Electronic Supplementary Information (ESI)

A Negatively Charged Pt(IV) Prodrug for Electrostatic Complexation with Polymers to Overcome Cisplatin Resistance

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1 Experimental materials and instruments

Materials

Methoxyl - poly (ethylene glycol) - block - poly (polylysine) (mPEG-b-PLL) was gifted by Professor Xuesi Chen (Key Laboratory of Polymer Ecomaterials Changchun Institute of Applied Chemistry). Cisplatin, sodium azide (NaN_3), sodium ascorbate (NaVc), chlorpromazine, methyl-β-cyclodextrin, wortmannin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Aladdin (Shanghai, China). 2-sulfobenzoic anhydride was purchased from TCI (Shanghai, China). Dodecyl isocyanate was purchased from Sigma (China). Hydrogen peroxide, *N, N*-Dimethylformamide (DMF), and acetonitrile were purchased from Beijing Chemical Works (Beijing, China). 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) was purchased from Sigma-Aldrich (Shanghai, China). Annexin-V-FITC apoptosis detection kit was purchased from Solarbio (Beijing, China). DMEM, RPMI 1640, fetal bovine serum (FBS), penicillin-streptomycin solution and trypsin were purchased from GIBCO (Beijing, China).

Instruments

Dynamic light scattering (DLS) was performed by Malvern Zetasizer NanoZS90. The transmission electron microscopy (TEM) were accomplished by using JEM-1011 electron microscope operated at 100 kV. All OD values were measured by SpectraMax M3. Flow cytometry was conducted by

Cytomics FC500 Flow Cytometry (Beckman Coulter Ltd.). Confocal laser scanning microscopy (CLSM) was performed with ZEISS LSM880. ^{1}H NMR spectra was recorded in DMSO-d_6 on a 400 MHz NMR spectrometer (Bruker) at 298 K. Inductively coupled plasma mass spectrometry (ICP-MS) was performed by Agilent technologies 7700 series. High resolution mass spectrometry (HR-MS) was conducted by Agilent 1290 UPLC/6540 Q-TOF; Reverse-phase high-performance liquid chromatography (RP-HPLC) was recorded by an Agilent 5 TC-C18(2) (4.6×250 mm) column. Eluent A: C_2H_3N, Eluent B: H_2O.

Synthesis of *Pt(IV)-OH [Pt(IV)-1]*

As reported[1], cisplatin (300 mg, 1 mM) was suspended in H_2O_2 (30% w/v, 36 mL). The mixture was placed in 55 °C and stirred for 4 h, and then the temperature was increased to 100 °C until the solution turned to clarified. Subsequently, the mixture was moved to 4 °C overnight after cooling down to room temperature, a large amount of yellow-green crystal was precipitated. The product was washed by acetone, ether and H_2O in turn and dried in vacuum oven. The Pt(IV)-1 was obtained and yielded 95%. ESI-MS (positive mode) for $Cl_2H_8N_2O_2Pt$: *m/z* $[M+H]^+$ Calcd: 332.96, Found: 333.0.

Synthesis of Pt(IV)-SO_3^- [Pt(IV)-2]

2-sulfobenzoicanhydride (184 mg, 1 mM) was added to Pt(IV)-1 (334 mg, 1 mM) and suspended in 10 mL DMF and the mixture was stirred at 30°C for

24 h. Clarified yellow solution was obtained. The crude product was purified by flash column chromatography on silica gel (DCM: MeOH =5:1) to give the product as a yellow solid (263 mg, 51 %). ^{1}H NMR: (400 MHz, DMSO-d_6, 25 °C): δ_H 7.71 (1 H, m), 7.41 (2 H, m), 7.23 (1 H, d, *J* 7.0), 6.64 (6 H, s).

Synthesis of C_{12}-Pt(IV)-SO_3^- [Pt(IV)-3]

Dodecyl isocyanate (105.5 mg, 0.5 mM) was added to Pt(IV)-2 (258 mg, 0.5 mM) and suspended in 10 mL DMF. After stirring at 65 °C for 12 h, the crude product was purified by flash column chromatography on silica gel (DCM: MeOH =20:1) to give the product as a yellow solid (152 mg, 42%). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ_H (ppm) 8.19 (1 H, s), 7.74 (1 H, s), 7.40 (2 H, dd, *J* 13.9, 7.4), 7.29 (1 H, d, *J* 6.9), 6.07-5.48 (5 H, m), 3.07 (2 H, d, *J* 20.7), 1.31 (19 H, d, *J* 51.8), 0.85 (3 H, d, *J* 6.9). HR-MS: calcd for $C_{20}H_{36}Cl_2N_3O_7PtS^-$: *m/z* calcd: 728.14 [$C_{20}H_{37}Cl_2N_3O_7PtS$]; found: 728.12926 [$C_{20}H_{37}Cl_2N_3O_7PtS$].

Preparation process of nanoparticles

As the following description, Pt(IV)-3 (10 mg) and mPEG-b-PLL (11 mg) were dissolved in DMF (1 mL) and H_2O (1 mL) respectively, mixed and stirred for 15 min, and then the de-ionized water (10 mL) was added dropwise to the mixture subsequently. Finally, the mixture was collected and dialyzed against a dialysis bag (MWCO: 3500 Da) for 48 h, and the supernatant was collected by centrifugation (4000 r, 5 min).

Study of the reaction of 5'-GMP with cisplatin and Pt(IV)-3 by MALDI/TOF-

MS

5'-GMP (2 mM, 50 μL) was used to react with cisplatin (1 mM,50 μL) or Pt(IV)-3 (1 mM, 50 μL) at 37 °C for 24 h. The products were studied by MALDI/TOF-MS (matrix: 2, 5-dihydroxybenzoic acid (DHB)).

Drug release of NPs in vitro studies

Drug release of NPs was studied at pH 7.4, pH 5.0 and in the presence of 5 mM NaVc respectively. Solutions of different pH were prepared in PBS. Nanoparticles solution of 1 mL was taken into dialysis bag (MWCO: 3500 Da) and then immersed in those solutions of different pH as soon as possible. Next, the systems were placed into an incubator shaking at 37 °C. At each specific point in time, 2 mL of sample solution was collected and replenish a considerable volume of solution immediately. All the samples were examined by ICP-MS. The cumulative Pt release was expressed as the percentage of the cumulative Pt in the dialysate to the total platinum in the nanoparticles.

Cell lines and cell incubation conditions

A549, A549/DDP (cisplatin resistant), A2780, and MCF7 cells were used in the following experiments. A549, A549/DDP, and MCF-7 cells were cultured in DMEM media, while A2780 cells were cultured in RPMI 1640 media. Culture medium were supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) 10 kU/mL penicillin and 10 mg/mL streptomycin. The cell lines were cultured in 37 °C with 5% (v/v) CO_2 atmosphere.

Preparation of Rhodamine B (RhB) loaded NPs [RhB@NPs]

RhB loaded NPs [RhB@NPs] were prepared. RhB (2 mg) and Pt(IV)-3 (10 mg) were dissolved in DMF (10 mL), and mPEG-b-PLL was dissolved in H_2O, then got the mixture stirred for 15 min, and the de-ionized water (10 mL) was added dropwise to it subsequently. Finally, the mixture was collected and dialyzed against a dialysis bag (MWCO: 3500 Da) for 48 h, and the supernatant was collected by centrifugation (4000 r, 5 min).

Intracellular uptake studies

Intracellular uptake studies of confocal laser confocal scanning microscopy (CLSM) The cell slides were used to put on the bottom of the six-well plates in advance, 2×10^4 A549 or A549/DDP cells in 2 mL media were added to each well and incubated in 37 °C for 12 h subsequently. Then the cells were treated with [RhB@NPs] at a final concentration of 2 μg/ml of RhB for 1 h, 4 h and 7 h. After washed with cold PBS, the cells were fixed with paraformaldehyde and nuclei were stained with DAPI. At last, images were performed with CLSM.

Intracellular uptake studies of flow cytometry A549/DDP cells were seeded in six-well plates at a density of 2×10^5 per well and incubated at 37 °C for 12 h. Then the cells were treated with [RhB@NPs] at a final concentration of 2 μg/ml of RhB for 1 h, 4 h and 7 h. Finally, the cells were harvested to examine by flow cytometry.

Platinum uptake in the cells

A549 and A549/DDP cells were seeded in six-well plates at a density of 1×10^6 cells per well and incubated at 37 °C for 12 h. Cisplatin and NPs were

treated with the cells at a final concentration of 20 μM of Pt. After treatment for 1 h, 4 h and 7 h, the cells were harvested in EP tubes and ICP-MS was performed to examine the Pt concentrations.

Platinum uptake inhibition in the cells

A549/DDP cells were seeded in six-well plates at a density of 1×10^6 cells per well and incubated at 37 °C for 12 h. Inhibitors chlorpromazine (CL) (20 μg/ml), methyl-β-cyclodextrin (Me-β-CD) (200μM), wortmannin (0.2 μM) and NaN_3 (120 mM) were added in plates and incubated for 2 h. The media was replaced with fresh media after the cells were washed 3 times with cold PBS. NPs were treated with the cells at a final concentration of 20 μM of Pt. Particularly, two groups of the cells were placed in 4 °C and 37 °C without inhibitors treatment, the other groups were placed in 37°C. After incubated for 4 h, the cells were harvested in EP tubes and ICP-MS was performed to examine the Pt concentrations.

Pt-DNA adducts in the cells

A549/DDP cells were seeded in six-well plates at a density of 1×10^6 per well and incubated at 37 °C for 12 h. Cisplatin and NPs were added to the wells at a final concentration of 20 μM. After treatment in 37 °C for 1 h, 4 h, and 7 h, the cells were washed three times with cold PBS, and DNA extraction kit (Solarbio) was used to extract DNA. DNA contents were tested by Micro spectrophotometer. Finally, 10 μL DNA was dissolved in EP tubes with 2 mL deionized water and ICP-MS was performed to examine the Pt concentrations.

Cell relative viability studies

MTT assay was used to examine the cell relative viability. A549, A549/DDP, A2780, and MCF7 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated at 37 °C for 12 h. Cells were treated with cisplatin, Pt(IV)-3 and NPs with final concentrations of 0.005, 0.05, 0.5, 5, 10, 20, 40 μM of Pt respectively. After treatment for 48 h or 72 h, 10% MTT diluted with DMEM was added in the wells. After incubation in 37 °C for 4 h, 10% SDS was added in the wells and cells were incubated in 37 °C for 12 h in the dark. The results were tested by Molecular Devices. Cell viability was expressed as the ratio of the absorbance of the test wells and control wells, and datas are shown as the mean ± standard deviation (S.D.).

Apoptosis studies

A549/DDP cells were seeded in six-well plates at a density of 2×10^5 per well and incubated at 37 °C for 12 h. Cisplatin, Pt(IV)-3 and NPs were added in the wells at the final concentration of 20 μM of Pt. After treatment for 24 h, the media was removed, and the cells were washed 3 times with cold PBS. Then the cells were harvested and strained with 5 μL FITC and 5 μL PI for 10 min in the dark at room temperature respectively. Finally, all the samples were tested by flow cytometry in 1 h.

Cell cycle studies

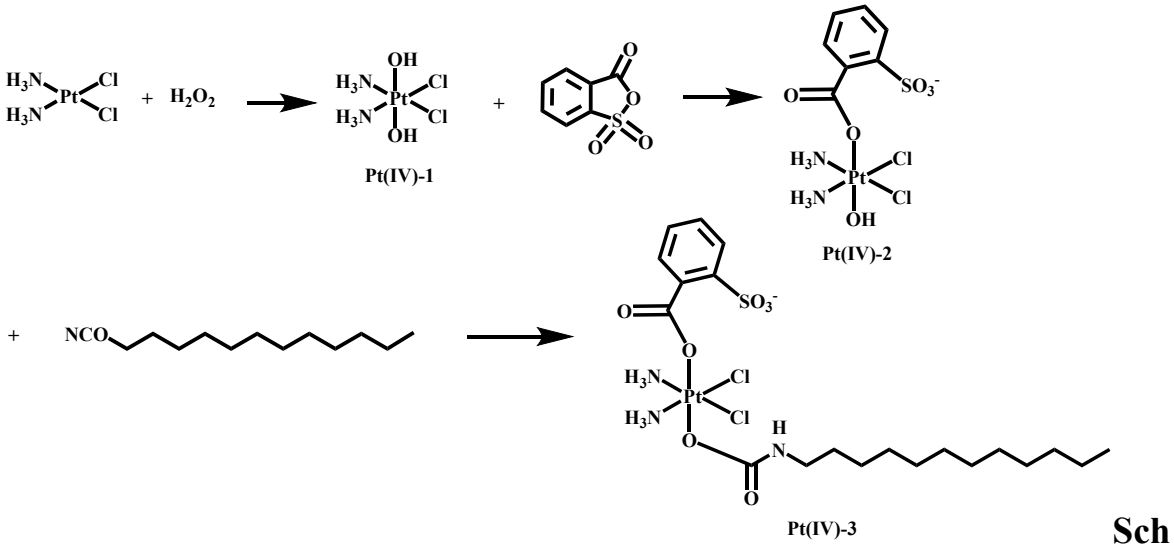
A549/DDP cells were seeded in six-well plates at a density of 2×10^5 cells per well and incubated at 37 °C for 12 h. Cisplatin, Pt(IV)-3 and NPs were added

in the wells at the final concentration of 20 μM of Pt. After treatment for 24 h, the media was removed, and the cells were washed three times with cold PBS. After fixed with 70% ethanol at 4 °C for 12 h, the cells were harvested and treated with RNAse (100 μg/ml) and propidium iodide (100 μg/ml) at 4 °C for 30 min. Finally, all the samples were tested by flow cytometry in 1 h.

References

1 T. Johnstone and S. Lippard, *J. Biol. Inorg. Chem.*, 2014, **19**, 667.

2 Supplementary figures



Scheme S1. Synthetic routes of Pt(IV)-3 prodrug.

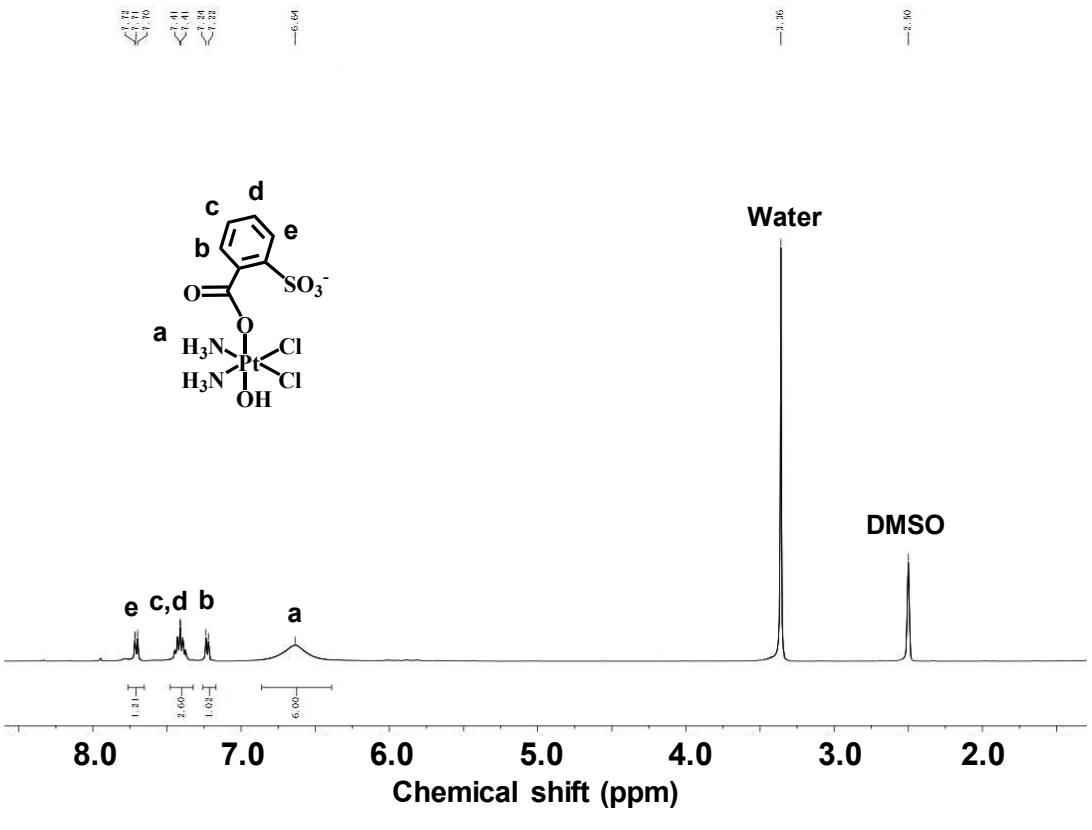


Fig. S1 ^{1}H NMR spectrum of Pt(IV)-2 prodrug in DMSO-d_6.

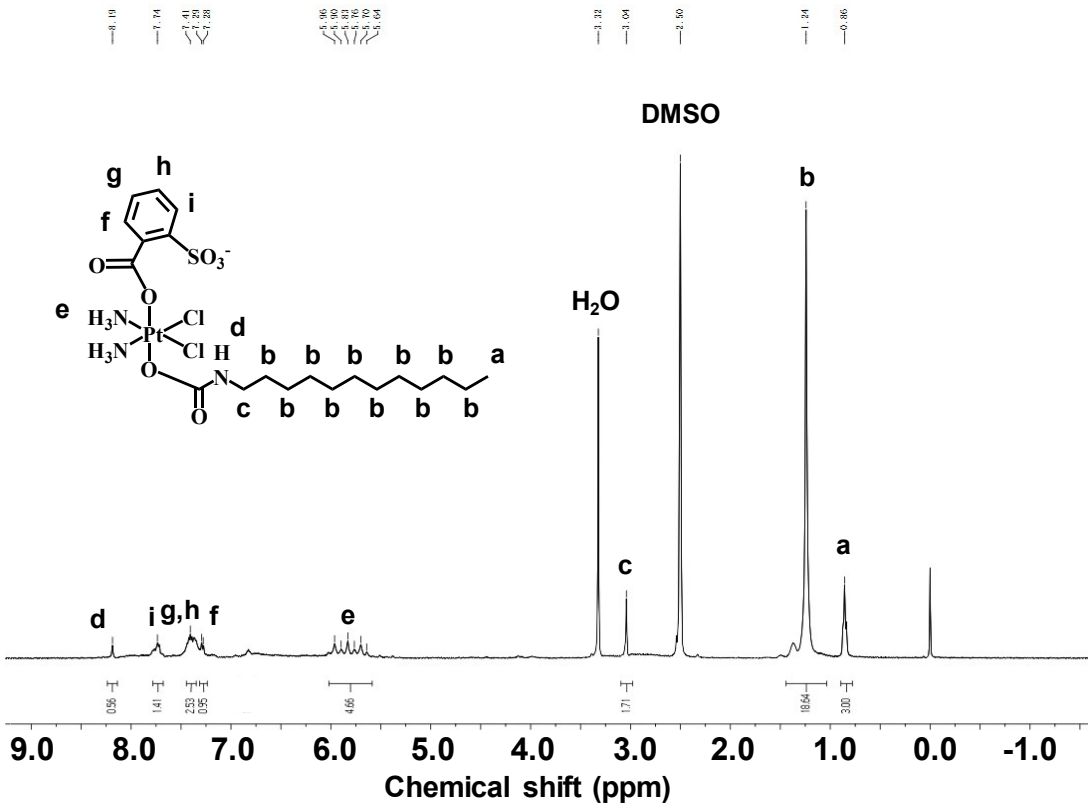


Fig. S2 ^{1}H NMR spectrum of Pt(IV)-3 prodrug in DMSO-d_6.

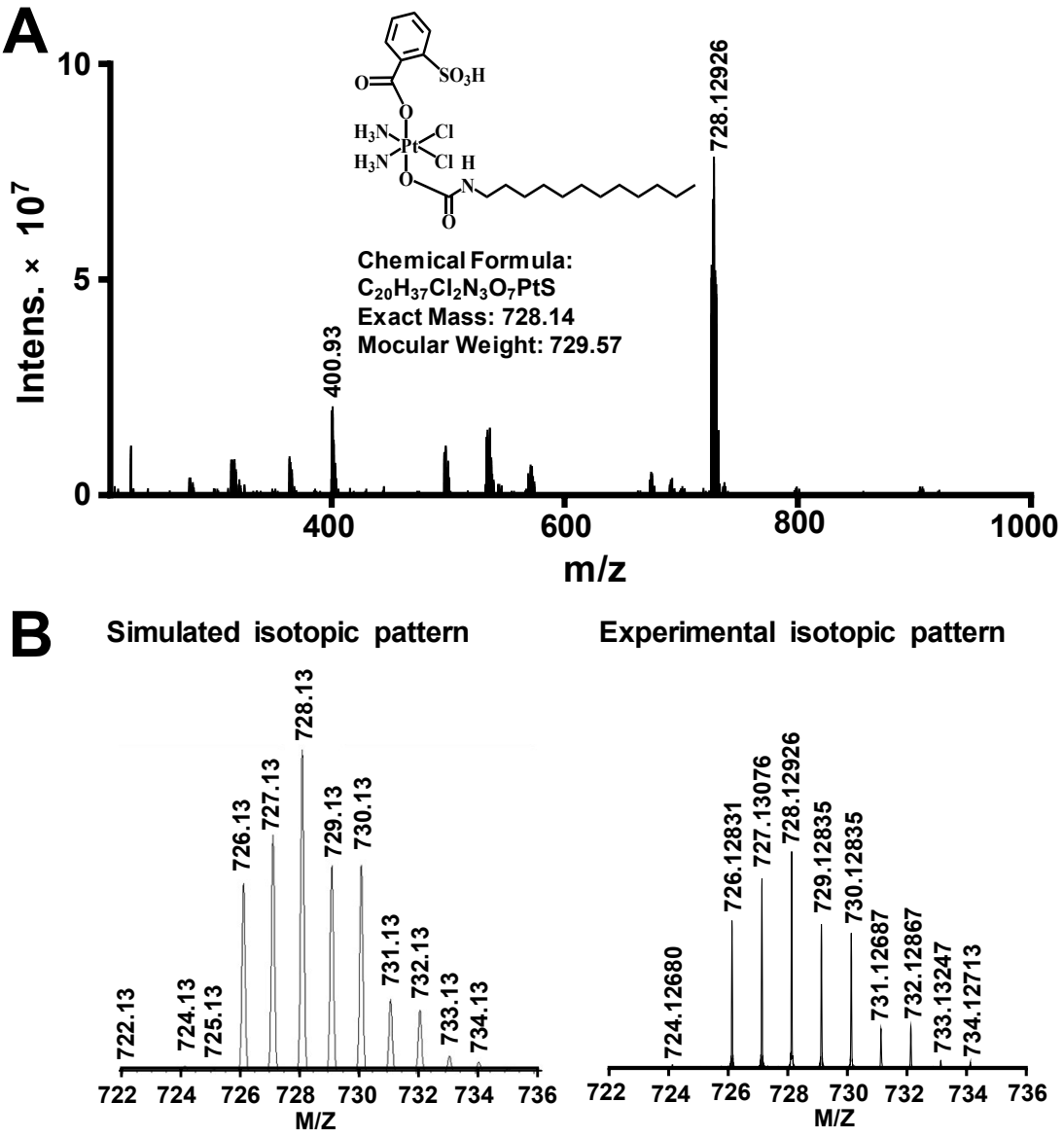


Fig. S3 Characterization of Pt(IV)-3 prodrug by ESI-MS (negative mode) (A). The simulated isotopic and experimental patterns of corresponding compound

(B).

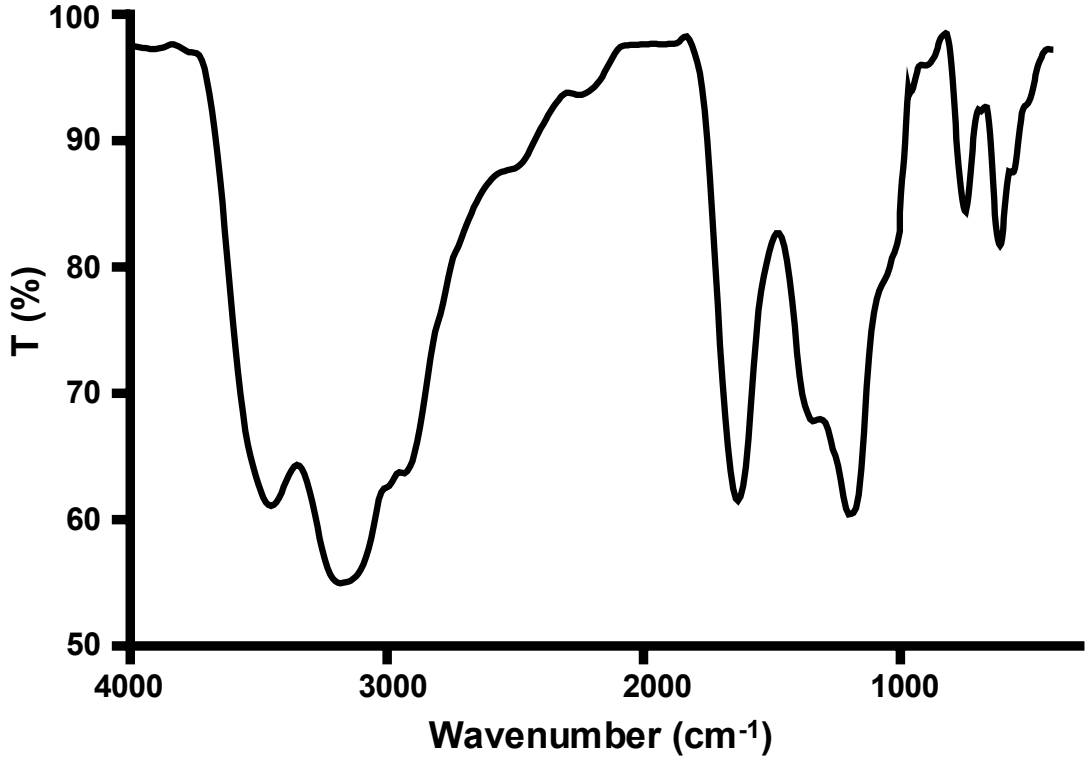


Fig. S4 Characterization of Pt(IV)-3 prodrug by FITR spectra.

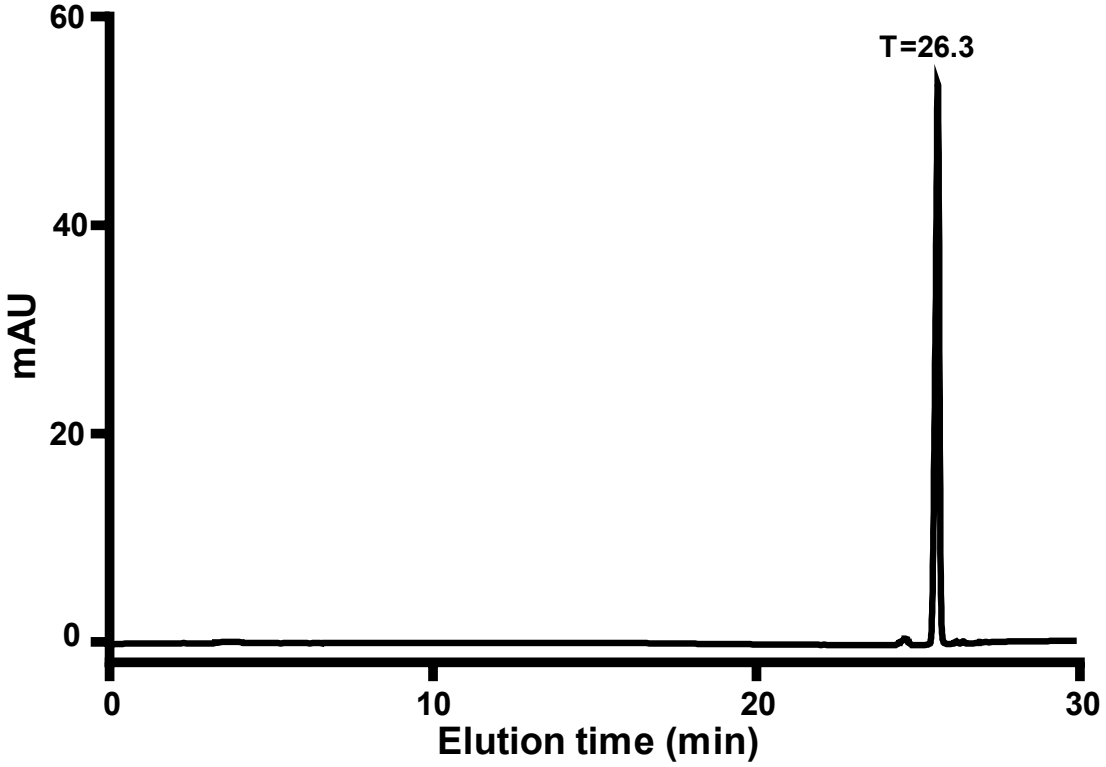
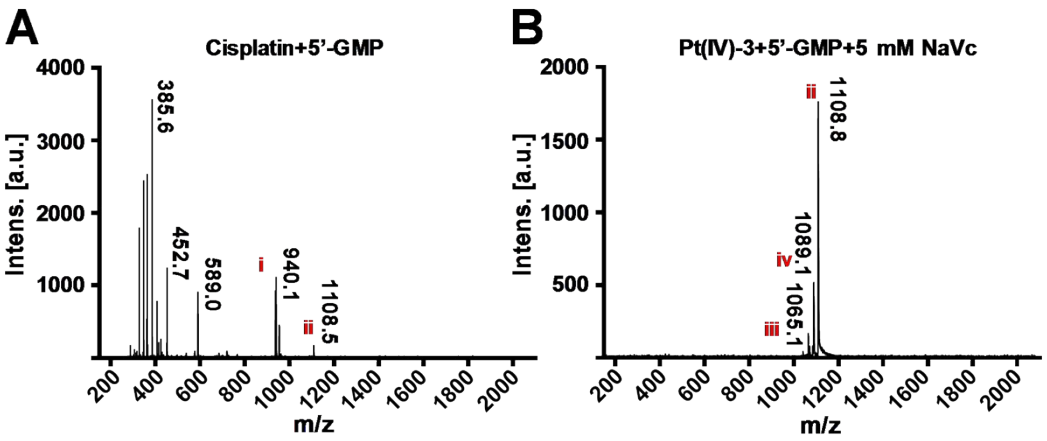


Fig. S5 HPLC characterization of Pt(IV)-3.



C

5'-GMP
Guanosine monophosphate

Guanosine

Guanine

Chemical Formula: $C_{10}H_{14}N_5O_8P$
Exact Mass: 363.06
Molecular Weight: 363.22

Chemical Formula: $C_{10}H_{13}N_5O_5$
Exact Mass: 283.09
Molecular Weight: 283.24

Chemical Formula: $C_5H_5N_5O$
Exact Mass: 151.05
Molecular Weight: 151.13

D

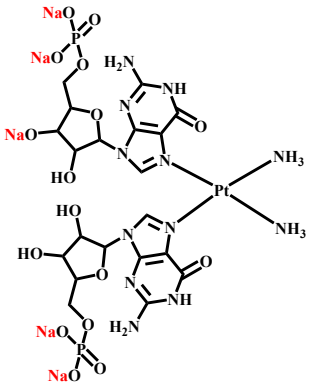
i

ii

Chemical Formula:
$C_{20}H_{30}N_{12}Na_3O_{13}PPt$
Exact Mass: 941.11
Molecular Weight: 941.56

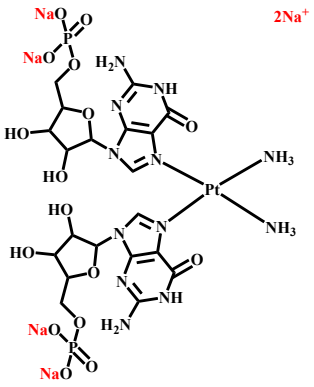
Chemical Formula:
$C_{20}H_{34}K_3N_{13}NaO_{16}P_2Pt^+$
Exact Mass: 1109.2
Molecular Weight: 1109.88

iii



Chemical Formula:
$C_{20}H_{29}N_{12}Na_5O_{16}P_2Pt$
Exact Mass: 1065.04
Molecular Weight: 1065.50

iv



Chemical Formula:
$C_{20}H_{30}N_{12}Na_6O_{16}P_2Pt^{2+}$
Exact Mass: 1089.06
Molecular Weight: 1089.52

Possible peak and assignment

Peaks	Exact mass	Observed m/z	Relative deviation (‰)
i	941.11	940.1	1.07
ii	1109.2	1108.5/1108.8	0.63
iii	1065.04	1065.1	0.06
iv	1089.06	1089.1	0.04

Fig. S6 Characterization of the reaction of 5'-GMP with cisplatin and Pt(IV)-3 by MALDI/TOF-MS (DHB). 5'-GMP was incubated with cisplatin (A) and Pt(IV)-3 (B) at 37 ℃ for 24 h followed by MALDI/TOF-MS measurements. Different corresponding chemical structures are postulated (C). Images of the reaction products of 5'-GMP with cisplatin and Pt(IV)-3 for 24 h (D). Various association complexes that may account for the peaks seen in D are summarized in the table.

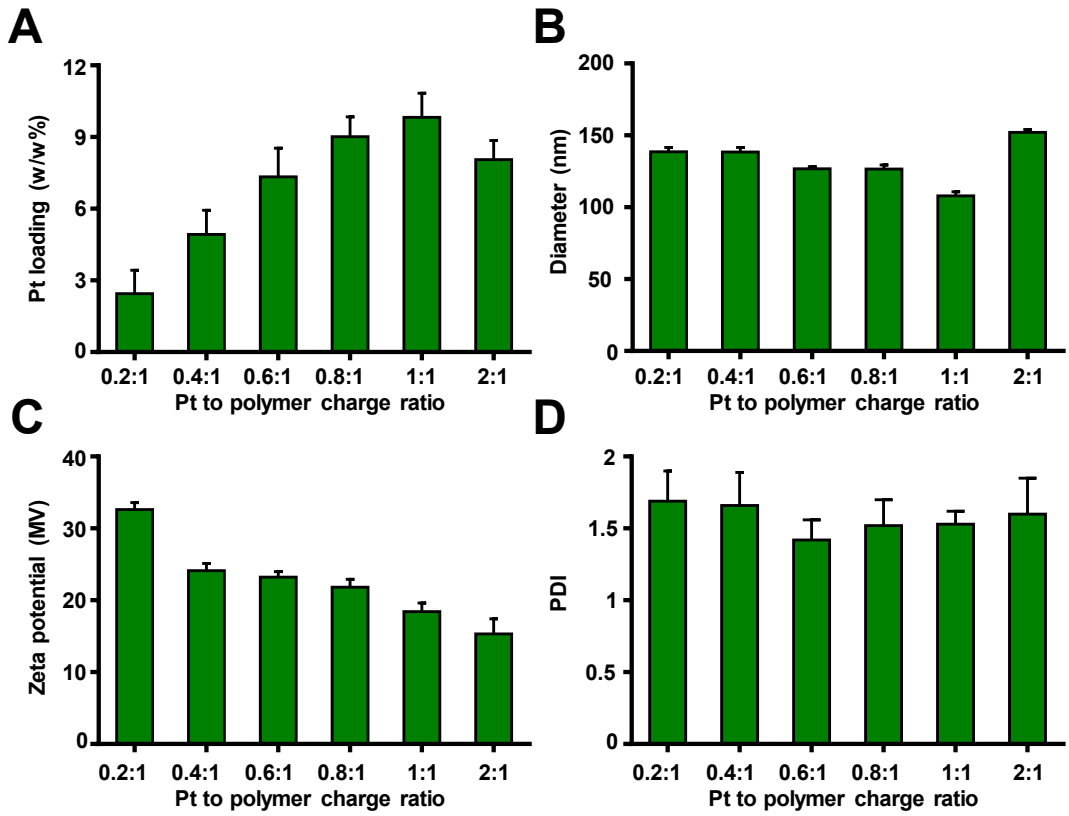


Fig. S7 Formulation optimization of the nanoparticles. Pt loading (A), diameter (B), zeta potential (C) and PDI (D) were shown according to various Pt to polymer charge ratios.

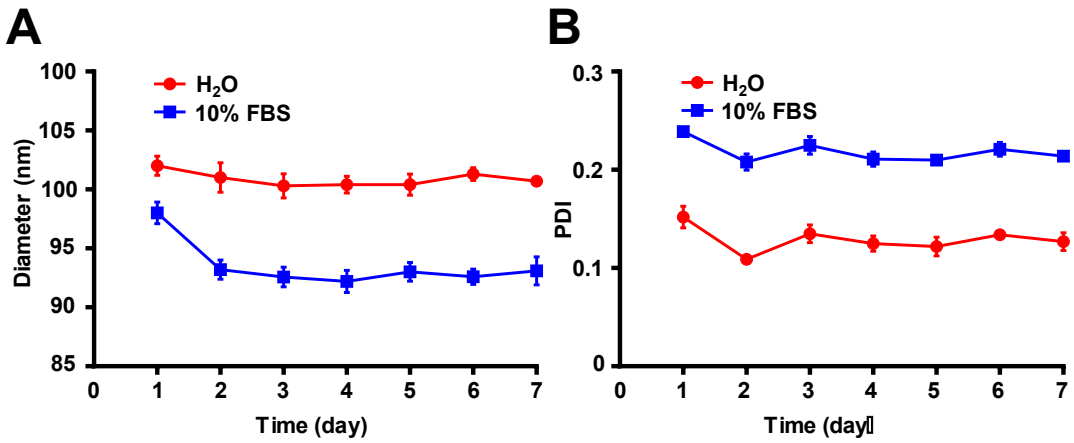


Fig. S8 Monitoring the stability of NPs. Size (A) and PDI (B) of NPs incubated with H_2O and 10% FBS were performed by DLS for 7 days.

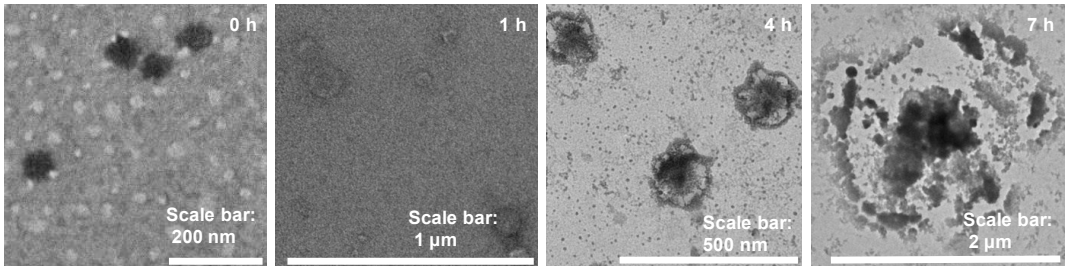


Fig. S9 TEM images of NPs incubated with 5mM NaVc for 0 h, 1 h, 4 h and 7 h.

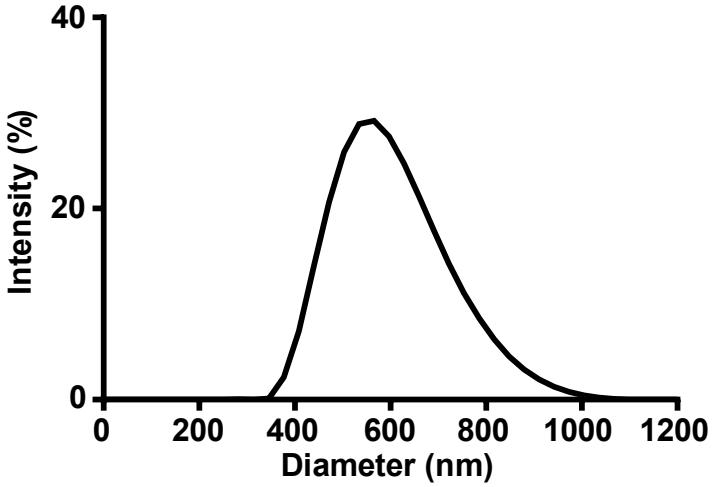


Fig. S10 The size report of NPs after incubation with 5 mM NaVc for 7 h.

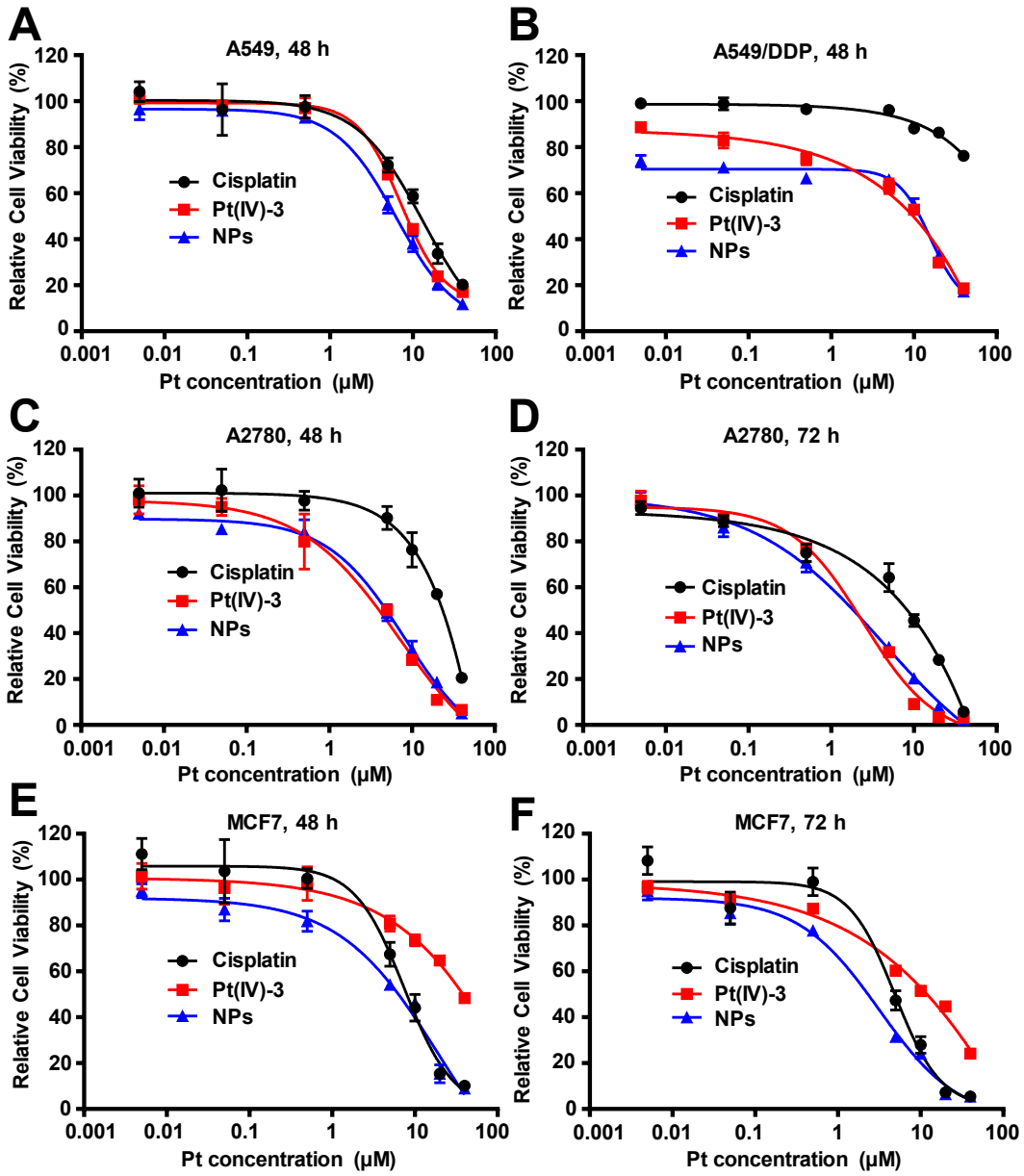


Fig. S11 Relative cell viability of serval cell lines treated with Cisplatin, Pt(IV)-3 and NPs for 48 h or 72 h. A549, A549/DDP, A2780, MCF7 treated with each experimental group for 48 h (A, B, C, E) and 72 h (D, F)

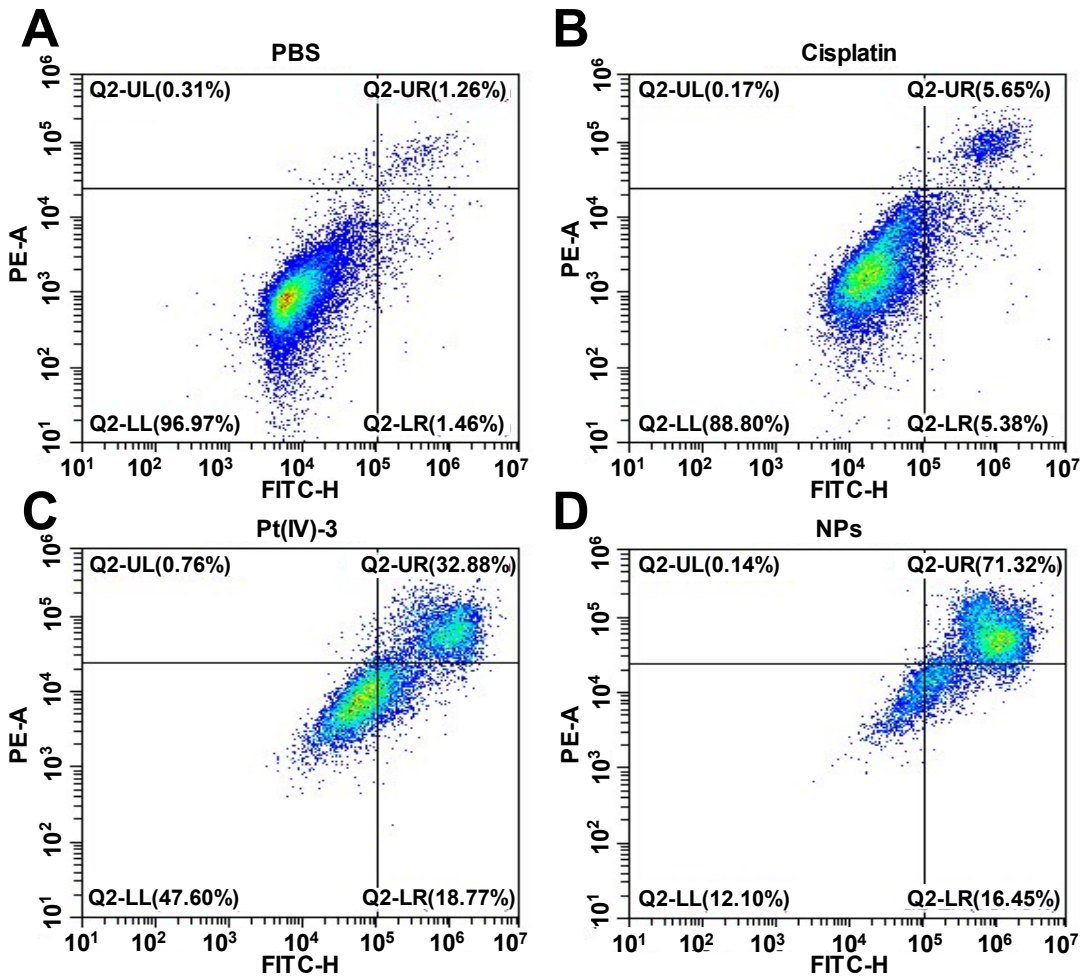


Fig. S12 Cell apoptosis images of A549/DDP cells treated with PBS (A), Cisplatin (B), Pt(IV)-3 (C) and NPs (D) for 24 h.

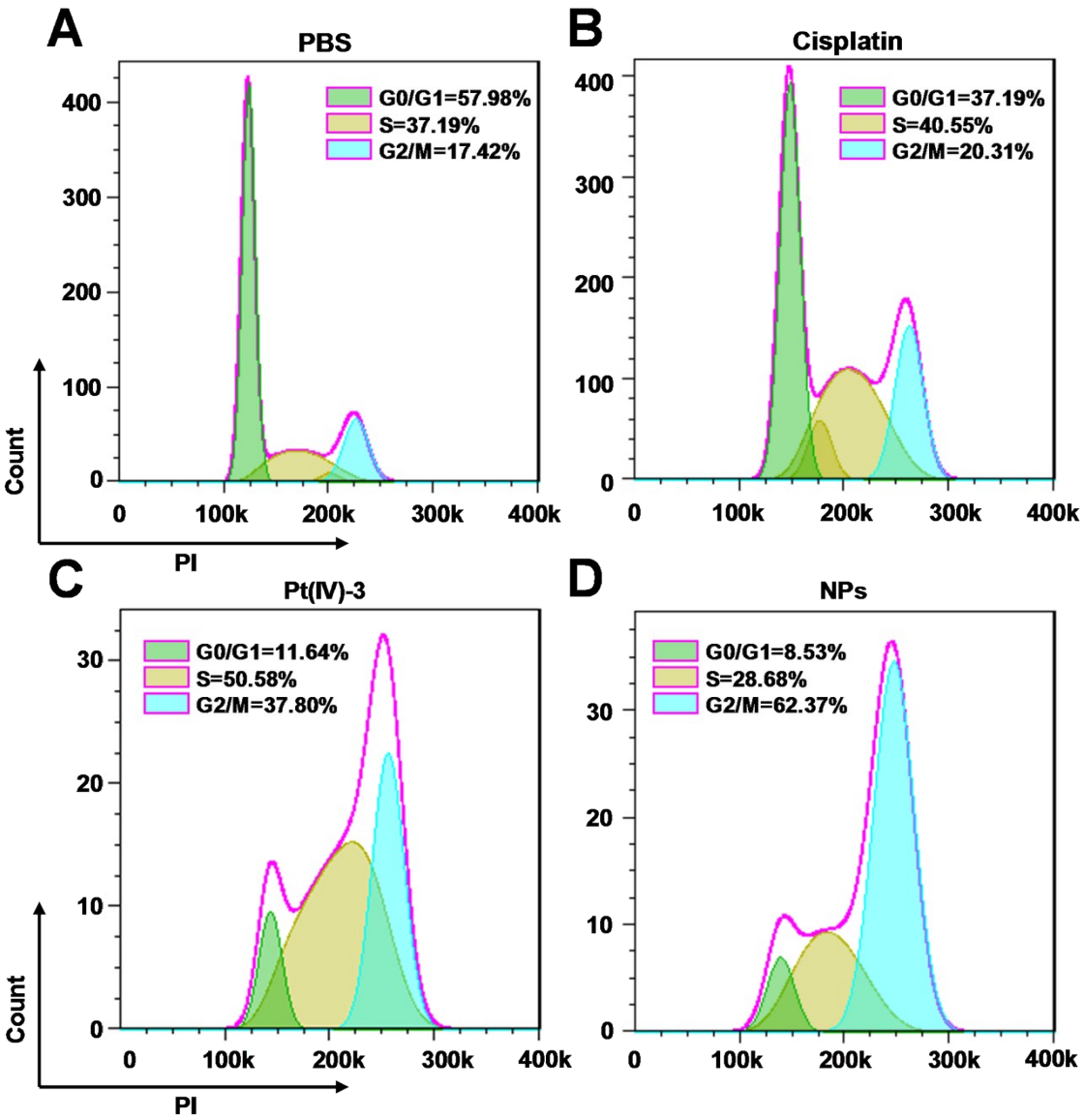


Fig. S13 Cell cycle images of A549/DDP treated with PBS (A), Cisplatin (B), Pt(IV)-3 (C) and NPs (D) for 24 h.