Electronic supplementary information

Redox-responsive cisplatin nanogel for anticancer drug delivery

*Weiqi Zhang and Ching-Hsuan Tung**

Molecular Imaging Innovations Institute, Department of Radiology, Weill Cornell Medicine, Cornell University

*To whom correspondence should be addressed.

413 East 69th Street, Box 290, New York, NY 10021, USA.

E-mail: cht2018@med.cornell.edu.

Materials

Hyaluronan (HA) of MW=1000 kDa was purchased from Lifecore Biomedical Co. (Chaska, MN, USA). Cisplatin and O-phenylenediamine (OPDA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin hydrochloride (Dox) was purchased from LC Laboratories (Boston, MA, USA).

Preparation of HA/Cis/Dox

Cisplatin, HA and Dox was dissolved in ddH_2O to acquire a 2.5, 5 and 10 mg/ml solution, respectively. The mixture of 360 μL cisplatin and 40 μL Dox was preheated at 90 °C for 2 min and then 800 μL of HA solution was added and mixed thoroughly. The solution was further heated at 90 °C for 2 hours, then cooled on ice for 15 min. The cooled solution was transferred to a dialysis tube (MWCO=3.5 kDa) and dialyzed against 1 L of ddH_2O in a beaker for 1 day. The dialysis water, in the beaker, was collected during the water change to quantify the free Dox based on its fluorescence. The Dox retaining in the nanogel was calculated by deducting the Dox in dialysis water from the Dox input during the synthesis. The cisplatin in the nanogel was quantified directly using the OPDA method. The drug incorporation efficiency was calculated as follow:

$$Incorporation\ efficiency = \frac{Drug\ in\ HA/Cis/Dox}{Drug\ input} \times 100\%$$

Dynamic light scattering (DLS) analysis

Around 50 μL of acquired HA/Cis/Dox solution was mixed with 1 ml of ddH_2O in a cuvette and subjected to hydrodynamic size measurement using a Malvern Zetasizer Nano-S machine (Malvern Instruments, Malvern, UK). The measurement was conducted

with 3 repeats. After that, 200 μL of extra HA/Cis/Dox was added in the cuvette and the zeta potential was measured using the Zeta PALS analyzer (Brookhaven Instruments, Holtsville, NY, USA). To probe the stability of HA/Cis/Dox in PBS, HA/Cis/Dox was diluted with PBS at the rate of 25 fold. The hydrodynamic size was monitored after 0 and 3 days incubation at 37 °C. To probe the effect of GSH on the DLS size distribution of HA/Cis/Dox, the nanogel was dispersed in PBS, PBS containing 0.5 mM and 5 mM GSH at 37 °C for 3 days. Then the DLS size was measured as described above.

Spectral characterization

The absorption spectrum of HA/Cis/Dox was collected on a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Briefly HA/Cis/Dox was dispersed in ddH_2O to attain an HA concentration of 0.1 mg/ml. Around 500 μL of diluted HA/Cis/Dox was subjected to measurement with a wavelength that ranged from 300 to 800 nm. To highlight the spectra shift between HA/Cis/Dox and free Dox, the absorption spectrum of free Dox was plotted together. To compare the fluorescence spectra of HA/Cis/Dox and free Dox, the Dox concentration was fixed at 10 μg/ml. The fluorescence spectra was collected on a plate reader (Tecan, Männedorf, Switzerland) with an excitation of 480 nm and emission of 520-720 nm, respectively.

Transmission electron microscopy (TEM)

Around 5 μL HA/Cis/Dox (~1.3 mg/ml HA) was dropped onto the copper grid and left at room temperature for 2 min. The solution on the grid was then carefully wicked away with a piece of filter paper. The grid was further stained by one drop of 1.5% uranyl

acetate solution for 1 minute and dried with filter paper. The sample was observed using a TEM microscope (JEM-1400, JEOL, Tokyo, Japan). To probe the effects of GSH on the HA/Cis/Dox morphology, HA/Cis/Dox dissolved in PBS containing 0, 0.5 and 5 mM GSH respectively, was incubated at 37 °C for 3 days. Then the TEM samples were prepared as described above.

OPDA method

Cisplatin was quantified using a modified OPDA method from that reported by Golla et al.[1, 2] Basically, HA/Cis/Dox nanogel was diluted in a 9% NaCl solution to attain an HA concentration of 33 μg/ml. Diluted HA/Cis/Dox (250 μL) was then mixed with an equal volume of the OPDA solution (1.2 mg/ml in DMF). The mixture was heated at 100 °C, for 10 min, followed by the absorbance determination (705 nm) on a plate reader (Tecan, Männedorf, Switzerland). Solutions with 0, 0.625, 1.25, 2.5, 5, 10 and 20 μg/mL cisplatin in 9% NaCl were prepared to determine the cisplatin standard curve. The final cisplatin in the nanogel was calculated based on its OD705 value, referring to the cisplatin standard. To verify the effects of GSH on cisplatin, 10 μg/ml cisplatin in 9% NaCl containing 0, 0.005, 0.005, 0.5 and 5 mM GSH was prepared. Then the cisplatin solution was reacted with an OPDA solution as described above. After heating, the absorption spectrum was monitored on the plate reader with a wavelength set from 600 to 800 nm. The mixture of OPDA, without cisplatin, was used as a background. To probe the cisplatin depletion by the GSH, HA/Cis/Dox was diluted in a 9% NaCl solution containing GSH; the mixture was then subjected to the OPDA reaction. OPDA only, GSH plus OPDA, cisplatin (12.5 μg/mL) plus GSH, and HA/Cis/Dox plus OPDA were included as controls. All GSH

concentrations were set at 5 mM and the HA concentration in the nanogel was fixed at 133 μg/ml. After the OPDA reaction, the absorption spectra were collected using a plate reader.

Drug release

One milliliter of HA/Cis/Dox was sealed in a dialysis tube (MWCO=3.5 kDa) and immersed in 80 ml PBS. The drug release was performed on a shaker with a rotation speed and temperature set at 100 rpm and 37 °C, respectively. At the time points of 1, 2, 4, 8, 12, 24, 48 and 72 hours, 1.6 ml of PBS was collected from the solution outside the dialysis bag; meanwhile, an equal volume of fresh PBS was added back into the dialysis bag. The cisplatin and Dox amounts in the collected samples were then quantified based on the OPDA method and specific absorbance, respectively. To check GSH's effect on the release of Dox in the nanogel, 1 mL of HA/Cis/Dox was sealed in a dialysis tube and dialyzed against PBS in a beaker containing 0, 0.5 and 5 mM of GSH. The experimental settings were kept the same as those used above. At pre-determined times, the sample from the beaker was collected and quantified.

Cell culture

Ovarian cancer cells A2780 and its cisplatin resistant cell line A2780cis were acquired from Sigma-Aldrich (St. Louis, MO, USA). The cells were cultured in RPMI160 medium (Cellgro, Manassas, VA, USA) containing 10% FBS supplemented with 100 U/ml penicillin and streptomycin. To maintain the resistance, 1 μM of cisplatin was added in

medium for A2780cis cells twice a week. The cells were maintained in an incubator at 37 °C with humidified air and 5% CO_2.

Fluorescent microscopy

Around 2 × 10^4 A2780 or A2780cis cells were seeded in a chamber slide (μ-slide, Ibidi GmbH, Munich, Germany) and left overnight to allow the cell attachment. The cells were then treated with medium only, free Dox or HA/Cis/Dox for 8 hours. The Dox concentration in the medium was kept at 13.5 μM. After the incubation, the cells were rinsed once with PBS and incubated with 2 μM of LysoTracker green (Life Technologies, New York, NY, USA) for 30 min. After a wash, the cells were stained by 0.1 μg/ml of Hoechst dye to highlight the nucleus. To mimic the extracellular GSH provided by stromal cells, 1 mM of GSH was added to the medium during the 8-hours of co-incubation. After the organelle staining, all samples were observed on an EVOS fluorescent microscope as soon as possible. The Dox, lysosome and nucleus signals were collected in the RFP, GFP and DAPI channels, respectively.

Flow cytometry (FACS)

A2780 and A2780cis cells (2 × 10^5) were seeded on 12-well plates and incubated overnight to allow cell adherence. Then the cells were subjected to the same treatments used for fluorescent microscopy. After 8 hours of incubation, the cells were washed twice and collected by trypsinization. The cells were further analyzed using a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) in FL3 channel. Each treatment was conducted in duplicate and at least 10000 cells were collected for each sample.

Cell viability assay

To evaluate the drug resistance of A2780 and A2780cis cells, 4500 cells in a 96-well plate were incubated with cisplatin and Dox with drug concentrations set from 16 to 48000 nM and 1 to 80000 nM, respectively. After 2 days of incubation, the cells were washed and the cell viability was evaluated by using the MTS assay kit (Promega, Madison, WI, USA). To verify the cytotoxicity of HA/Cis/Dox, cells (4500 cells/well) were treated by medium only, cisplatin, Dox, cisplatin + Dox and HA/Cis/Dox. The cisplatin and Dox dose concentrations were standardized to 24 μM and 13.5 μM, when used. For the GSH positive group, 1mM of extra GSH was added in the medium to mimic extracellular GSH. After 1 day challenge, the cell's viability was measured by MTS assay. To check the cytotoxicity of cisplatin and HA/Cis/Dox in different GSH concentrations, A2780cis cells were seeded as described above. Then cells were incubated free cisplatin and HA/Cis/Dox with cisplatin set at 16 and 32 μM and meanwhile 0, 0.05 and 0.5 mM GSH were supplemented in the medium. After 2 days incubation, the cell viability was evaluated using the MTS assay. Following the manufacture's instruction, absorbance at 490 nm of cells with no treatment ($A490_{con}$), with treatments ($A490_{treatments}$) and medium only (no cells) ($A490_{background}$) were used to calculate the cell viability. Cell viability of those incubated in medium alone was set as 100% and each treatment was conducted in triplicate.

$$Relative\ cell\ viability = \frac{A490treatments - A490background}{A490con - A490background} \times 100\%$$

Statistical analysis

All data were indicated as Mean ± SD. Statistical analysis was performed using the 2-sided Student's t test. $p < 0.05$(*), $p < 0.01$(**) and $p < 0.001$(***), represents statistically significant difference between tested groups.

References

1. E. D. Golla and G. H. Ayres, *Talanta*, 1973, **20**, 199-210.
2. W. Zhang, Z. Zhang and C. H. Tung, *Chem. Commun.*, 2017, **53**, 779-782.

Table S1 Characterization of HA/Cis/Dox.

	HA/Cis/Dox
Zeta potential	-24.2 ± 4.3 mV
Hydrodynamic diameter (PDI)	195.8 ± 10.5 nm (0.199 ± 0.002)
Dox concentration (Incorporation efficiency)	0.307 ± 0.001 mg/ml (92.1 ± 0.3%)
Cisplatin concentration (Incorporation efficiency)	0.291 ± 0.035 mg/ml (38.9 ± 4.7%)
Molar ratio of Cis to Dox	1.8:1

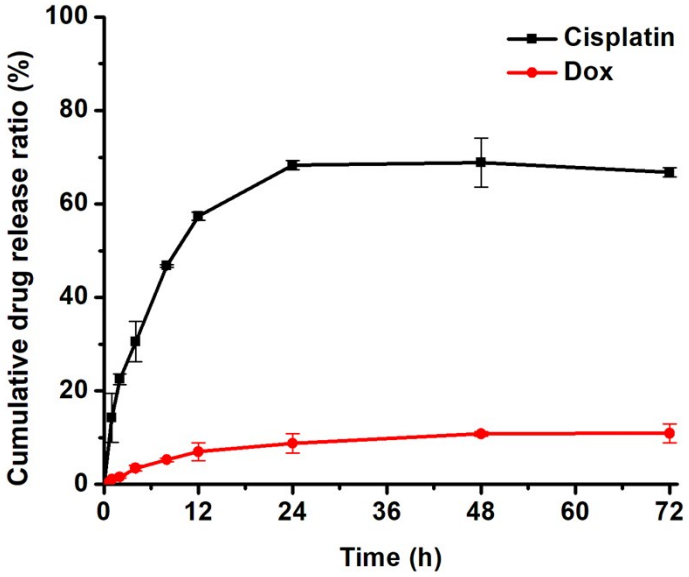

Fig. S1 Cisplatin and Dox release from HA/Cis/Dox in PBS at 37 °C.

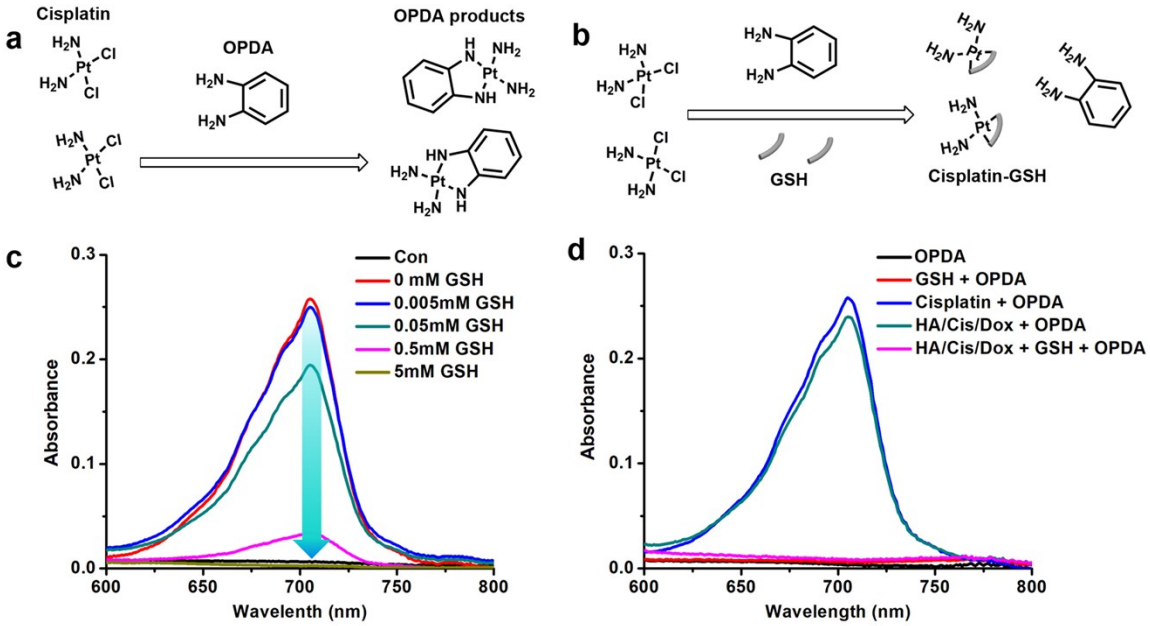

Fig. S2 Binding of GSH and cisplatin. a) Scheme of OPDA reaction. b) Scheme of GSH inhibiting the OPDA reaction. c) Absorption spectra of the OPDA products with the presence of different concentrations of GSH (0 to 5 mM). The cisplatin was fixed at 10 μg/ml, except the control group containing no cisplatin. d) Cisplatin depletion by GSH (5 mM) in HA/Cis/Dox monitored by OPDA reaction.

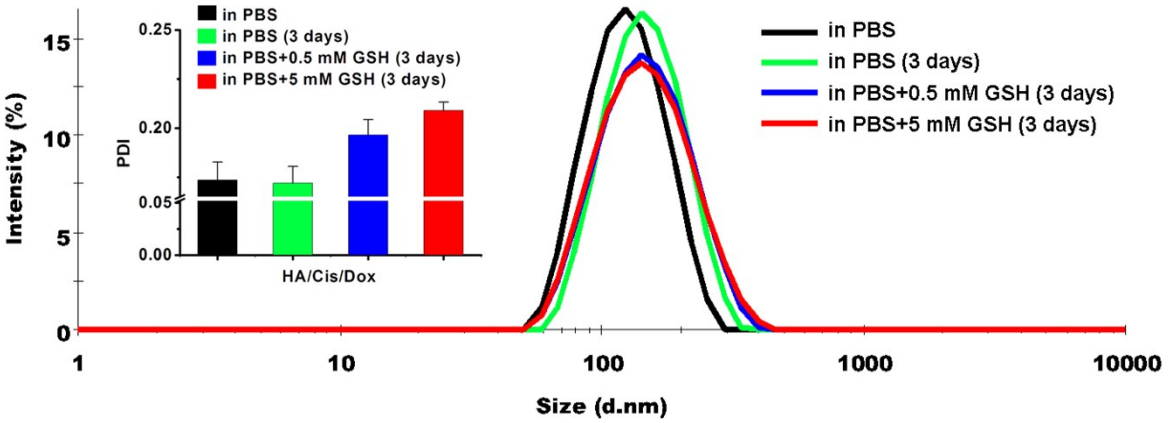

Fig. S3 DLS size distribution of HA/Cis/Dox in the presence of GSH. HA/Cis/Dox was dispersed in PBS, PBS containing 0.5 and 5 mM GSH at 37 °C for 3 days. The inserted bar graph presents the PDI value from at least 3 measurements.

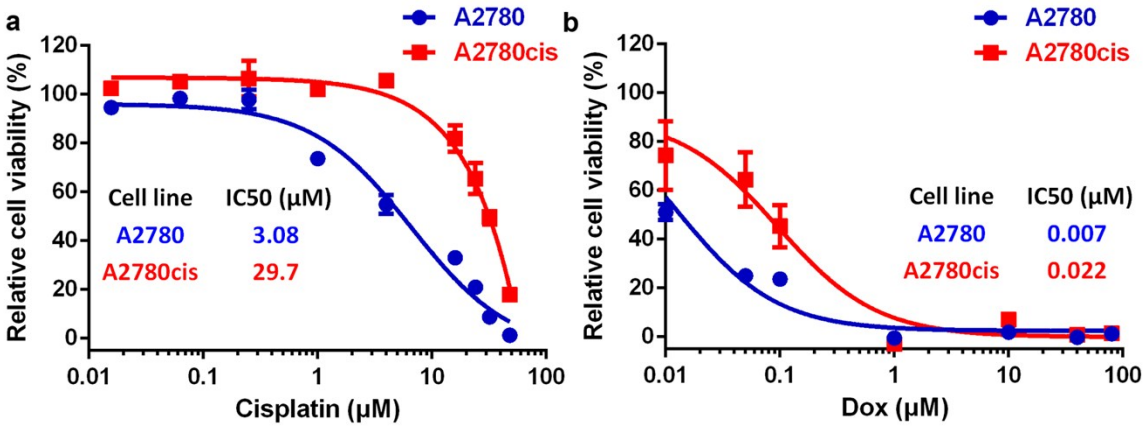

Fig. S4 Dose response of drugs on A2780 and A2780cis cells. a) cisplatin. b) Dox. Cells were incubated with different drug concentrations of for 2 days.

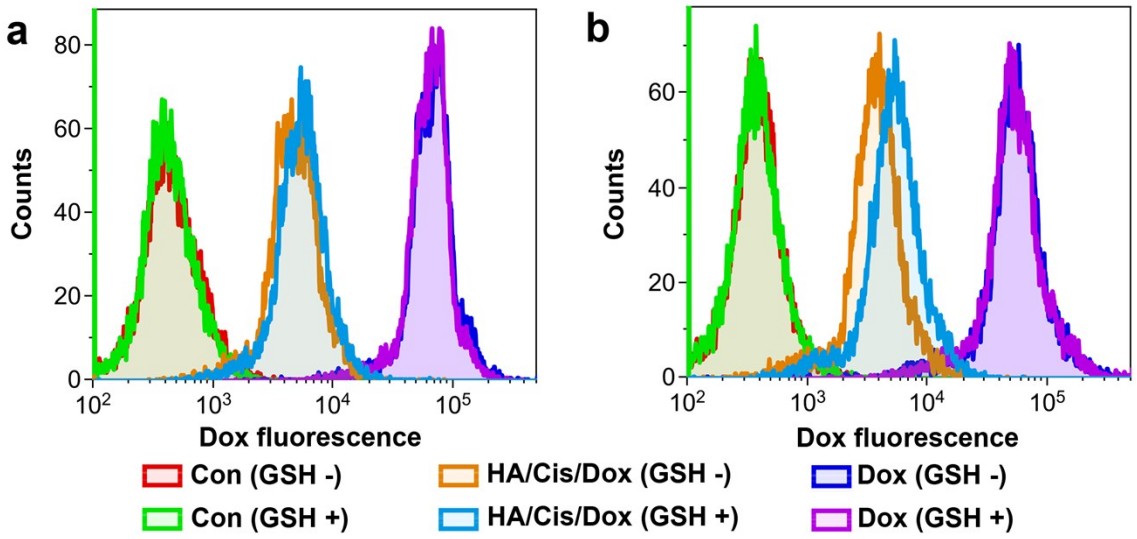

Fig. S5 Representative graphs of FACS analysis of cells with different treatments. a) A2780 cells. b) A2780cis cells. Cells were incubated with different formulations in absence and presence of GSH (1 mM) for 8 hours. Dox concentration was set at 13.5 μM.

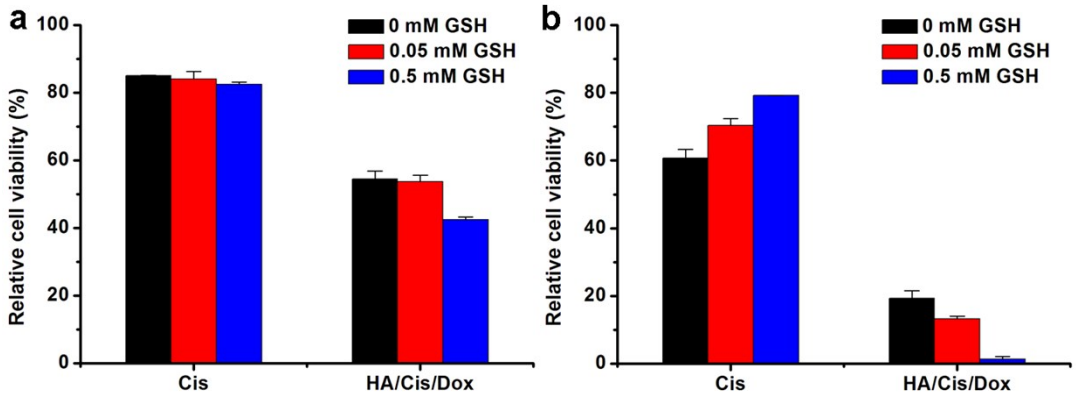

Fig. S6 Cytotoxicity of Cisplatin and HA/Cis/Dox in presence of GSH. A2780cis cells were incubated with free cisplatin or HA/Cis/Dox in presence of GSH (0, 0.05 and 0.5 mM) for 2 days. The cisplatin concentration was set at (a) 16 μM or (b) 32 μM.