Electronic Supplementary Information (ESI)

An imidazole modified Pt(IV) prodrug-loaded multi-stage pH responsive nanoparticle to overcome cisplatin resistance

Xiaoxu Kang[a]‡, Yushu Wang [b]‡, Zhigang Chen[c], Yixin Wu[a], Hao Chen[a], Xi Yang[d]* and Changyuan Yu[a]*

a. College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, P. R. China
b. Department of Biomedical Engineering, Tufts University, 02155, USA
c. Research Center for Human Tissue and Organs Degeneration, Institute of Biomedical and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, P. R. China
d. Department of Neurourgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, P. R. China
‡ These authors contributed equally to this work.

1 Experimental materials and instruments

Materials

Methoxyl-poly(ethylene glycol)-block-poly(glutamic acid) (mPEG-b-PGA) was gifted by professor Xuesi Chen. Cisplatin, sodium azide (NaN_3), glutathione (GSH), chlorpromazine, methyl-β-cyclodextrin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Aladdin (Shanghai, China). Hexadecyl isocyanate was purchased from Sigma-Aldrich (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 (Gran Island, NY, USA). Cell culture vessels were purchased from Corning (Corning, NY, USA).

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 were measured by a 400 MHz or 300 MHz NMR spectrometer (Bruker) at room temperature. 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;

Synthesis of Cis*Pt(IV)-OH*

According to report[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 CisPt(IV)-OH was obtained and yielded 93%. ESI-MS (positive mode) for $Cl_2H_8N_2O_2Pt$: *m/z* $[M+H]^+$ Calcd: 332.96, Found: 333.0.

Synthesis of Cis*Pt(IV)-Suc*

CisPt(IV)-OH (334 mg, 1.0 mmol) was suspended in 300 mL anhydrous DMF. Hereafter, succinic anhydride (100 mg, 1.0 mmol) was added to the above mixture, and then stirred at room temperature for 48 h. The filtrate

was evaporated under reduced pressure and the crude product was dispersed in methanol and precipitated in ether, then dried under the vacuum to obtain CisPt(IV)-Suc as a white solid (yield, 51%). ^{1}H NMR (400 MHz, DMSO-d_6) 6.29 – 5.63 (6 H, m), 2.38 (4 H, dd, J 13.5, 5.6).

Synthesis of C16-CisPt(IV)-Suc

Cisplatin(IV)-Suc (434 mg, 1.0 mmol) was suspended in 10 mL anhydrous DMF. Then hexadecyl isocyanate (267 mg, 1.0 mmol) was added in the above mixture. The solution was stirred overnight at 65 ℃ until to be clarified. The solvent was removed under reduced pressure, C16-CisPt(IV)-Suc was recrystallized by MeOH to give a yellow solid (yield, 70%).^{1}H NMR (300 MHz, DMSO-*d6*) 6.56 (7 H, d, J 25.5), 2.46 (2 H, s), 2.34 (2 H, s), 1.23 (26 H, s), 0.85 (3 H, s).

Synthesis of C16-Pt(IV)-API

C16-CisPt(IV)-Suc (200 mg, 0.286 mmol), EDC (109 mg, 0.572 mmol), and NHS (66 mg, 0.572 mmol) were suspended in 10 mL anhydrous DMF.

The mixture was stirred for 4 h, then N-(3-Aminopropyl)-imidazole (72mg, 0.572 mmol) was added. The reaction was continued for 2 days at room temperature. The solvent was removed under reduced pressure, the product (termed C16-Pt(IV)-API) was recrystallized by MeOH to give a

yellow solid and yielded 70%. ^{1}H NMR (400 MHz, DMSO-*d6*) 7.88 (1 H, s), 7.72 (1 H, s), 7.22 (1 H, s), 6.94 (1 H, s), 6.55 (5 H, d, J 41.6), 3.97 (2 H, t, J 6.9), 3.01 (4 H, dd, J 13.2, 6.4), 2.45 (2 H, d, J 7.1), 2.28 (2 H, t, J 7.0), 1.28 (28 H, d, J 38.9), 0.85 (3 H, t, J 6.5). HR-MS (ESI) m/z calcd for (C27H54Cl2N6O5Pt): 808.75, found: ($[M+H^+]^+$, 100%): 809.32445, ($[M+Na^+]^+$,100%): 831.30605.

Preparation process of nanoparticles

As the following description, C16-Pt(IV)-API (2 mg) and mPEG-*b*-PGA (8 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).

Zeta potential variation of NPs

To explore the surface charge variation of NPs, the zeta potential of NPs was measured following incubation with buffer solutions at the different pH values (pH 7.4, 6.5,5.5, 4.5). 1 mL NPs was incubated with 2 mL buffer solutions at 37 ℃ for 4 h. The zeta potentials of the resulting samples at the different pH values were measured three times by a Malvern Zetasizer NanoZS90.

Drug release of NPs in vitro studies

Drug release of NPs was studied at pH 7.4, pH 6.5, pH 5.5 and in the presence of 10 mM GSH 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

7404, 7404 DDP (cisplatin resistant), A549, A549 DDP (cisplatin resistant), A2780, and A2780 DDP (cisplatin resistant) cells were used in the following experiments. A549, A549 DDP cells were cultured in DMEM media, while 7404, 7404 DDP, A2780, A2780 DDP 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 was used to label NPs to form RhB@NPs. RhB (2 mg) and C16-Pt-API (2 mg) were dissolved in DMF (10 mL), and mPEG-*b*-PGA (8 mg) 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 mL 7404 and 7404 DDP cells at a density of 2×10^5 were added to each well and incubated in 37 °C overnight. Then RhB@NPs was added to each well at a final concentration of 2 μg/ml of RhB for 1 h, 4 h and 7 h. After washed with cold PBS thrice, the cells were fixed with paraformaldehyde and the nuclei were stained with DAPI. At last, images were performed with CLSM.

Intracellular uptake studies of flow cytometry 7404 DDP cells were seeded in six-well plates at a density of 2×10^5 per well and then incubated at 37 °C overnight. Subsequently, 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 test by flow cytometry.

Platinum uptake in the cells

7404 and 7404 DDP cells were seeded in six-well plates at a density of 1×10^6 per well and then incubated at 37 °C overnight. Cisplatin and NPs were added to each well at a final concentration of 10 μM of Pt. The cells were harvested in EP tubes after treated for 1 h, 4 h and 7 h, and ICP-MS was performed to test the Pt concentrations.

Platinum uptake inhibition in the cells

7404 DDP cells were seeded in six-well plates at a density of 1×10^6 per well and then incubated at 37 °C overnight. Inhibitors chlorpromazine (CL) (20 μg/ml), methyl-β-cyclodextrin (Me-β-CD) (200μM), and NaN_3 (120 mM) were added to each well and incubated for 2 h. Afterwards, the cells were washed thrice with cold PBS and fresh media was added to the plates. NPs were added to the wells at a final concentration of 10 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 test the Pt concentrations.

Pt-DNA adducts in the cells

7404 DDP cells were seeded in six-well plates at a density of 1×10^6 per well and then incubated at 37 °C overnight. Cisplatin and NPs were added to each well at a final concentration of 10 μM. The media was removed

after incubation for 1 h, 4 h and 7 h, then the cells were washed thrice with cold PBS. Hereafter, DNA extraction kit (Solarbio) was used to extract DNA of the above cells. 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 relatively cell viabilities.7404, 7404 DDP, A549, A549 DDP, A2780, and A2780 DDP cells were seeded in 96-well plates at a density of 5×10^3 per well and then incubated at 37 °C overnight. Each Cell was incubated with cisplatin, C16-Pt-API and NPs with final concentrations of 0.005, 0.05, 0.5, 5, 10, 20, 40 μM of Pt for 48 h, respectively. Then, 10% MTT diluted with DMEM was added in the wells. After incubation in 37 °C for 4 h, 10% SDS was added to each well and incubated at 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

7404 DDP cells were seeded in six-well plates at a density of 2×10^5 per well and then incubated at 37 °C overnight. Cisplatin, C16-Pt-API and NPs were added to each well at the final concentration of 10 μM of Pt. The media was removed after incubation for 24 h, then the cells were washed thrice 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.

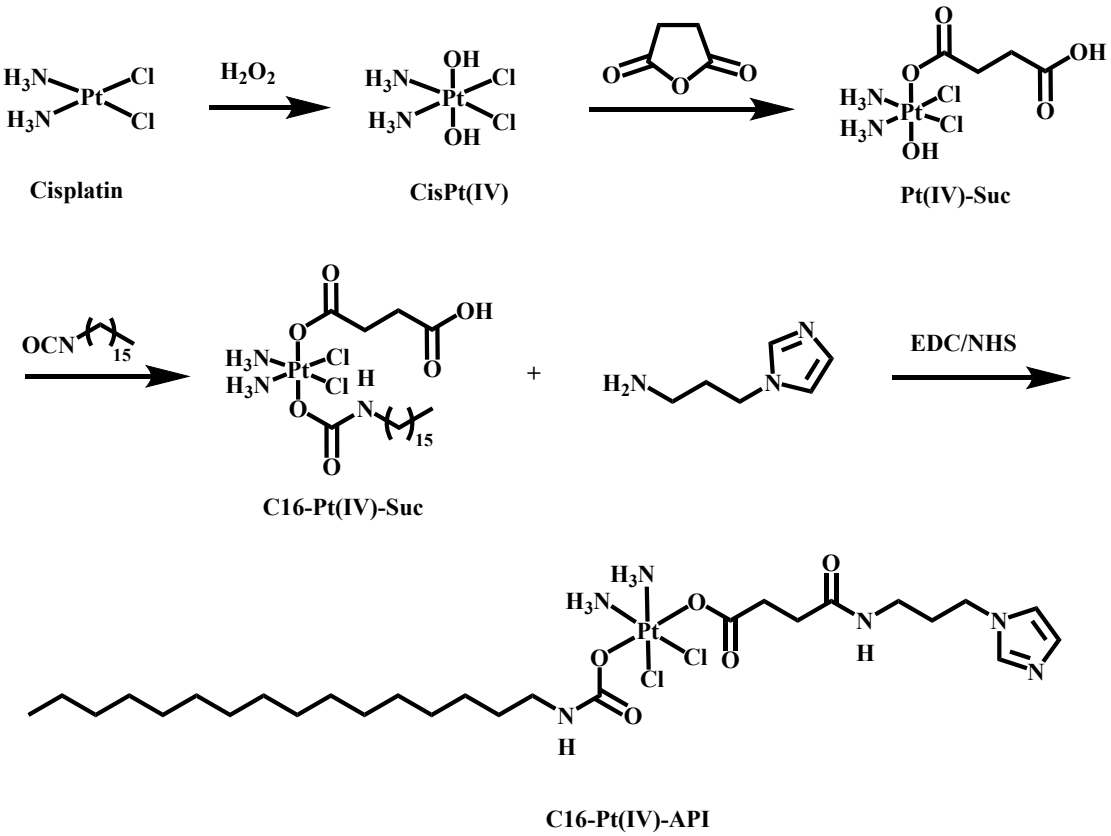
Cell cycle studies

7404 DDP cells were seeded in six-well plates at a density of 2×10^5 per well and then incubated at 37 °C overnight. Cisplatin, C16-Pt-API and NPs were added to each well at the final concentration of 10 μM of Pt. The media was removed after incubation for 24 h, then the cells were washed thrice with cold PBS. After fixed with 70% ethanol at 4 °C for 12 h, the cells were harvested and treated with RNase A (100 μg/ml) and PI (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 route of C16-Pt-API.

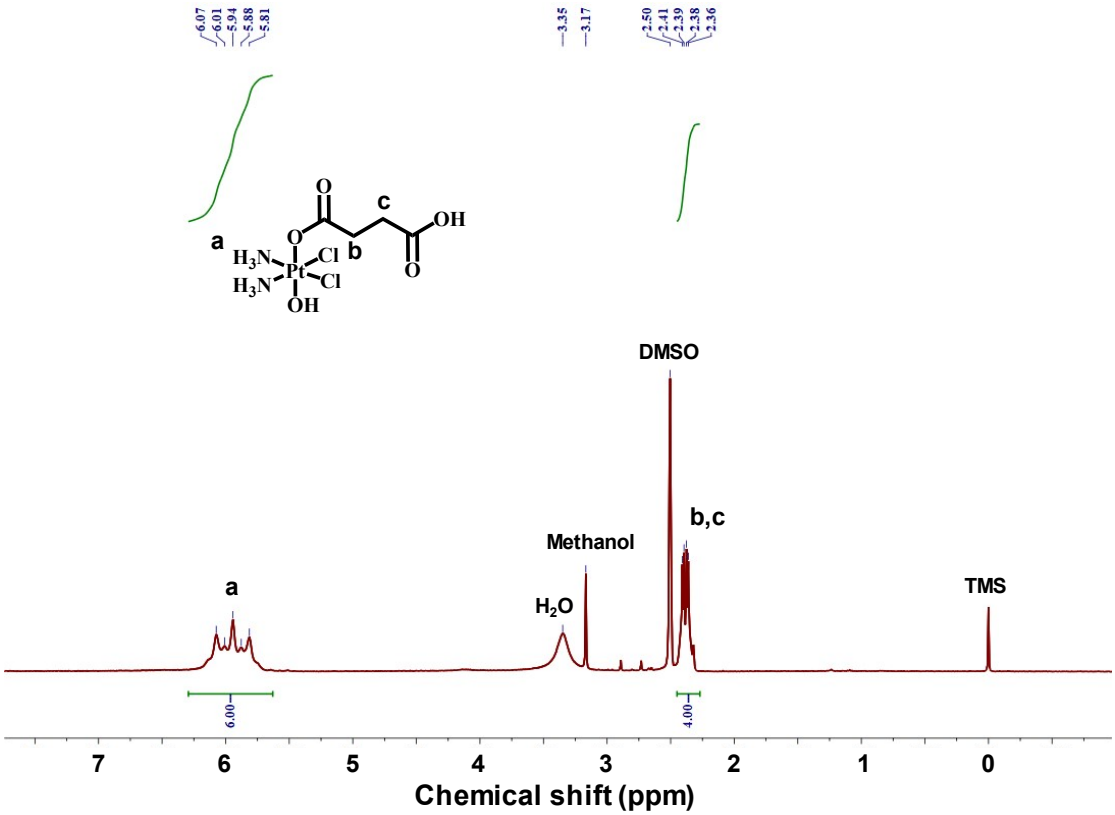


Fig. S1 ^{1}H NMR spectrum of CisPt(IV)-Suc in DMSO-*d6*.

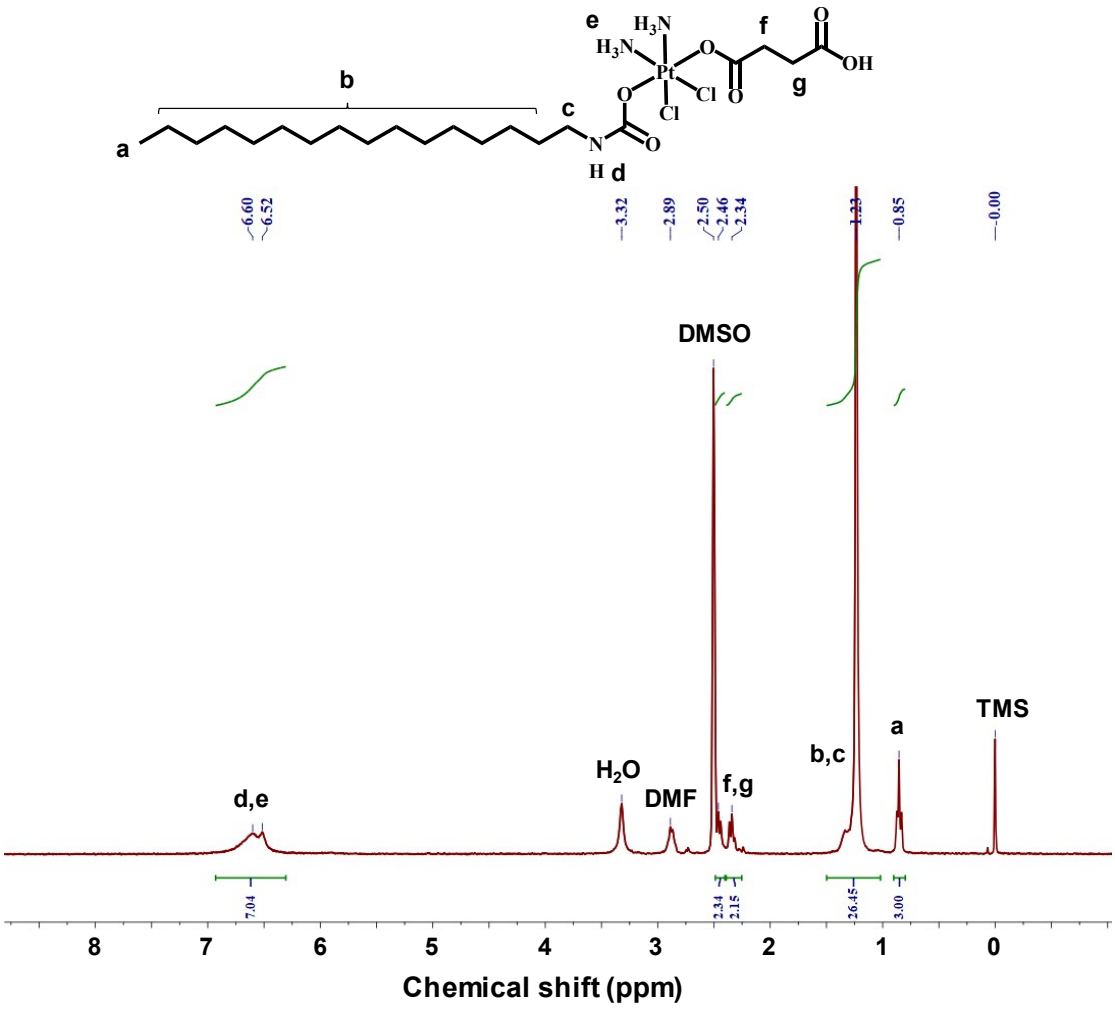


Fig. S2 ^{1}H NMR spectrum of C16-CisPt(IV)-Suc in DMSO-*d6*.

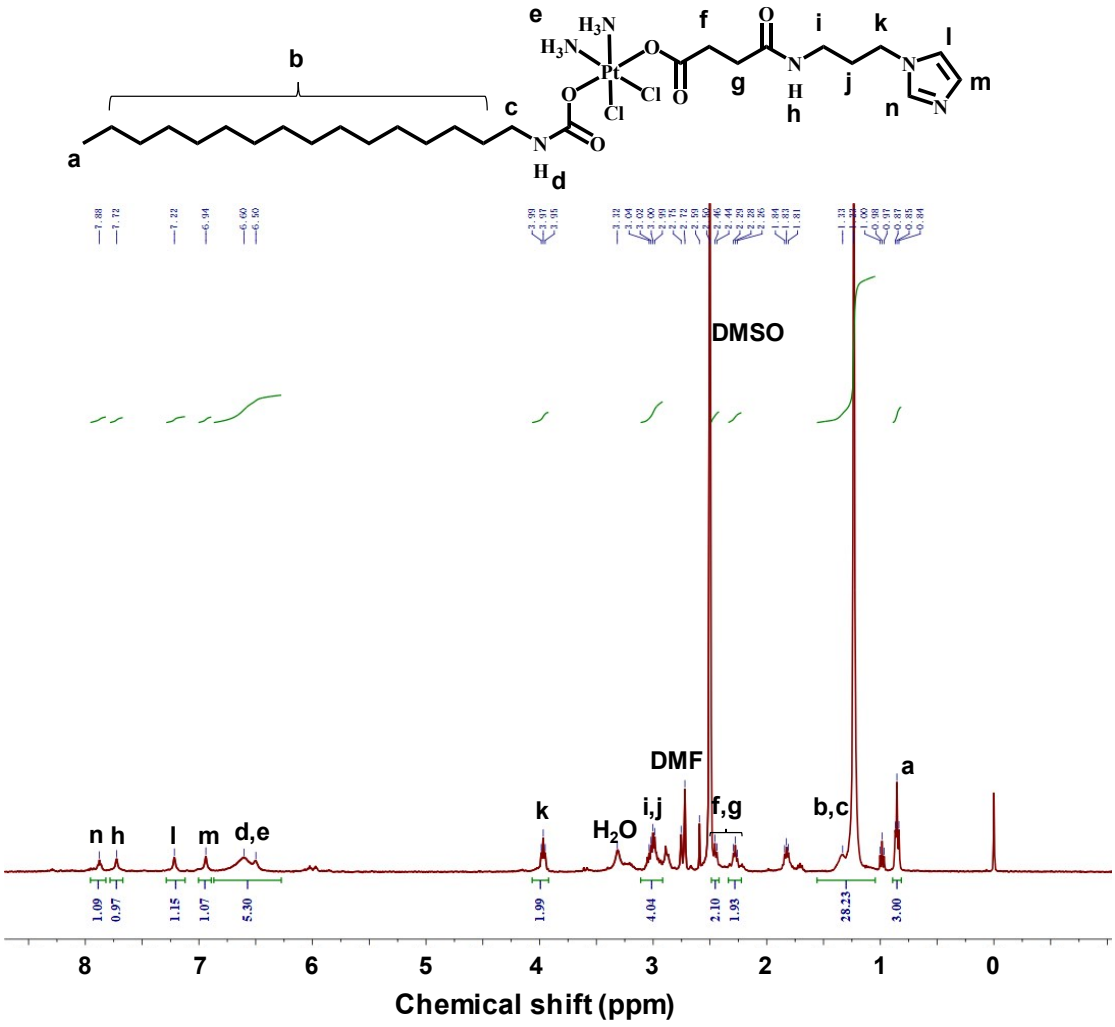


Fig. S3 ^{1}H NMR spectrum of C16-Pt(IV)-API in DMSO-*d6*.

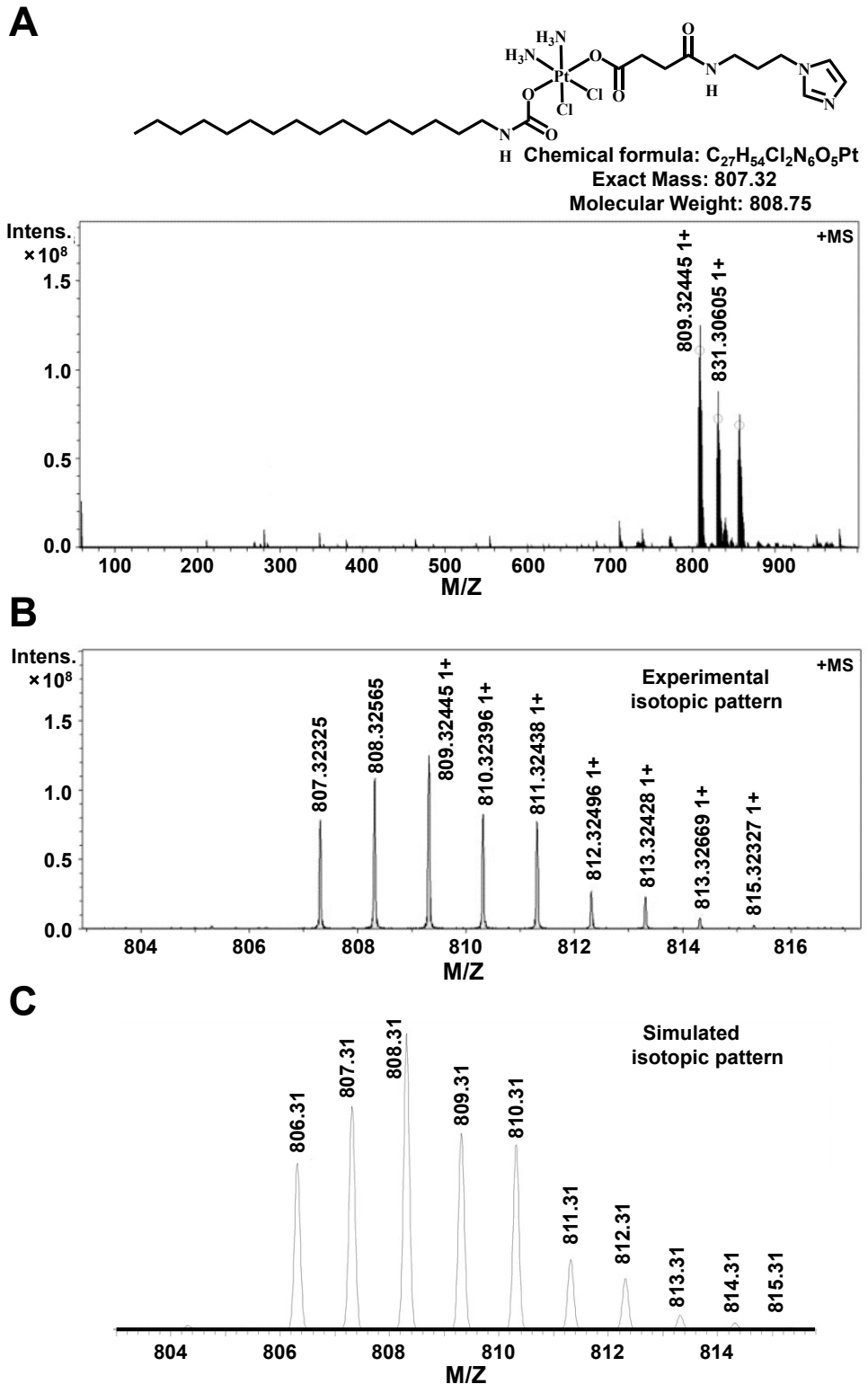


Fig. S4 Characterization of C16-Pt-API by HR-MS (positive mode) (A). The simulated experimental (B) and isotopic (C) patterns of corresponding compound.

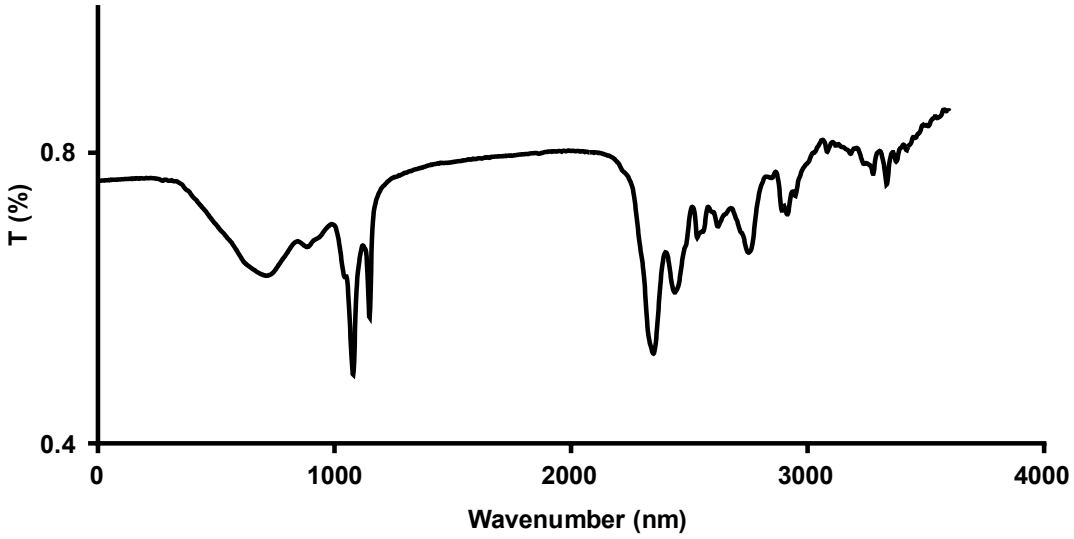


Fig. S5 Characterization of C16-Pt-API by FT-IR spectra.

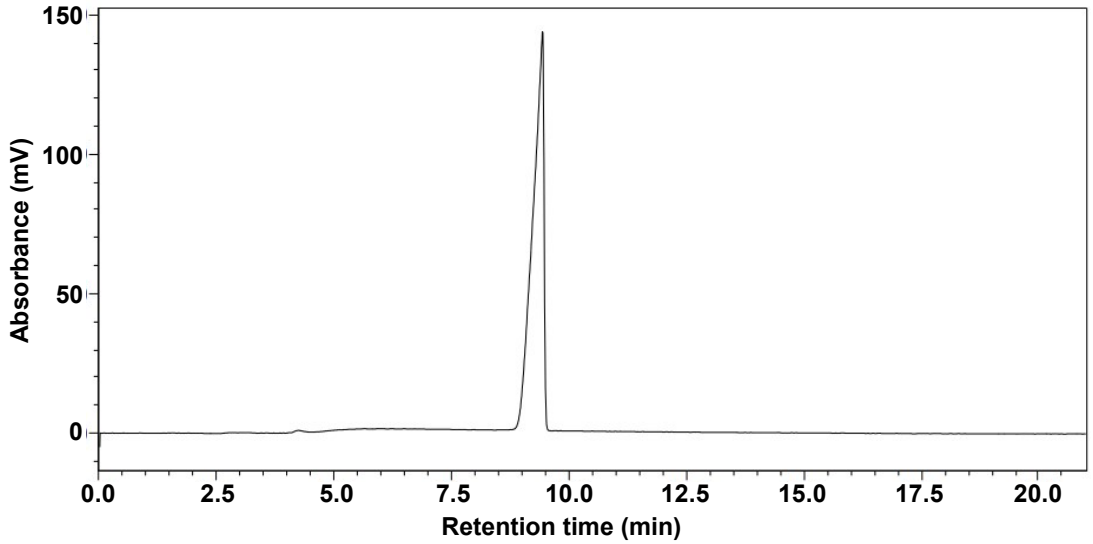


Fig. S6 Reversed phase HPLC chromatogram of C16-Pt-API. An Agilent ZORBAX 300SB-C18 column (5μm, 250mm × 4.6mm) was used for analytical HPLC of purified C16-Pt-API. Eluent A: water (with 0.1% TFA), Eluent B: 80% acetonitrile (with 0.1% TFA). A gradient was used as follows, %B (min.): 5 (0), 50 (10), 100 (20), 50 (21). Retention time for C16-Pt-API was 9.43 min.

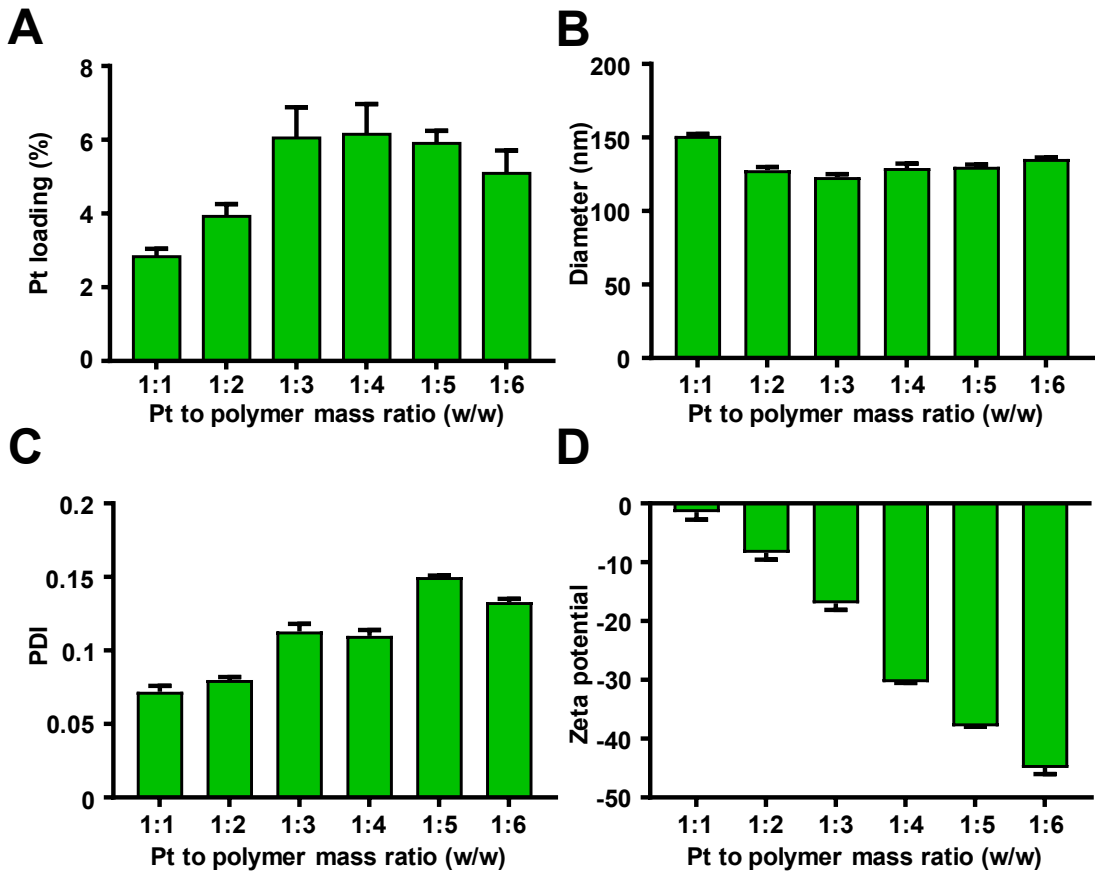


Fig. S7 Formulation optimization of the nanoparticles. Pt loading (A), diameter (B), zeta potential (C) and PDI (D) were shown according to various Pt to polymer mass ratios.

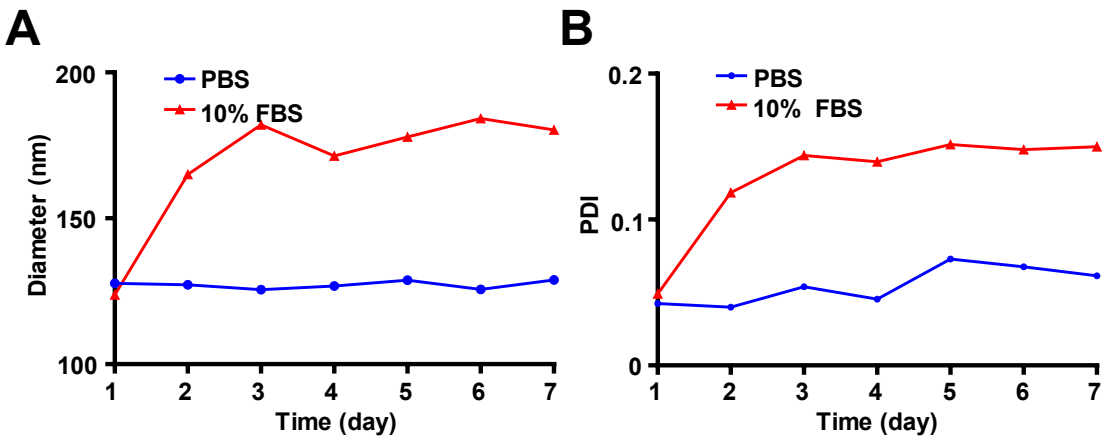


Fig. S8 The stability of NPs monitored by DLS. Diameter (A) and PDI (B) of NPs incubated with PBS and 10% FBS were monitored for 7 days.

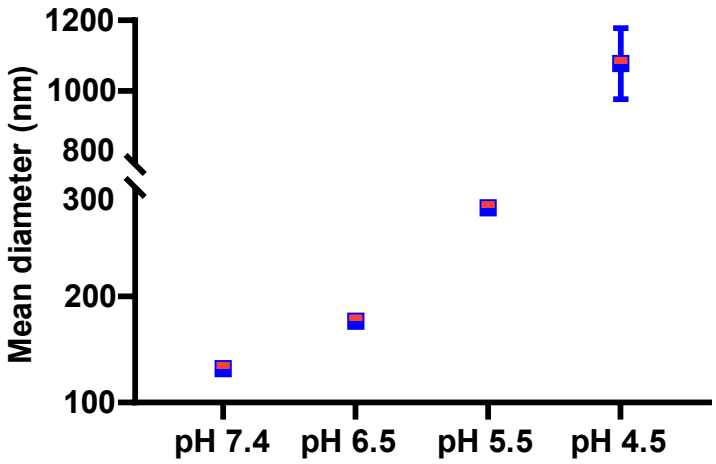


Fig. S9 The mean diameter of NPs at different pH conditions.

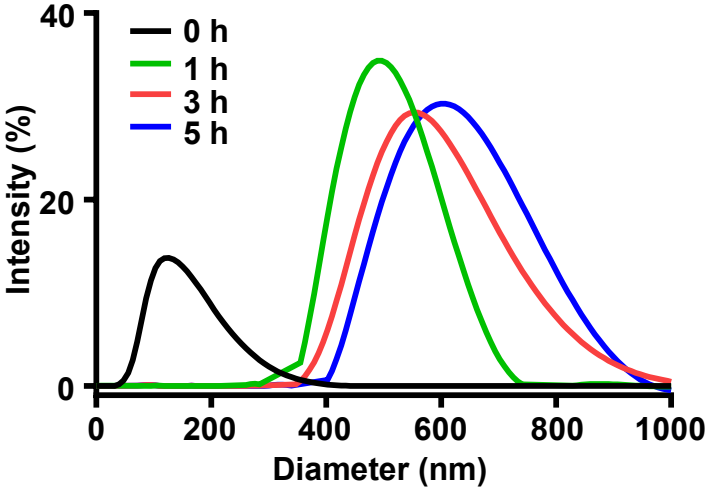


Fig. S10 The size distribution of NPs incubated in reductive condition.

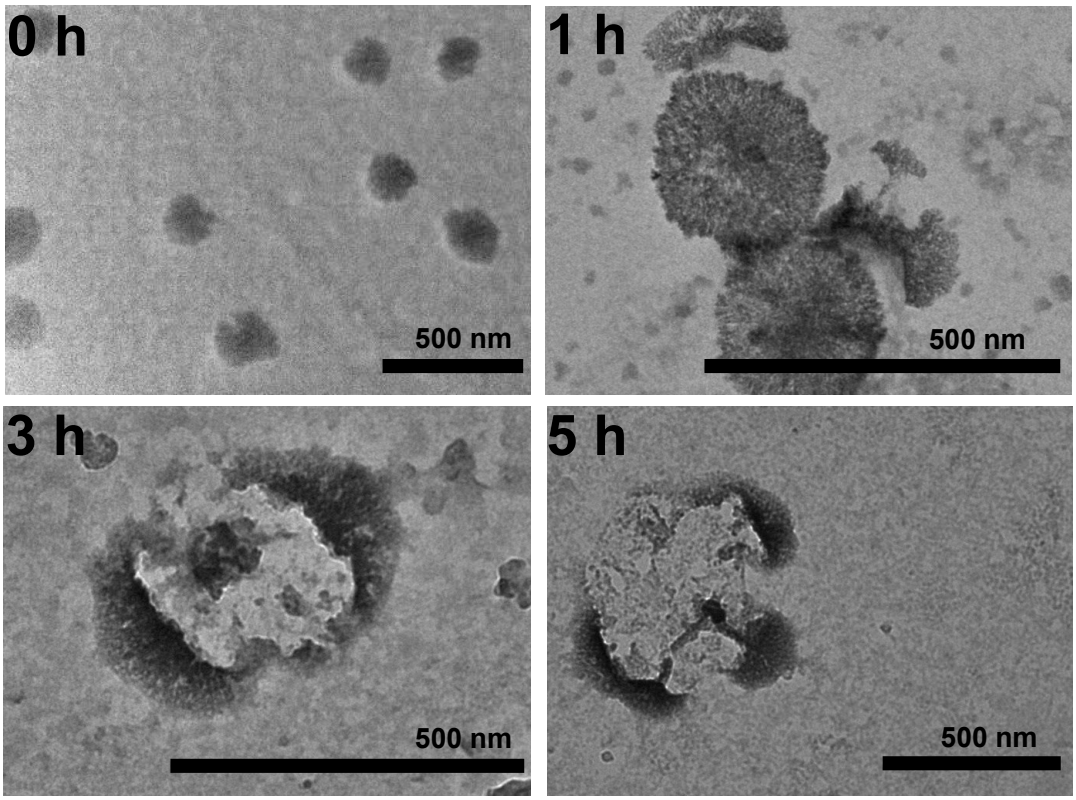


Fig. S11 TEM images of NPs incubated with 10mM GSH for 0 h, 1 h, 3 h and 5 h.

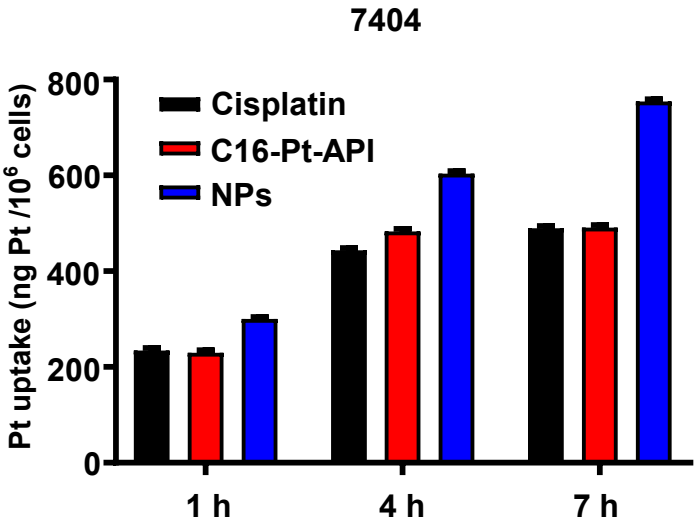


Fig. S12 Pt uptake of 7404 cells treated with Cisplatin, C16-Pt-API and NPs for 1 h, 4 h and 7 h utilizing ICP-MS.

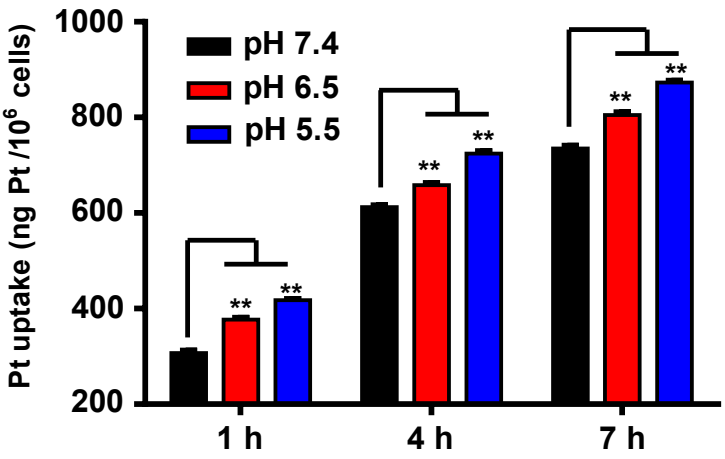


Fig. S13 The intracellular Pt content of 7404 DDP cells treated with NPs in different pH conditions. Significance is defined as **P＜0.01.

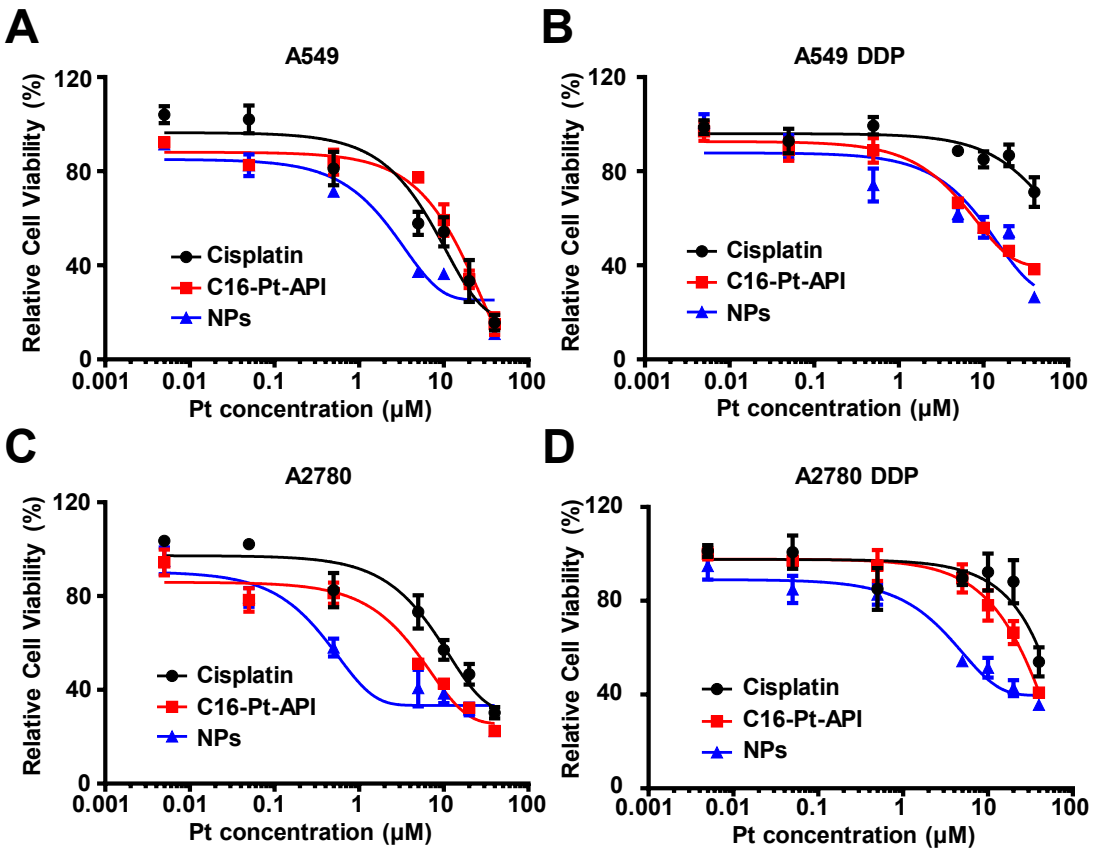


Fig. S14 Relative cell viability of A549 (A), A549 DDP (B), A2780 (C), A2780 DDP (D) cells treated with Cisplatin, C16-Pt(IV)-API and NPs for 48 h.

Table S1 IC_{50} (μM) values of Cisplatin, C16-Pt-API and NPs and on different cell lines.

Cell lines	A549	A549/DDP	A2780	A2780/DDP	7404	7404/DDP
Cisplatin	7.035	>40	14.850	>40	2.446	>40
C16-Pt-API	8.987	17.218	4.153	>40	1.021	23.064
NPs	2.085	12.322	2.081	10.017	0.630	4.095

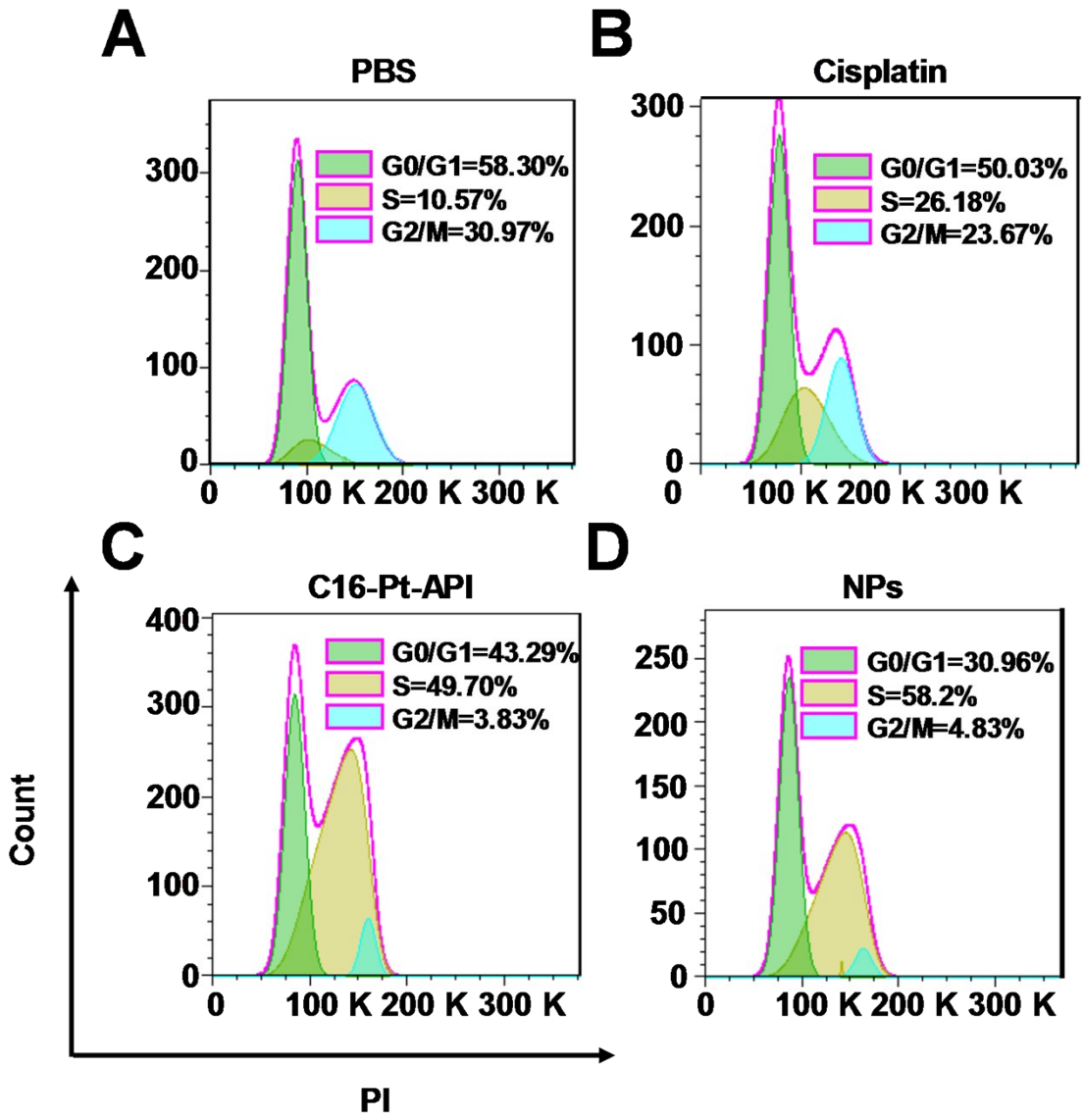


Fig. S15 Cell cycle study of 7404 DDP cells treated with PBS (A), Cisplatin (B), C16-Pt-API (C) and NPs (D) for 24 h.

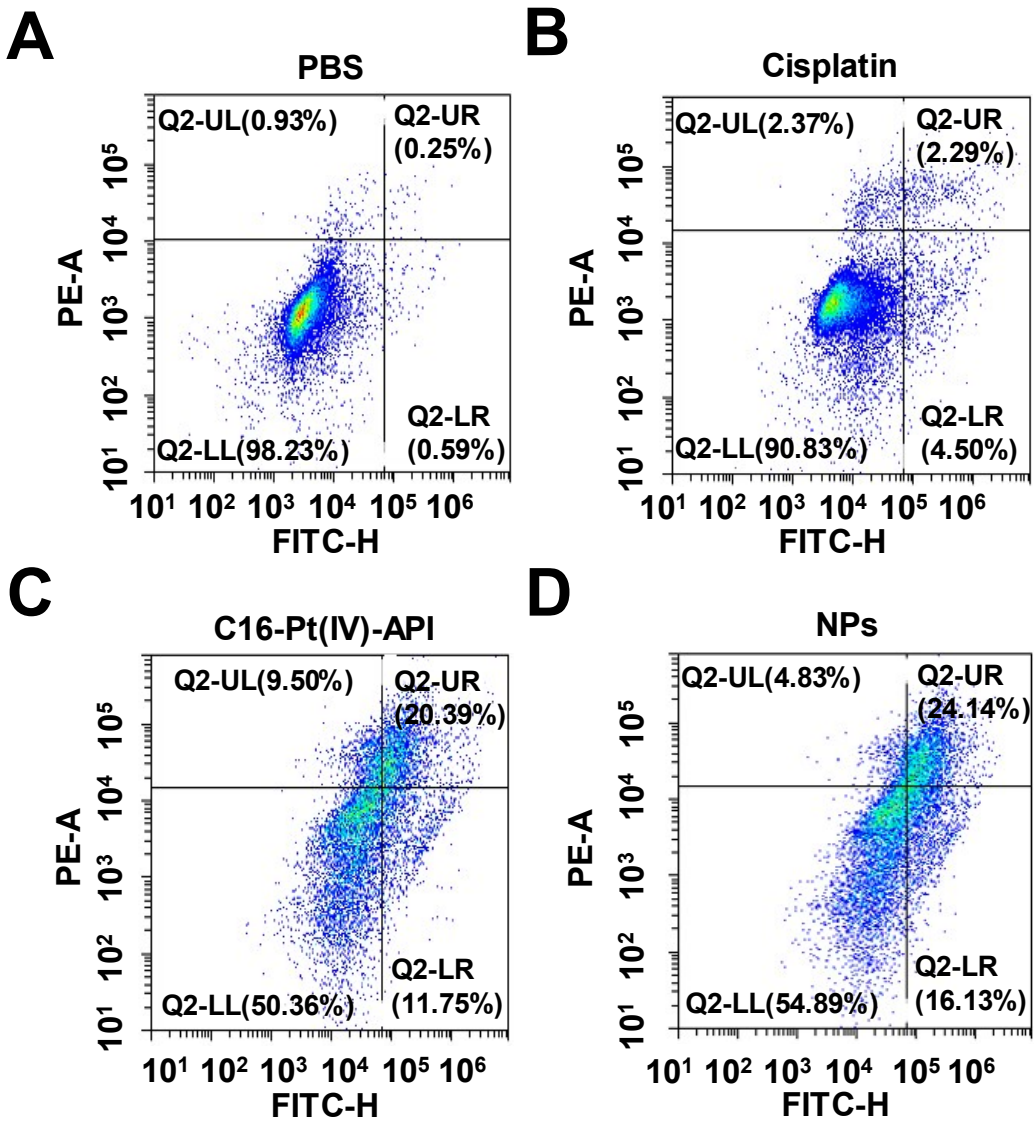


Fig. S16 Cell apoptosis assay of 7404 DDP cells treated with PBS (A), Cisplatin (B), C16-Pt-API (C) and NPs (D) for 24 h.

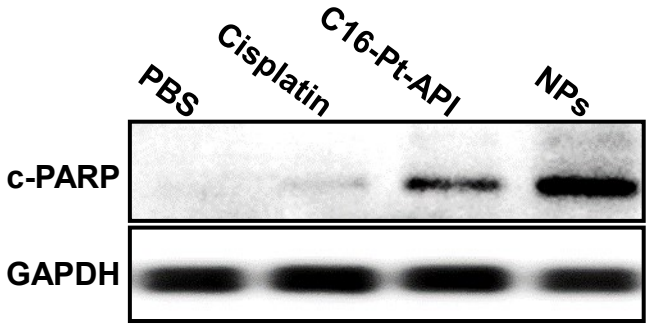


Fig. S17 Expression patterns of apoptosis related protein of 7404 DDP cells treated with PBS, Cisplatin, C16-Pt-API and NPs by Western Blot.