (heart, liver, spleen, lung and kidney) were collected for hematoxylin and eosin (H&E) staining.

Statistical analysis.

Experimental data were presented as the mean ± standard deviation (SD) of at least three independent experiments. Analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Statistical analysis was conducted using one-way ANOVA or Student's t-test. Statistical significance is indicated as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

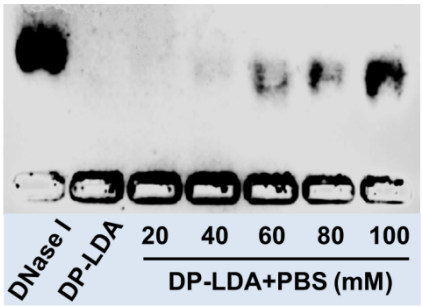

Fig. S1. Intact DNase I release in PBS with different ionic strength.

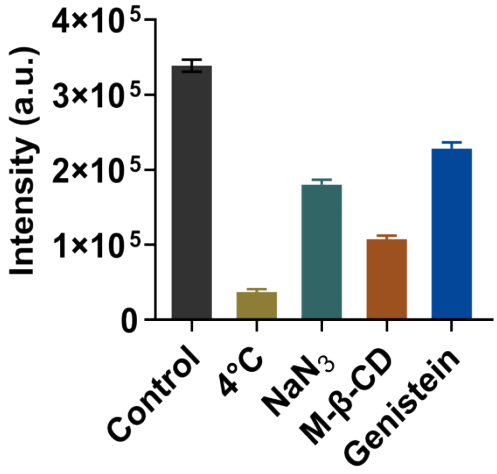

Fig. S2. Effects of endocytosis inhibitors on cellular uptake of DP-LDA nanoparticles.

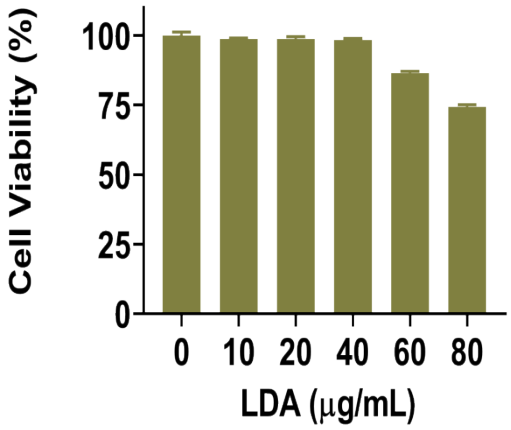

Fig. S3. Relative viabilities of 4T1 cells after incubation with different concentrations of LDA nanoparticle carriers.

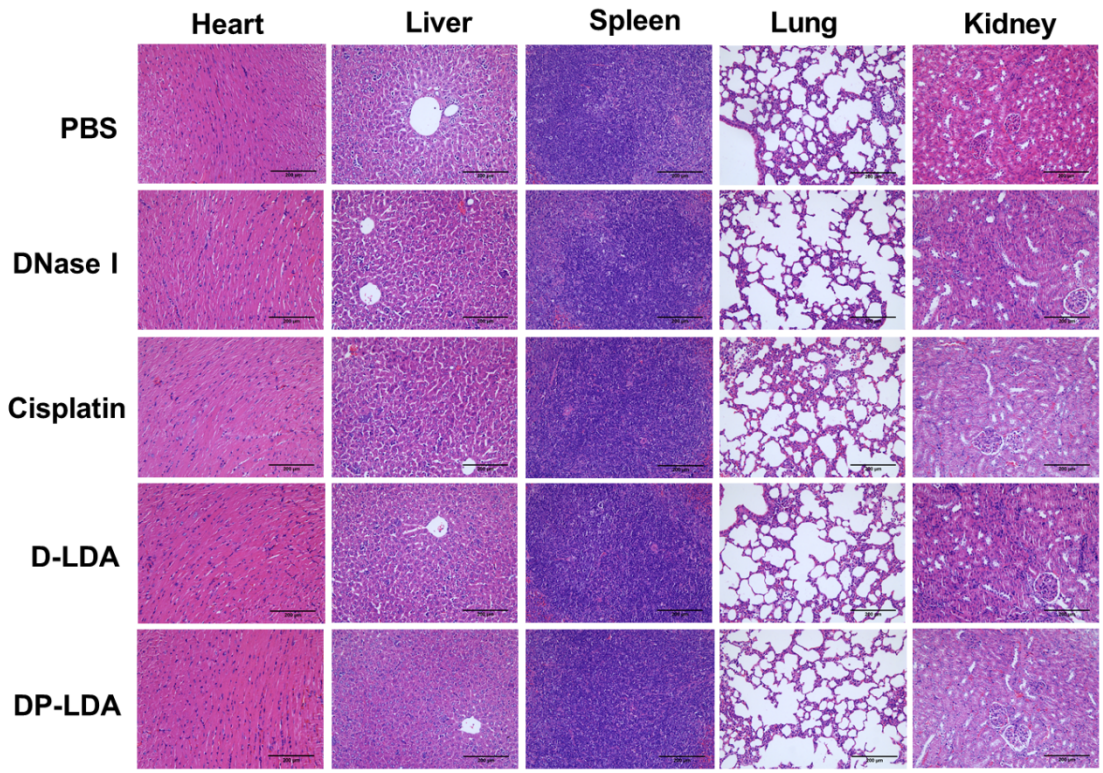

Fig. S4. H&E staining of the main organs after different treatments. Scale bars: 200 μm.

1 H. Xiao, Y. Lan, S. Hang, L. Shan, Q. Dongmei, C. Hui, Q. Yunfei, L. Libing and F. Xuli, *J Control Release.*, 2021, **337**, 306-316.