Supplementary Information for

Combined chemotherapy and photodynamic therapy using a nanohybrid based on layered double hydroxides to conquer cisplatin resistance

Zhigang Wang, Rong Ma, Li Yan, Xianfeng Chen, and Guangyu Zhu

Experimental Details

1. Material and general measurements

All the chemicals were received from commercial sources and used directly. Cisplatin was obtained from Boyuan Technology (Shandong, China). Chlorine e6 (Ce6) was from J&K chemical (China).

The morphology of the nanoparticles was observed by a Philips Technai 12 Transmission Electron Microscope at 120 kV. Malvern Zetasizer Nano ZS was used to determine the zeta potential and zeta size of the nanoparticles. Samples were tested for three times with 15 runs each time. The Pt content was measured by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (PE 2100DV) or Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, PE Elan 6100 DRC). UV-Vis spectra were recorded on a Ultra-Violet Visible Scanning Spectrophotometer (Shimadzu 1700).

2. Synthesis of Pt(IV)/LDH, Ce6/LDH, and Ce6-Pt(IV)/LDH

LDH nanoparticles, DSCP, and Pt(IV)/LDH were synthesized following our previous work.¹ For the synthesis of Ce6/LDH, Ce6 (400 μg/mL, pH 7.0, 400 μL) was added to a suspension of LDH nanoparticles (4.0 mg/mL, 1.0 mL), and the reaction was stirred at room temperature for 16 h in dark. After loading, the mixture was centrifuged at
10,000 rpm and the precipitate was washed twice with degased water. Finally, the precipitate was resuspended in degased water and the suspension was stored at 4 °C in dark. Ce6-Pt(IV)/LDH was synthesized by following the same method, except that 250 μL Ce6 together with various amount of DSCP were added. The Pt content in the nanoparticles was determined by ICP-OES. UV-vis spectroscopy was used to quantify the concentration of Ce6 in the nanoparticles. Before each measurement, the sample was digested by 0.1 N HCl for 5 min to digest LDH NPs into free ions. The concentration of Ce6 was calculated from the absorbance peak of Ce6 at 411.5 nm in the digested solution.

3. Ce6 photo-stability assay
Ce6, Ce6/LDH, and Ce6-Pt(IV)/LDH (10 μM Ce6, 200 μL in PBS) were added to 96-well plates and the absorbance was measured by a microplate reader (Biotek, Powerwave xs). The plates were irradiated under 650 nm at 7 mW/cm² for 5 min before measurements.

4. Measurements of Pt binding to CT-DNA in vitro
CT-DNA (80 μg) was mixed with DSCP, Pt(IV)/LDH, or Ce6-Pt(IV)/LDH (final Pt concentration = 40 μM) with and without the presence of ascorbic acid (2 mM) in a phosphate buffer (10 mM phosphate, 150 mM NaClO4, pH 7.4). The mixture was incubated at 37 °C with shaking for 4 h and 8 h. After incubation, the samples were centrifuged at 12,000 rpm for 10 min to collect the nanoparticles. The suspension was transferred to another eppendorf tube and 0.7× volume of isopropanol was added to precipitate the CT-DNA. The CT-DNA pellet was collected by centrifugation (15,000 rpm, 20 min), washed twice with 70% ice-cold ethanol, and dried by spin vacuum. CT-DNA was resuspended in TE buffer. The DNA concentration was measured by Nanodrop (Thermo Scientific ND-1000) and the Pt content was ascertained by ICP-OES.

5. Detection of singlet oxygen by DPBF assay
A freshly prepared DPBF solution (0.05 mg/mL in DMSO, 100 μL) was added into each well in a 96-well plate. Ce6, Ce6/LDH, and Ce6-Pt(IV)/LDH containing 50 pmol Ce6 was added to different wells. Pt(IV)/LDH was also added as a negative control. The absorbance of each well at 410 nm was measured by a microplate reader (Biotek Powerwave xs). The plate was irradiated by a 650 nm LED lamp (7 mW/cm²) for 1 min before each measurement.

6. Cell lines and cell culture
A2780, A2780cisR, and A549cisR cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640) supported with 10% fetal bovine serum (FBS), 50 U/mL penicillin and streptomycin, and 2 mM L-glutamine. A549 cells were grown in Dulbecco's modified Eagle medium (DMEM) supported with 10% FBS and 50 U/mL penicillin and streptomycin. For the cisplatin-resistant cells, 5 μM cisplatin was added to the culture media every two passages to maintain the resistance. All the cells were cultured in a humidified incubator at 37 °C with 5% carbon dioxide.

7. Cell viability assay
Cell viability of cancer cells exposed to the compounds was examined by MTT assay. Cells were seeded in 96-well plates at a density of 2,000 cells per well and incubated until the cell confluency reached 50%. Then the medium was aspirated and fresh medium containing different concentration of compounds was added to each well. After 24 h, the medium was removed and cells were washed twice with PBS. Then, fresh complete medium was delivered to each well, and the cells were irradiated for 30 min under a 650 nm LED lamp at a density of 7 mW/cm². Cells were further incubated for 24 h, and then the medium was changed to serum-free medium containing 3 mg/mL MTT. After 3 h incubation at 37 °C, the medium was removed and DMSO (200 μL) was added to each well to dissolve the formed purple formazan. The absorption at 570 nm and 730 nm was measured by a microplate reader.

8. Cell cycle arrest analysis
A2780 cells were plated in 6-well plate at a density of 100,000 cells per well and incubated for 36 h. After that, medium was replaced with fresh medium containing different concentration of compounds and further incubated for 24 h. Then, medium was removed and cells were washed twice with PBS. Fresh medium was added to each well and the cells were irradiated at 650 nm for 30 min at 7 mW/cm². Then, cells were further incubated for 24 h, collected by trypsinization, washed with PBS, and fixed with 70% ethanol overnight at 4 °C. Before flow cytometric measurements, cells were spun down and stained by a PI staining solution (10 μg/mL PI, 100 μg/mL RNAse, and 0.1% Triton-X100 in PBS) for 15 min at 37 °C. Cell cycle distribution was analyzed by a BD flow cytometer with 10,000 cell counts for each sample. The data were analyzed by a Modifit 1.2 software.

9. Cellular uptake of platinum
A2780 cells were seeded in 100 mm dishes and incubated until the cell confluency reached 80%. The medium was changed to fresh medium containing cDDP, Pt(IV)/LDH, and Ce6-Pt(IV)/LDH at the concentration of 5 μM Pt. After 24 h, cells were collected and washed 3 times with ice-cold PBS. Cell pellet was resuspended in 2 mL PBS and divided into two portions. One portion of cells was lysed in a radioimmunoprecipitation assay buffer (RIPA) supported with 1 mM phenylmethanesulfonyl fluoride (PMSF), and the protein concentration in the cell lysate was determined by a BCA kit (Biyun Tian, China). The other portion of cells was digested by 30% nitric acid and 15% H₂O₂ overnight at 65 °C, and the Pt content was measured by ICP-MS. The platinum levels in cells was expressed as pmol Pt per mg protein.

10. Apoptosis measurements
Cells were seeded in 6-well plates at a density of 2×10⁵ cells per well and incubated for 24 h. Then, the cells were treated with different complexes for 24 h. After that, the medium was change to fresh medium, and the cells were irradiated at 650 nm for 30 min at 7 mW/cm². The cells were further incubated for 10 h before cell collection by
trypsinization. The cells were subsequently washed with cold PBS and annexin binding buffer, and the cell density was determined and adjusted to 1 x 10^6 cells per mL. Annexin V-FITC conjugate and PI solution were added to 100 μL cells suspension. The cells were stained at RT for 15 min, then 400 μL annexin binding buffer was added and mixed gently. The as prepared samples were kept on ice and analyzed immediately by a flow cytometer.

11. Intracellular ROS detection

A2780 cells were seeded on cover slips in 35 mm dishes. After the cells grown to 50% confluency, the medium was changed to fresh medium containing Ce6-Pt(IV)/LDH with a final concentration of Ce6 at 1 μM. A control group of cells without the treatment of Ce6-Pt(IV)/LDH was also prepared. After 24 h, the cells were stained with 10 μM H2DCFDA (Life Technologies) in PBS for 40 min. Then, the staining solution was removed and the complete medium was added to each dish. The cells treated with Ce6-Pt(IV)/LDH were irradiated at 650 nm for 15 min at 7 mW/cm² or covered by aluminum foil during irradiation. The positive control cells were treated with 3 mM H2O2 for 15 min in PBS. After that, the cover slip was mounted on a glass slide and the cells were observed by a confocal microscope (Lecai SPE) with the excitation at 488 nm and emission at 517 to 527 nm.
Table S1. Hydrodynamic size and polydispersity index of LDH and Ce6-Pt(IV)/LDH.
The data are presented as the average value±SD from three independent experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average hydrodynamic size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH</td>
<td>52.8±2.2</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td>Ce6-Pt(IV)/LDH(0.81)</td>
<td>152.3±6.5</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>Ce6-Pt(IV)/LDH(1.92)</td>
<td>184.6±2.8</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td>Ce6-Pt(IV)/LDH(4.99)</td>
<td>2633.7±685.8</td>
<td>0.31±0.06</td>
</tr>
</tbody>
</table>

Figure S1. (A-C) Dose-dependent cell killing effect of Ce6/LDH, Pt(IV)/LDH, and Ce6-Pt(IV)/LDH loaded with different ratios of Ce6 and Pt(IV); (D) Combination index (CI) values of Ce6-Pt(IV)/LDH calculated from the dose-response curves.
**Figure S2.** UV-vis spectra of Ce6, Ce6/LDH, and Ce6-Pt(IV)/LDH in PBS buffer. The concentration of Ce6 was 10 µM in each of the samples.

**Figure S3.** Photo-stability of Ce6 upon irradiation at 650 nm. The concentration of Ce6 was 10 µM in each of the samples.
**Figure S4.** DNA binding ability of Pt in DSCP, Pt(IV)/LDH, and Ce6-Pt(IV)/LDH with and without the presence of AsA at (A) 4 h and (B) 8 h.

**Figure S5.** Fluorescent images of H$_2$DCFDA-stained A2780 cells treated with 3 mM H$_2$O$_2$ for 20 min or 1 µM Ce6-Pt(IV)/LDH for 24 h followed by 15 min irradiation at 650 nm. Scale bar: 250 µm.
Figure S6. Fluorescent images of H$_2$DCFDA-stained A2780cisR cells treated with 3 mM H$_2$O$_2$ for 20 min or 1 µM Ce6-Pt(IV)/LDH for 24 h followed by 15 min irradiation at 650 nm. Scale bar: 500 µm.

Figure S7. Singlet oxygen generation ability of different complexes upon 650 nm irradiation. The amount of singlet oxygen was determined by a DPBF assay.
Figure S8. Cell cycle distribution of (A) A2780 and (B) A2780cisR cells after treatment with different complexes for 48 h.

Reference