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Platinum (IV) Prodrugs with Long Lipid Chains for Drug Delivery and Overcoming Cisplatin Resistance

Supporting Information

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Materials and methods

Materials

Methoxyl - poly (ethylene glycol) - block - poly (lactic acid) ($mPEG_{5000}$-b-PLA_{6000}) was synthesized as previously reported by Jing and his coworkers from State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, China[1]. Hydrogen peroxide (H_2O_2), hexadecyl isocyanate, sodium azide, chlorpromazine, genistein and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Aladdin, Shanghai, China. Cisplatin and oxaliplatin (purity 99%) were bought from Kunming Institute of Precious Metals, Yunnan, Chian. Annexin V-FITC apoptosis detection kit was purchased from Beyotime Institute of Biotechnology, Jiangsu,

China. 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI), propidium iodide, Rhodamine B (RhB) and glutathione were purchased from Sigma-Aldrich, Shanghai, China.

General measurements

^{1}H NMR spectra were measured by a 400 MHz NMR spectrometer (Bruker) at room temperature. Matrix-assisted laser-desorption ionization and time-of-flight mass spectroscopy (MALDI-TOF-MS, Waters, USA) was used to determine the mass spectrometry. Inductively coupled plasma mass spectrometry (ICP-MS, Xseries II, Thermoscientific, USA) and Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA) were used for quantitative analysis the total platinum contents in the nanoparticles, *in vitro* drug release samples and cells. The morphology and size of nanoparticles were obtained by Transmission electron microscopy (TEM) carried out on a JEOL JEM-1011 electron microscope. Localization of RhB labeled M(CisPt) were performed on confocal laser scanning microscope (FLV-1000, Olympus, Japan). Size and zeta potential measurements were conducted on a Malvern Zetasizer (Nano ZS, UK).

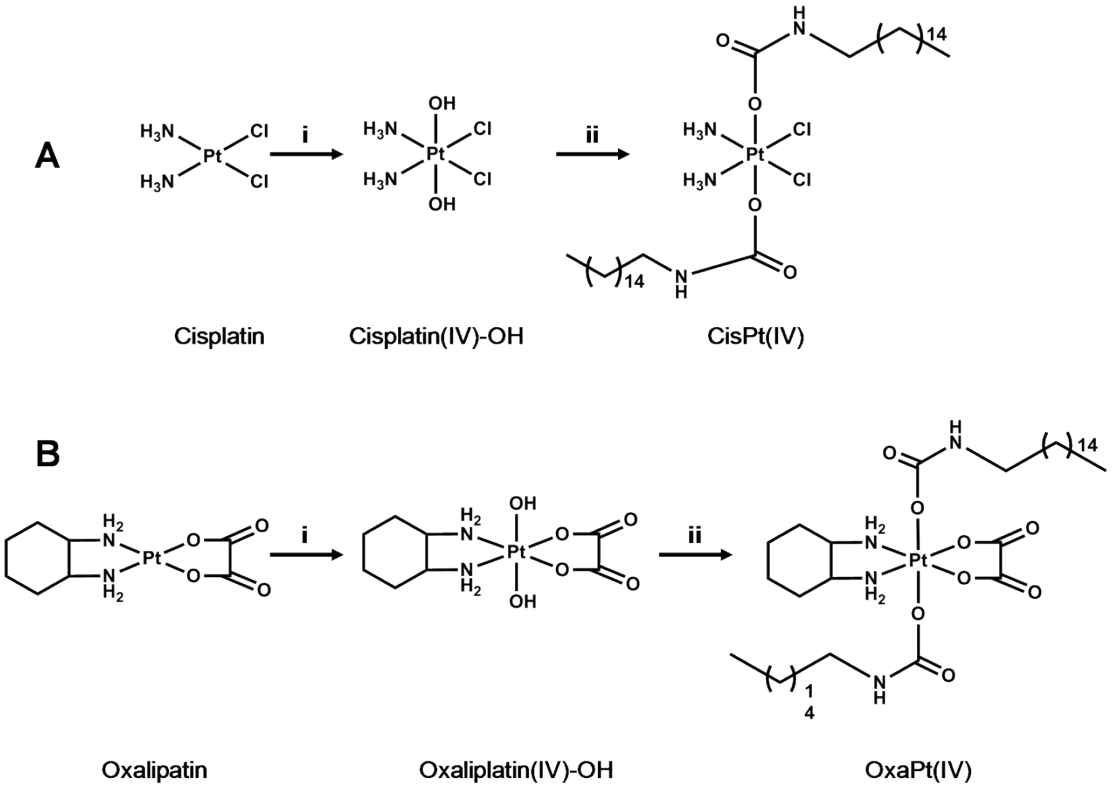


Figure S1. The synthesis of Pt(IV). The synthesis of CisPt(IV) (A) and OxaPt(IV) (B).

Synthesis of Cisplatin(IV)-OH

Cisplatin (0.5 g, 1.65 mmol) was suspended in H_2O_2 (30% w/v, 20 mL). The mixture was stirred at 50 ^oC overnight and clear solution resulted. After cooled down to room temperature, a large amount of needle-like crystal was precipitated. The product was washed several times with acetone and dried with a desiccator. The Cisplatin(IV)-OH was isolated and yielded79%. ESI-MS (positive mode) for $Cl_2H_8N_2O_2Pt$: *m/z* $[M+H]^+$ Calcd: 332.96, Found: 333.0.

Synthesis of Oxaliplatin(IV)-OH

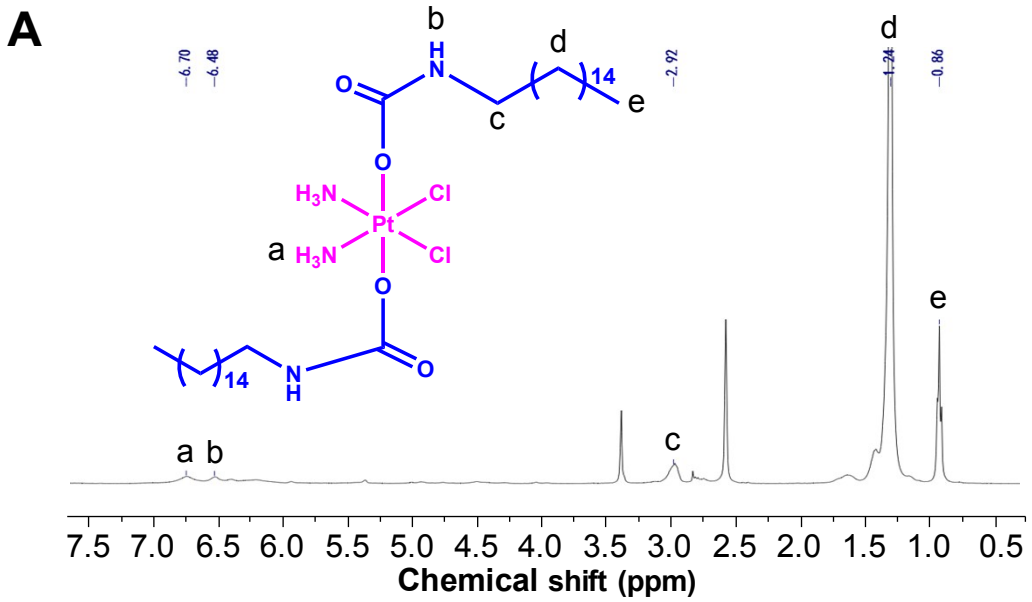
Similarly, oxaliplatin (0.5 g) was suspended in H_2O_2 (30% w/v, 20 mL). The mixture was stirred at 50 ^oC overnight and clear solution resulted. After cooled down to room temperature, a large amount of needle-like crystal was precipitated. The resulted product was washed several times with acetone and dried with a desiccator. The Oxaliplatin(IV)-OH was isolated and yielded 81%. ESI-MS (positive mode) for Oxaliplatin(IV)-OH: *m/z* $[M+H]^+$ Calcd: 431.07, Found: 431.31.

Synthesis of CisPt(IV)

Cisplatin(IV)-OH (400 mg, 1.2 mmol) was suspended in 5mL anhydrous DMF. Then hexadecyl isocyanate (0.744 mL, 2.4 mmol) was added to the above mixture, and reacted at 110 ^{o}C under stirring until a clear yellow solution resulted. The solution was poured into ice water to precipitate the crude product. The crude product was washed for several times with acetone and diethyl ether and dried under the vacuum to obtain CisPt(IV) (28%) as light yellow solid. ^{1}H NMR (DMSO-d_6): δ=6.70 (m, 6H, 2NH_3), δ=6.48 (d, 2H, 2NHcarbamate), δ=2.92 (m, 4H), δ=1.24 (m, 56H), δ=0.86 (t, 6H); ESI-MS (negative mode) for $C_{34}H_{74}Cl_2N_4O_4Pt$: *m/z* $[M-H]^-$ Calcd: 867.47, Found: 867.4. IR (KBr): 2901, 2800, 1650, 1600, 1560, 1310, 790, 720 cm^{-1}.

Synthesis of OxaPt(IV)

Similar with the synthesis of CisPt(IV), OxaPt(IV) yielded 36% as a light yellow solid. ^{1}H NMR (DMSO-d_6): δ=6.70 (m, 4H, 2NH_2), δ=6.24 (d, 2H, 2NHcarbamate), δ=2.92 (m, 4H), δ=2.76 (2H), δ=2.22 (m, 4H), δ=2.19 (m, 4H), δ=1.24 (m, 56H), δ=0.86 (t, 6H); ESI-MS (negative mode) for $C_{34}H_{74}Cl_2N_4O_4Pt$: *m/z* $[M-H]^-$ Calcd: 964.57, Found: 965.8. IR (KBr): 2980, 2850, 1740, 1650, 1600-1500, 1480, 1370, 1280, 800, 720 cm^{-1}.



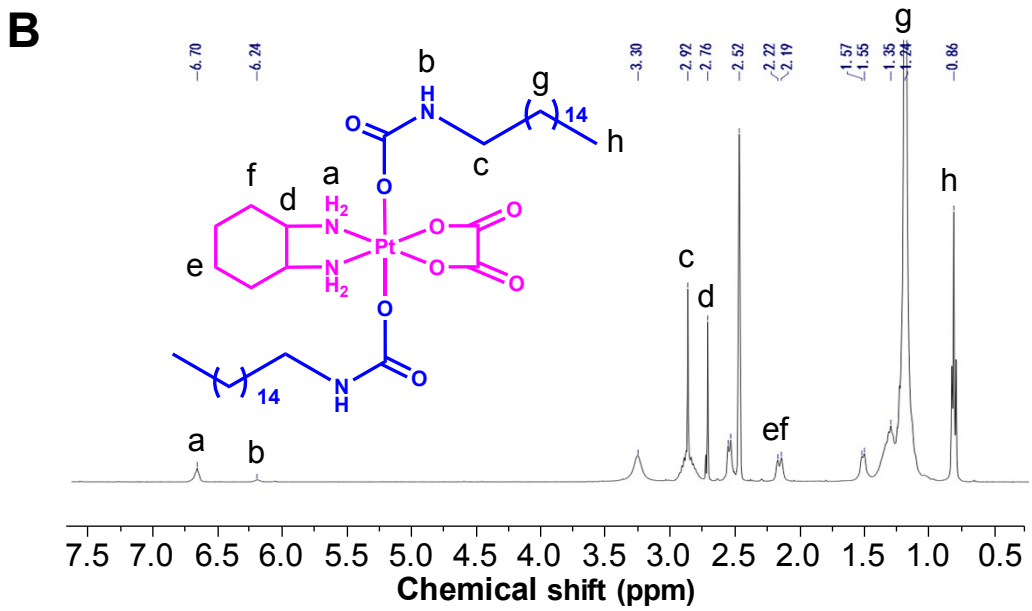
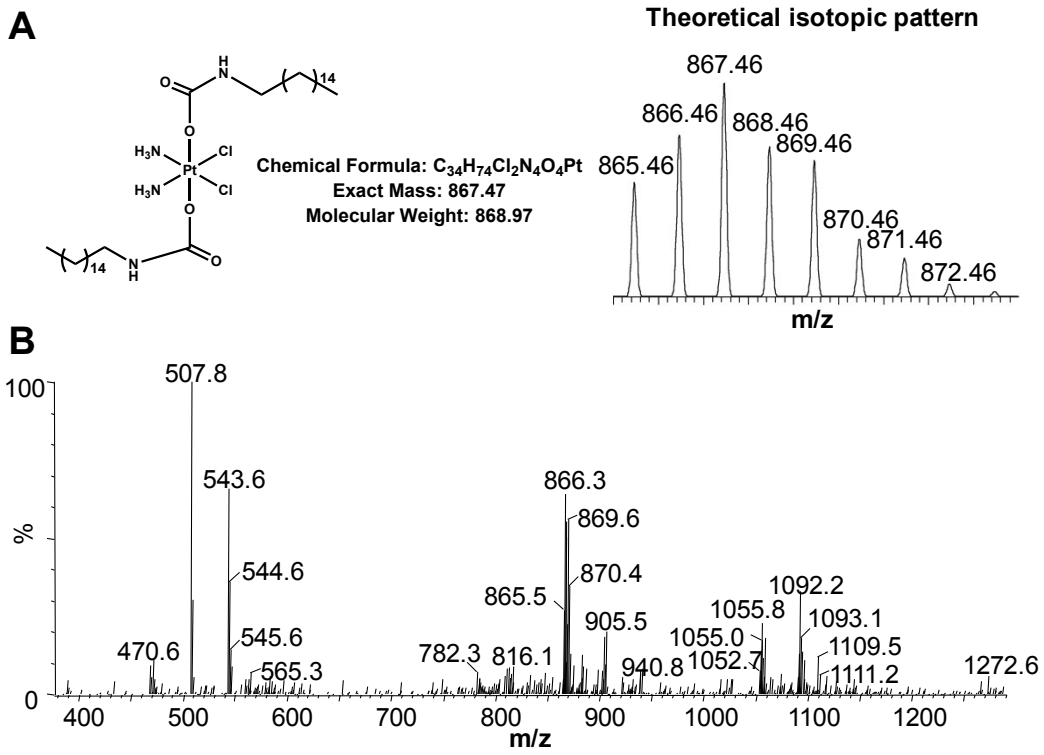


Figure S2. Characterization of Pt(IV) by ^{1}H NMR. The ^{1}H NMR spectra of CisPt (IV) (A) and OxaPt (IV) (B) in the mixture of DMSO-d_6 and $CDCl_3$ (6:1, V/V).



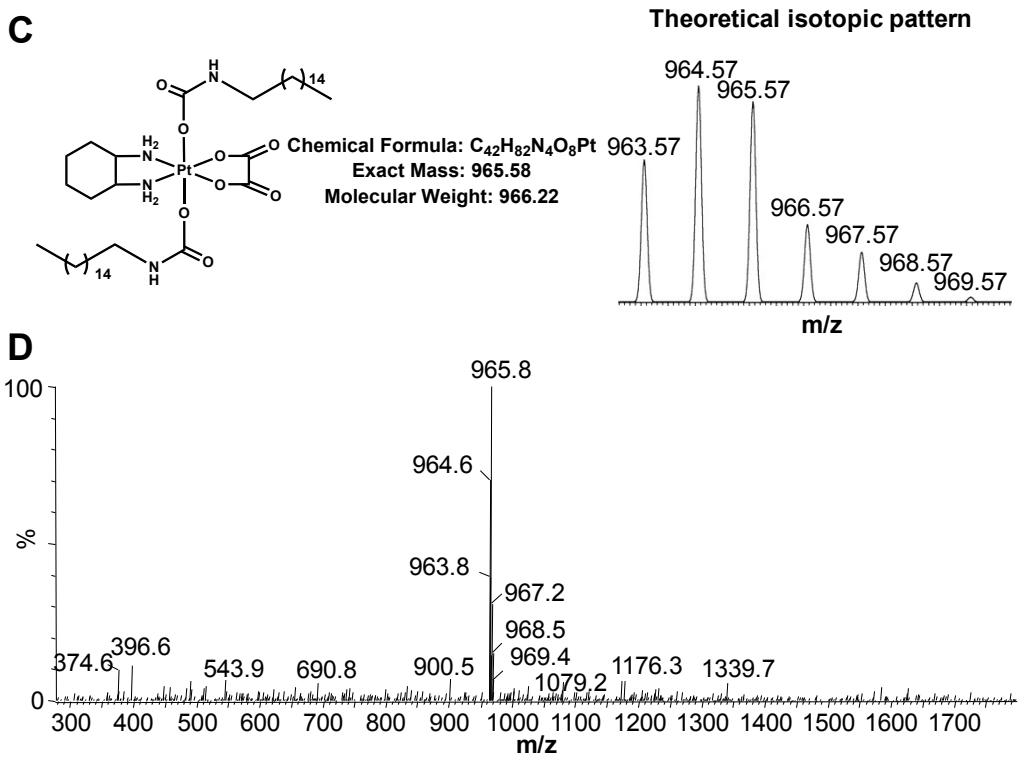


Figure S3. Characterization of Pt(IV) by ESI-MS. Simulated mass by Chemical Draw software and Isotope Viewer software (left panel) and their simulated isotopic pattern (right panel) of CisPt(IV) (A) and OxaPt(IV) (C); ESI-MS spectra of CisPt(IV) (B) and OxaPt(IV) (D) in negative mode.

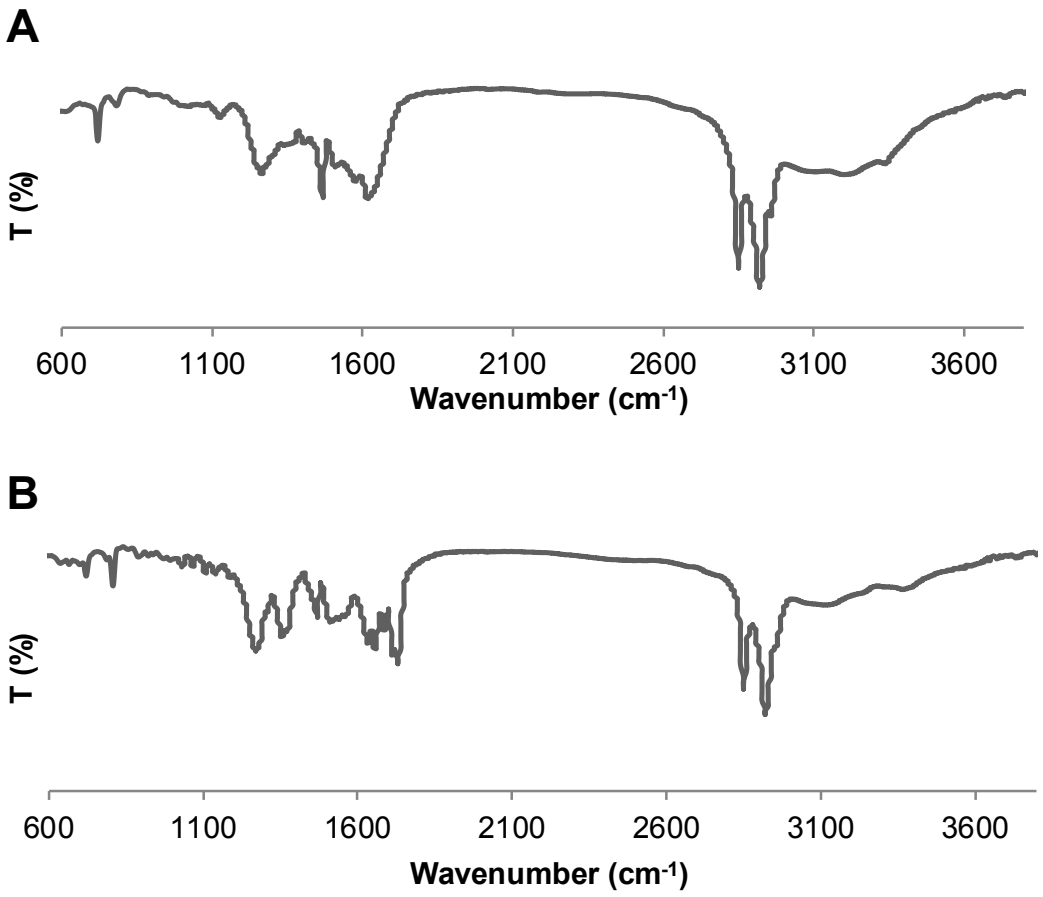


Figure S4. Characterization of Pt(IV) by IR spectra. The IR spectra of CisPt(IV) (A) and OxaPt(IV) (B) in KBr pellet.

Nano-micelle formulation of M(CisPt) and M(OxaPt)

The micellar nanoparticles of CisPt or OxaPt (M(CisPt) or M(OxaPt)) were prepared by a nano-precipitation method[2]. Briefly, the DMF (5mL) solution of Pt(IV) (2 mg) and $mPEG_{5000}$-b-PLA_{6000} (10 mg) were added de-ionized water (10 mL) dropwise. The mixture was dialyzed in a dialysis bag (MWCO: 3500 Da) for 48 h and lyophilized to obtain a white powder.

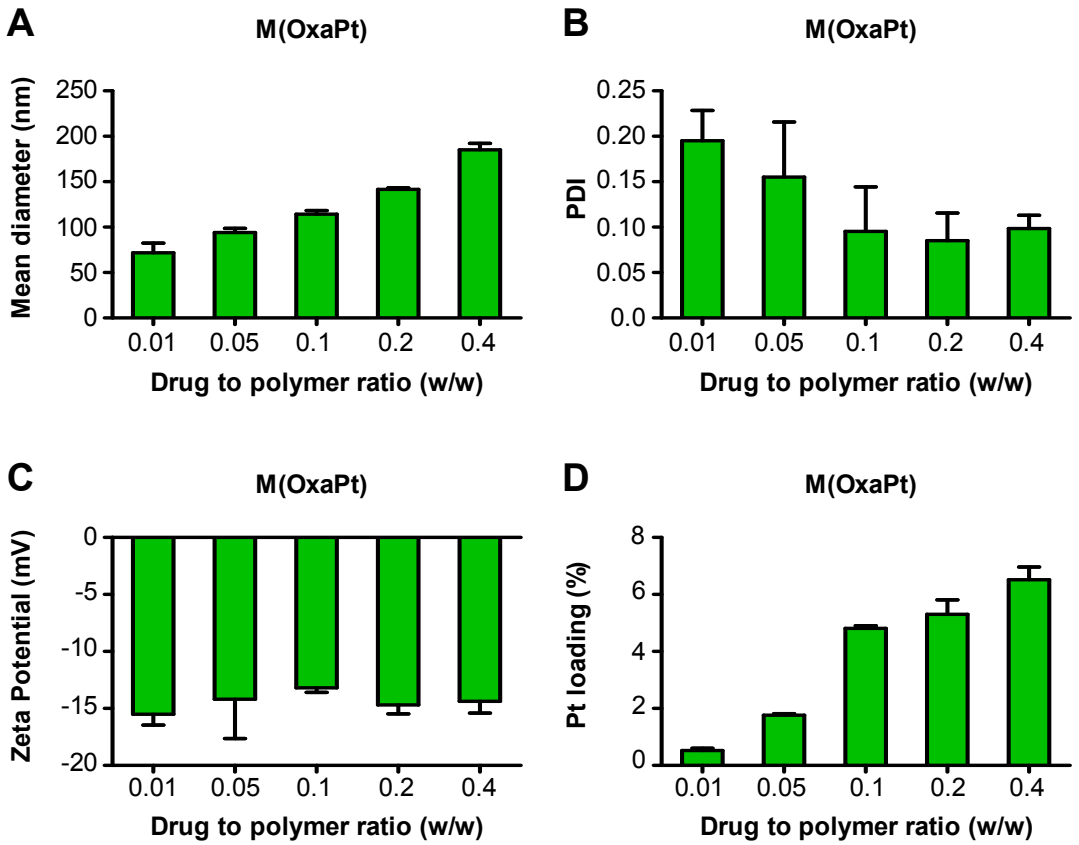


Figure S5. Formulation optimization of the micelles of M(OxaPt). Mean diameter (A), PDI (B) Zeta potential (C) and Pt loading at various drug to polymer ratios (D) of M(OxaPt) were shown.

Drug Release of M(CisPt)

In vitro release study at different media was described as following: M(CisPt) (5 mg) was dissolved in PBS (10 mL, pH 7.4), PBS (10 mL, pH 5.0), or PBS (10 mL, 10 mM GSH). Then every solution was transferred into a pre-swelled dialysis bag (MWCO: 3500 Da), which was then immersed into 100 mL corresponding media (PBS pH7.4, pH5.0 or with 10 mM GSH respectively) in a shaking culture incubator at 37 °C. At certain points in time, 1.5 mL of sample solution was withdrawn from the dialysate and fresh PBS (1.5 mL) was immediately added into the dialysate. All the samples were tested by ICP-OES. The platinum released from the micelles was expressed as the percentage of cumulative platinum in the dialysate to the total platinum in the micelles.

Cell lines and cell incubation conditions

The cisplatin sensitive ovarian cancer cell line A2780, cisplatin resistant ovarian cancer

cells A2780DDP were kindly supplied by the Medical Department of Jilin University in China and cultured in RPMI 1640 media (HyClone, USA) supplemented with 10% (V/V) fetal bovine serum (FBS, HyClone, USA), 100 U/mL penicillin and 100 ug/mL streptomycin (Ameresco) at 37 °C with 5% (V/V) CO_2 atmosphere.

Cell viability studies

The cells harvested in logarithmic growth phase were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated in for at least 12 h. The medium was then replaced with culture medium contained platinum-based drugs (cisplatin, oxaliplatin, M(CisPt) and M(OxaPt)) (concentration range based on Pt: 0.00125 to 50 μM for cisplatin and M(CisPt), 0.00625 to 50 μM for oxaliplatin and M(OxaPt) respectively). Incubating for a certain time, 5 mg/mL MTT solution in PBS was added and the plates were incubated for another 4 h at 37 °C. Followed by removal of the culture medium containing MTT, 150 μL of DMSO was added to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 min, and the absorbance of formazan product was measured at 492 nm by a microplate reader (Infinite M1000 Pro, Tecan, Switzerland). Cell viability was expressed as a percentage of OD value of the test well to the control well, and data are shown as the mean ± standard deviation (S.D.).

Platinum uptake in the cell

A2780 or A2780DDP cells (1×10^6) were seeded in 6-well plates and cultured for at least 12 h. Then the cells were treated with cisplatin, oxaliplain, M(CisPt) and M(OxaPt) (concentration: 40 μM Pt) in the culture medium at 37 °C for 5 h and 9 h. The cells were then washed with PBS for three times and collected to lyse with cell lysis buffer. Thereafter, the Pt content in the cancer cells was measured by ICP-OES.

Platinum uptake inhibition by various inhibitors

A2780 (1×10^6) were seeded in 6-well plates and cultured for at least 12 h. Then the cells were treated with medium containing sodium azide (10 mM), genistein (200 μg/ml) and chlorpromazine (50 μg/mL) at 37 °C respectively with M(CisPt) (40 μM Pt) for 4 h. The uptake with no inhibitors at 37 °C was set as a positive control and the group treated at 4 °C was set as a negative control. The cells were then washed with PBS three times and then harvested to

lyse with cell lysis buffer. Thereafter, the Pt content in the cancer cells was measured by ICP-MS.

Platinum uptake in the cell

A2780 (1×10^6) were seeded in 6-well plates and then treated with M(CisPt/RhB) with the Pt concentration in the culture medium of 40 μM at 37 °C for 2 h and 4 h. The cells were then washed with PBS three times and digest the cells with trypsin. The harvested cells were re-suspended twice in ice-cold PBS buffer (pH 7.4). After two cycles of washing and centrifugation, cells were resuspended with 500 μL of PBS. Flow cytometry was completed a FACS calibur flow cytometer (Becton Dickinson and Company, USA).

Cell cycle analysis

The population of cells in different phases of the cell cycle was detected by flow cytometer. Cell cycle distribution was determined by staining DNA with PI. A2780 cells were seeded in 6-well plates at a density of 1×10^6 cells/well and cultured for at least 12 h. After treatment with M(CisPt) (0.1 μM) for 48 h, cells were collected, washed and re-suspended in ice-cold PBS buffer (pH 7.4) for two cycles. After fixed with 50% alcohol at 4 °C overnight, the cells were stained with PI (1 mg/mL) and RNase A (10 mg/mL) for at least 30 min at room temperature. The percentages of cells in specific phases of the cell cycle were determined by a FACS calibur flow cytometer (Becton Dickinson and Company, USA).

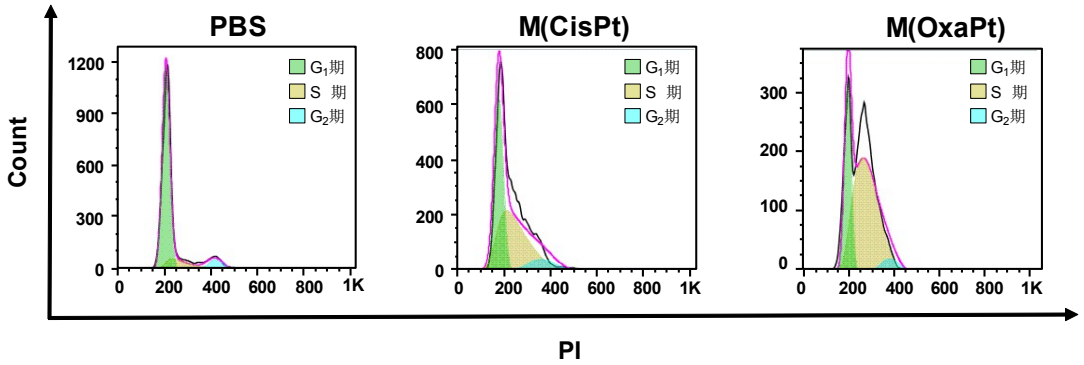


Figure S6. Cell cycle arrest induced by micelles of Pt(IV). Cell cycle arrest induced by PBS, M(CisPt) and M(OxaPt) for 48 h of A2780.

Apoptosis analysis

A2780 and A2780DDP cell apoptosis induced by M(CisPt) and M(OxaPt) was conducted using Annexin V/PI staining. The extent of apoptosis was measured with the annexin V-FITC apoptosis detection kit following the manufacturer's instruction. The samples were finally analyzed with MultiCycle software.

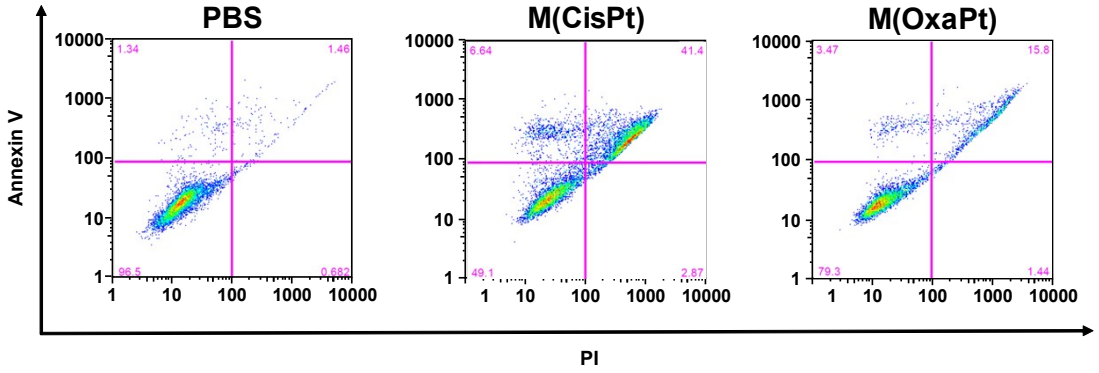


Figure S7. Flow cytometry apoptosis analysis of A2780 cells. A2780 cell apoptosis were induced by PBS, M(CisPt) and M(OxaPt) after 72 h and the treated cells were stained with Annexin V/PI.

References

1. J. Yue, X. Li, G. Mo, R. Wang, Y. Huang and X. Jing, *Macromolecules*, 2010, **43**, 9645-9654.
2. S. Dhar, F. X. Gu, R. Langer, O. C. Farokhzad and S. J. Lippard, *Proceedings of the National Academy of Sciences of the United States of America*, 2008, **105**, 17356-17361.