Nanoparticle Q1 Q2 delivery of sterically hindered platinum(IV) prodrugs shows 100 times higher potency than that of cisplatin upon light activation

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Materials and Methods

Materials

$MPEG_{114}$-b-PCL_{20}-b-PLL_{10} was synthesized as previously described in our lab[1]. TP1 was synthesized as previously described [2,3]. Rhodamine B (RhB), 4',6-diamidino-2-phenylindole (DAPI), N hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), 2′-deoxyguanosine 5′-monophosphate sodium salt hydrate (5'-dGMP) (D9500-100MG) and sodium azide were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Pharmaceutical Co. Ltd., China. All other chemicals and solvent were used as received.

General Measurements

^{1}H NMR spectra were measured by a Unity-300MHz 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 study the chelation of t,t-Pt(NH_3)(pyridine)Cl_2 and MPEG-PyPt conjugate in the presence of 5'-GMP upon UVA irradiation. Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA) was used to determine the total platinum contents in PyPt-NP and samples obtained outside of the dialysis bags in drug release experiments. Size and size distribution of micelles were determined by DLS with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology, USA). The morphology of PyPt-NP was measured by TEM performed on a

JEOL JEM-1011 electron microscope. Particle size and zeta potential measurements were conducted on a Malvern Zetasizer Nano ZS.

UVA light source and irradiation conditions

A bank of six UVA light tubes bought from Huaqiang Eletronics (Nanjing, China) with 18 W light sources (1.8 mW/cm^2, λ_{max} = 365 nm) were used for all *in vitro* irradiation including the drug releases, irradiated sample preparation for MALDI-TOF-MS and *in vitro* MTT assays.

Synthesis of TPt1, TPt2, PyPt1

TPt1, TPt2, PyPt1 were synthesized as previously described [2,3].

Synthesis of PyPt2

PyPt1 (0.409 g, 1 mmol) was dissolved in 5 ml DMSO in a flask protected from light, to which 0.1 g succinic anhydride were added. The reaction mixture was kept stirred overnight and then DMSO was removed by lyophilization to get the crude product PyPt2. PyPt2 was then dissolved in 5 ml methanol and precipitated by excess of ether, washed 3 times with ether (3× 50 mL), and re-crystallized in acetone.

Reaction of cisplatin and PyPt2 with HSA

Cisplatin and PyPt2 dissolved in water and mixed with HSA at a final concentration of 5 μM Pt and 5 μM HSA respectively (Total volume at 2 mL). The reaction mixture was put into an incubator shaker at 37 °C for 6 h, 12 h, 24 h and 48 h respectively. After desirable time, the reaction mixture was thoroughly transferred to a centrifugation tube with separation membrane (molecular cutoff at 3k Da) and centrifuged at 5000 rpm for 5 minutes. The protein was left in the tube and was washed for 5 times by water by centrifugation.

Reaction of cisplatin, TPt2 and PyPt2 with GSH

Cisplatin, TPt2 and PyPt2 dissolved in water and mixed with 5 mM GSH respectively. The reaction mixture was put into an incubator shaker at 37 °C for a period of 1000 min and monitored by UV-vis. 5 mM GSH was measured as a background. The peak absorbance at 286 nm was plotted against time to get the reaction rate of GSH with PyPt2.

Conjugation of PyPt2 onto biodegradable polymers

PyPt2 (0.509 mg, 1 mmol) was dissolved in the 10 ml pure water in a flask, to which 2 mmol EDC and 2 mmol NHS were added and the reaction mixture were vigorously stirred for 10 min at room temperature. $MPEG_{114}$-b-PCL_{20}-b-PLL_{10} (500 mg) in 1 mL DMF was added to the flask. The reaction mixture was stirred at room temperature for another 4 h and then subjected to filtration and the filtrate

was dialyzed against water to remove unreacted platinum drugs within a dialysis bag (molecular weight cutoff: 3500 Da) and byproducts of coupling agents. The aqueous solution of the polymer-drug conjugate was then lyophilized for use.

Preparation of PyPt-NP

The micellar nanoparticles of PyPt-NP were prepared by a nano-precipitation method. Briefly, polymer-PyPt conjugate (2 mg) was dissolved in DMF (1 ml) and then de-ionized water (5 ml) was added in drop-wise. The mixture was put in dialysis bag (molecular weight cut off: 3500 Da) and dialyzed against de-ionized water for 1 day and lyophilized to powder.

Preparation of Rhodamine B labeled PyPt-NP

PyPt-NP (10 mg) was first dissolved in 2 ml DMF in a glass vial, to which 0.2 mg Rhodamine B was added. EDC (5 mg) and NHS (4 mg) was also added to the solution. The reaction mixture was stirred for 6 h and then was dialyzed against water for 3 days (dialysis bag molecular weight cutoff: 3000 Da). The Rhodamine B labeled Polymer conjugates of PyPt were then lyophilized to powder for usage.

Drug release from PyPt-NP

PyPt-NP (5 mg) was dissolved in PBS **(**10 mL, 0.1 M, pH 7.4), and then put into a pre-swelled dialysis bag (3500 Da, MWCO), that was then immersed into PBS (100 mL) at 37 °C. Samples were kept in the dark or under UVA lamps. At desirable time intervals, 1.5 mL was withdrawn from the dialysate and measured for Pt using ICP-OES. After sampling, fresh PBS (1.5 mL) was added to the dialysate.

Study of the chelation of t,t-Pt(NH_3)(pyridine)Cl_2 and MPEG-PyPt conjugate with 5'-dGMP Pupon UVA irradiation

a) For t,t-Pt(NH_3)(pyridine)Cl_2: t,t-Pt(NH_3)(pyridine)Cl_2 (0.5 mM) was dissolved in an aqueous solution of 5'-dGMP (1 mM, 10 ml);

b) For MPEG-PyPt, MPEG-PyPt (5 mg) was dissolved in an aqueous solution and put in a dialysis bag with molecular cut off at 1000 Da with 5'-dGMP (1 mM, 10 ml) under UVA irradiation for 1 h;

Aliquots of the reaction mixture in both a) and b) were collected for MALDI-TOF-MS study in positive mode.

Cell source and growth conditions

HepG2 (liver cancer) and SKOV-3 (cisplatin intrinsic resistant ovarian cancer) were obtained from ATCC and grown as previously described [4].

Photo-toxicity of light source

The light source and intensity was described in previous section. HepG2 and SKOV3 were seeded in 96-well plate at a density of 5000 cells/well overnight. UVA light was used for irradiation of the 96-well plates for 1 h with one plate in the dark as a control. The cells were then left growing for another 48 h. After that, 20 μL MTT (5 mg/ml) were added to each well and the cells were further incubated for another 4 h. Then 100 μL acidified SDS solution was added to each well overnight. Absorbance at 570 nm was read.

Cell viability studies

HepG2 and SKOV3 cells were seeded in 96-well plate at a density of 5000 cells/well overnight. All the drugs including cisplatin, transplatin, TPt1, TPt2, PyPt1, PyPt2, TPt-NP and PyPt-NP were freshly prepared in RPMI-1640 culture media and filtered by 0.2 μm filter. Then the drug concentrations were measured by ICP-OES. The cells were treated with cisplatin, TPt-NP and PyPt-NP at a concentration range from 0.1 to 400 μM in triplicates. For transplatin, TPt1, TPt2, PyPt1, PyPt2, and TP2, the range was set from 0.1 to 800 μM. All the drug concentrations above were based on platinum. For the UVA irradiation, cells plates were incubated for 4 h in the dark, UVA irradiated for 1 h, then incubated in the dark for an additional 43 h. For control samples, incubation were performed in the dark for 48 h. Cytotoxicity was then assessed using an MTT assay as described previously [5]. The above MTT assay was repeated for 3 times. Then the IC_{50} values were derived from the dose-response curve of each drug. IC_{50} values were shown as mean value ± standard deviation.

Confocal laser scanning of Rhodamine B labeled PyPt-NPs

A2780DDP cells were seeded in 6 well plates at a density of 2×10^5 cells/well and then the cells were incubated with RhB labeled M(Pt) at 5 μg/ml (based on RhB) for 4 h at 37 °C. After that, cells were thoroughly washed with PBS, fixed, stained by DAPI (2-(4-amidinophenyl)-1H -indole-6-carboxamidine, 1 μg/ml) at 37 °C for 10 min , sealed with glycerin and observed by CLSM (Olympus, FV-1000).

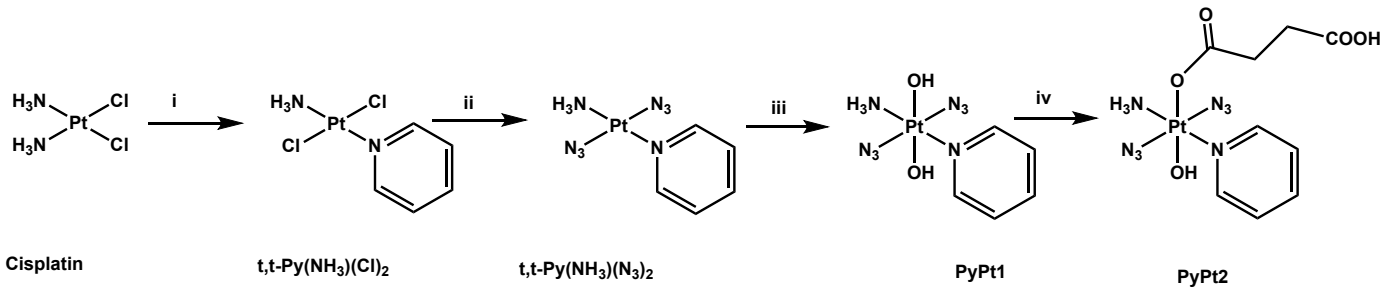
Intracellular uptake of drugs

HepG2 were seeded in 6-well plates at a density of 1×10^6 cells/well overnight, and then treated with Pt drugs which were pre-dissolved in RPMI-1640 culture media (cisplatin, transplatin, TPt1, TPt2, PyPt1, PyPt2, TPt-NP and PyPt-NP) in triplicates at an equal concentration of 5 μM based on platinum at 37 °C for 5 h. The cells were then washed with PBS for 5 times, counted and lysed. Thereafter, the

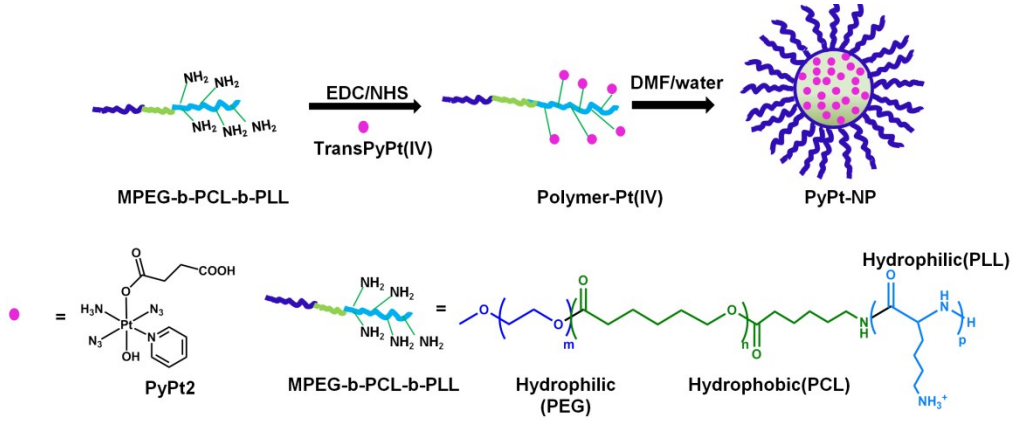
protein content was measure by BCA kit as previously described [6]. The Pt content in the cancer cells was measured via ICP-MS and the uptake of Pt was expressed as ng Pt/mg protein. The above drug uptake experiments were repeated for 3 times. Pt uptake values were shown as mean value ± standard deviation.

Pt-DNA adducts in the dark and upon UVA irradiation

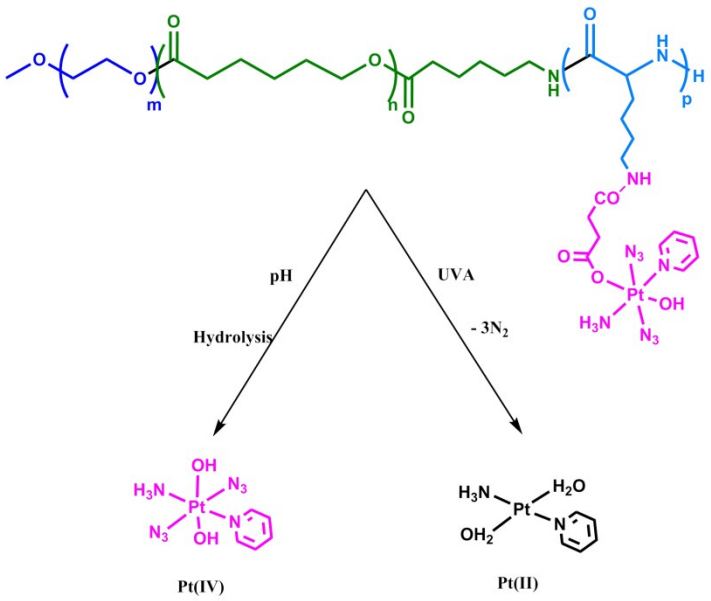
HepG2 were seeded in 6-well plates at a density of 1×10^6 cells/well overnight, and then treated with Pt drugs which were pre-dissolved in RPMI-1640 culture media (cisplatin, transplatin, TPt1, TPt2, PyPt1, PyPt2, TPt-NP and PyPt-NP) in triplicates at an equal concentration of 2 μM based on platinum at 37 °C for 24 h. During this process, one set of the cells were UVA irradiated for 1 h at 4 h after adding the drugs. For controls, another set of the cells were put in the dark. The cells were then washed with PBS for 5 times, counted and lysed. Thereafter, the Pt content in the cancer cells was measured via ICP-MS and the DNA was extracted and measured via Nano-drop. The Pt-DNA adducts were expressed as ng Pt/μg DNA. The above drug experiments were repeated for 3 times. Results were shown as mean value ± standard deviation.



Scheme S1. Synthesis route of PyPt2.



Scheme S2. Conjugation of PyPt2 to tri-block copolymer MPEG-b-PCL-b-PLL and self-assembly of this conjugate to PyPt-NP.



Scheme S3. Possible drug release and cleavage mechanism

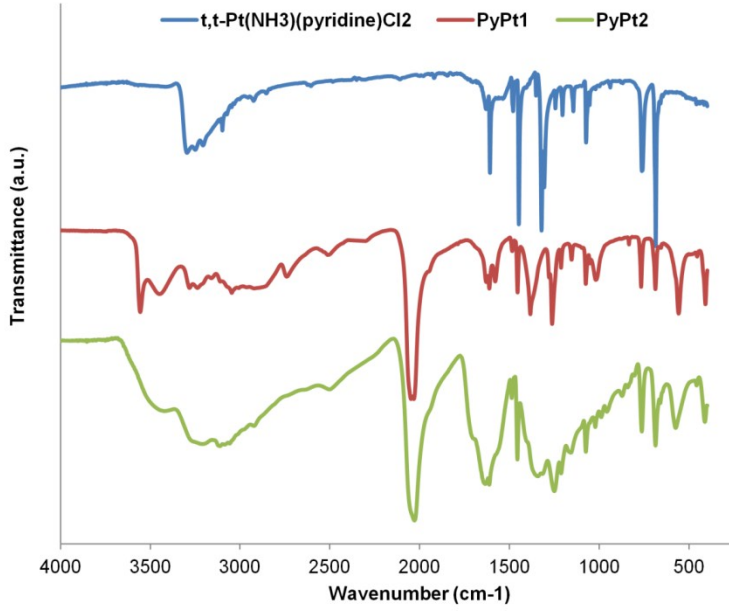


Figure S1. IR spectra of t,t-Pt(NH_3)(pyridine)Cl_2 (blue), PyPt1 (red) and PyPt2(green).

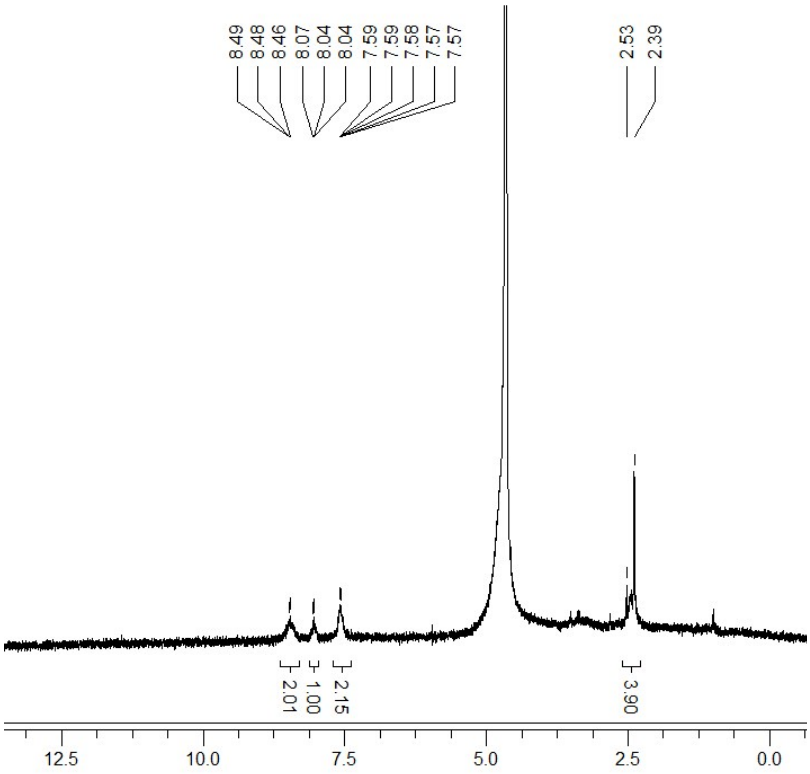


Figure S2. Characterization of the PyPt2 by ^{1}HNMR in D_2O. Chemical shift of each peak was assigned as shown in the insets.

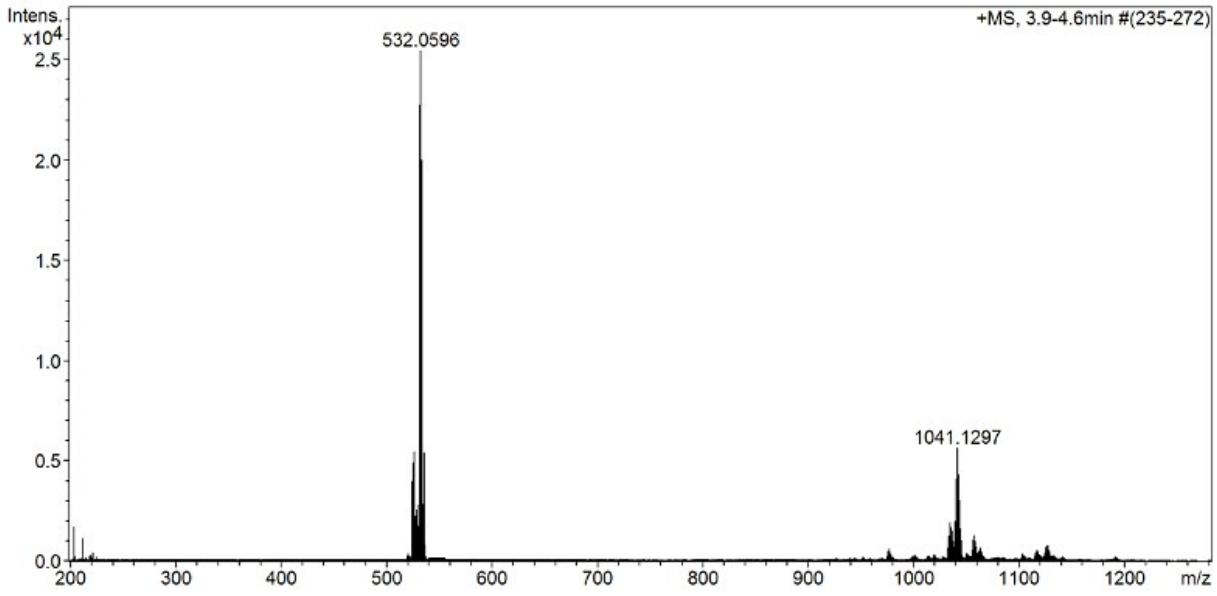


Figure S3. ESI-MS spectra of PyPt2 (positive mode). The peak at m/z=532.0596 could be attributed to the sodium adducts of PyPt2.

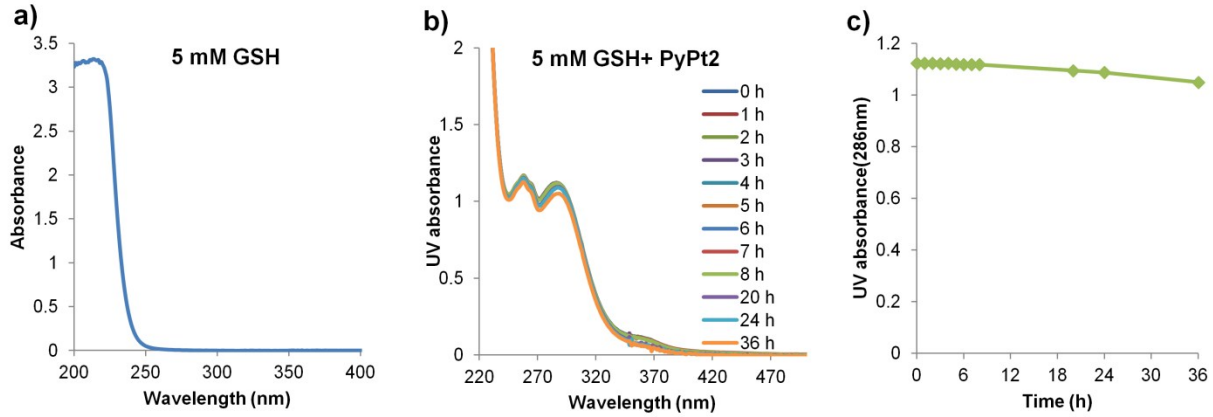


Figure S4. Reduction of PyPt2 by intracellular level of 5 mM GSH in the dark. UV-vis spectrum of (a) 5 mM GSH in water and (b) 5 mM GSH plus PyPt2 at different time points were recorded. The reduction kinetics was plotted by collecting the change of the peak UVA absorbance at 286 nm versus incubation time.

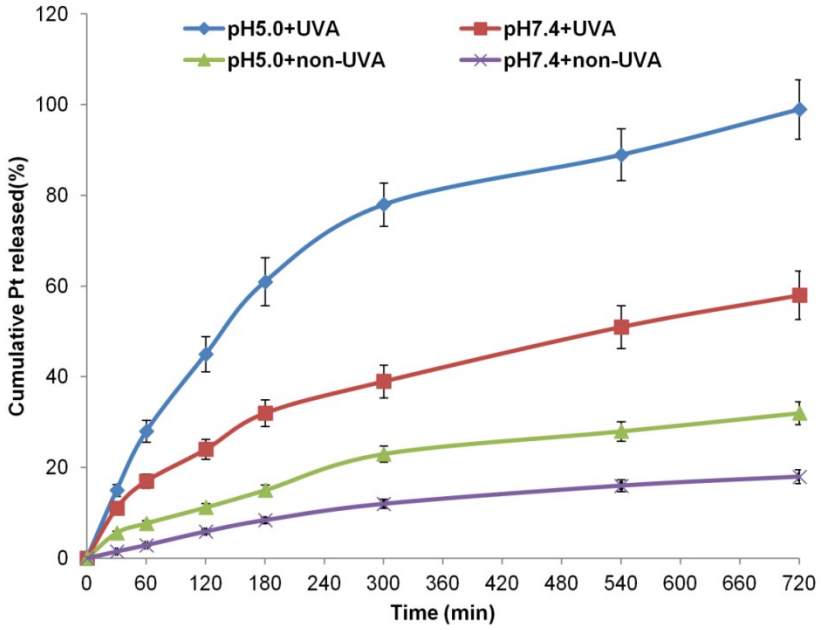


Figure S5. Drug release of PyPt-NP at pH5.0 and pH7.4 in the dark or upon continuous UVA irradiation (UV365, 1. 8 mW/cm^2) at 37 °C. Data were shown as mean values ± S.T.D (n=3).

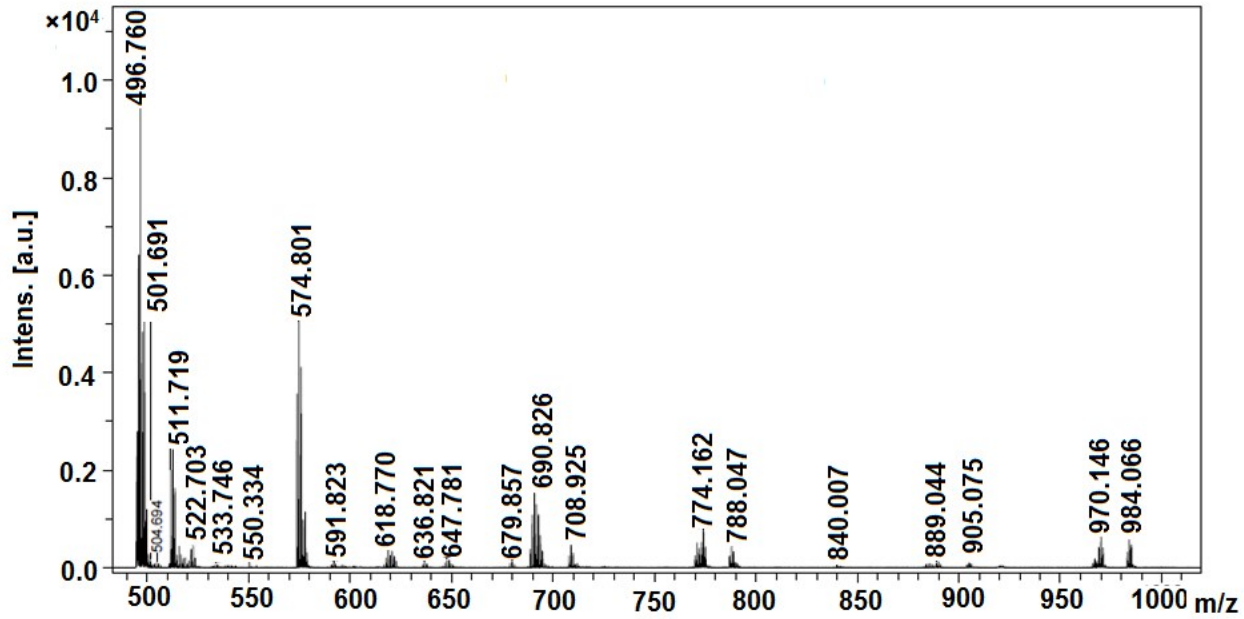
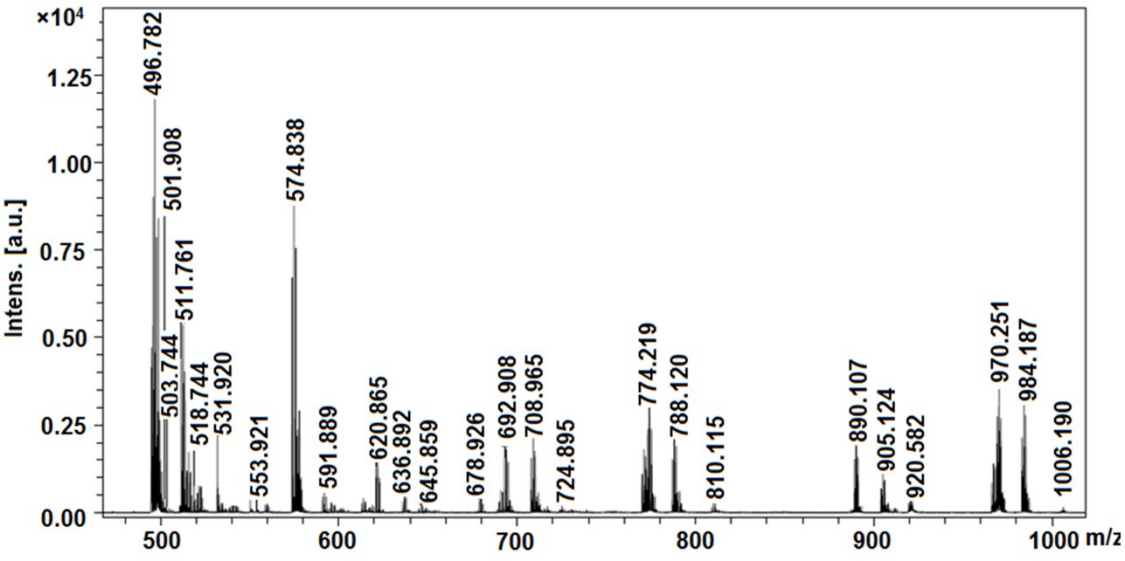


Figure S6. Reaction of t,t-Pt(NH_3)(pyridine)Cl_2 (1 mM in water) with 5'-GMP(2 mM in water) at 37 °C as monitored by MALDI-TOF-MS. The peak at m/z equal to 984.066 and 970.0146 can be assigned to the bis-adducts of t,t-Pt(NH_3)(pyridine)(5'-GMP)$_2$ and t,t-Pt(pyridine)(5'-GMP)$_2$(loss of one NH_3 in the mass spectroscopy).

a)



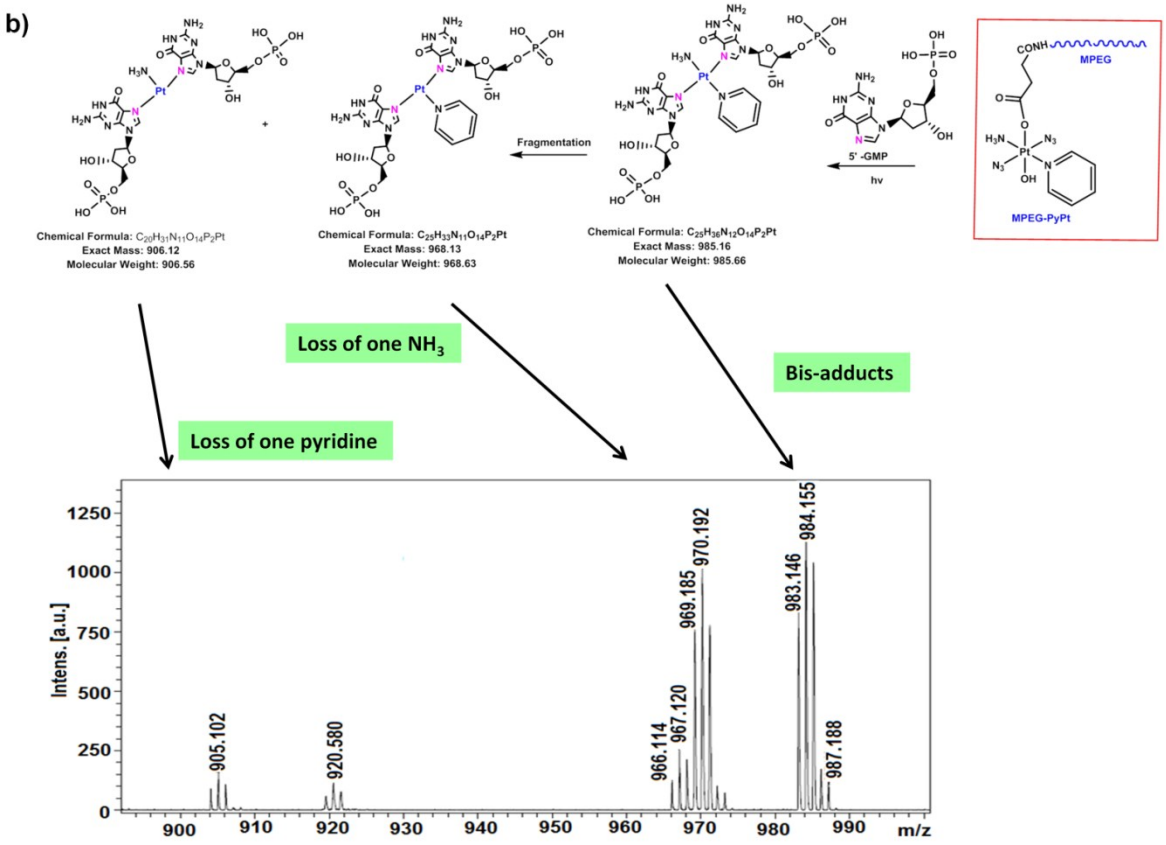


Figure 7. Reaction products of mPEG-PyPt conjugate (5 mg conjugate /ml in water) with 5'-GMP (2 mM in water) upon UVA irradiation. The MALDI-TOF-MS images of the products outside the dialysis bag were shown in (a). Possible UVA activation process and binding with 5'-GMP as well as the expanded view of the major peaks were shown in (b). The peak at m/z equal to 984.187 and 970.251 can be assigned to the bis-adducts of t,t-Pt(NH_3)(pyridine)(5'-GMP)$_2$ and t,t-Pt(pyridine)(5'-GMP)$_2$(loss of one NH_3 in the mass spectroscopy)

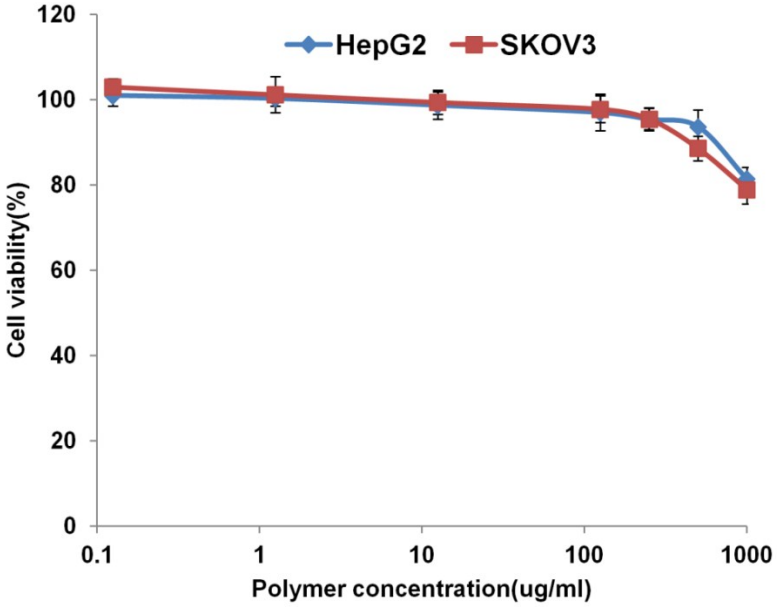


Figure S8. Polymer toxicity against HepG2 and SKOV3 cancer cells at a concentration from 0.1 μg/ml to 1000 ug/ml at 48 h. Cell viability was measured by MTT assay. Polymer was dissolved in culture media and dosed directly to the cancer cells. Data were shown as mean values ± S.T.D (n=4). As high as 1 mg/ml, the polymer showed limited toxicity, denoting the relative compatibility of the polymeric carriers.

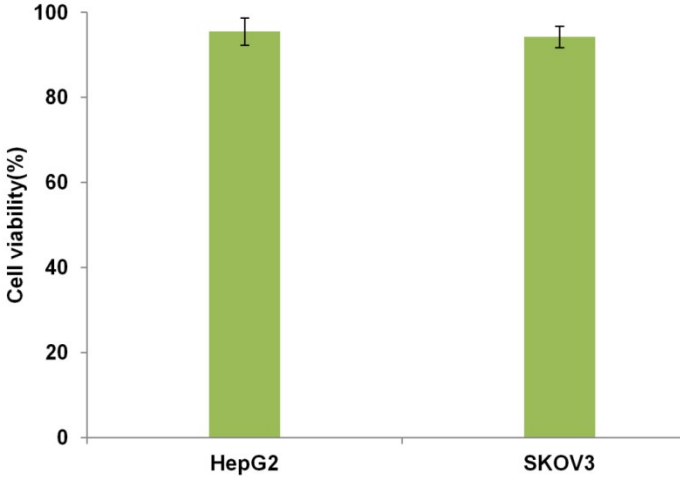


Figure S9. UVA Light toxicity against HepG2 and SKOV3 cancer cells. UVA light (UV365, 1.8 mW/cm2) was used to irradiate the cancer cells for 1 h, then the cells were left to growth for another 47 h. The cell viability was then tested by standard MTT assay. Data were shown as mean values ± S.T.D (n=4).

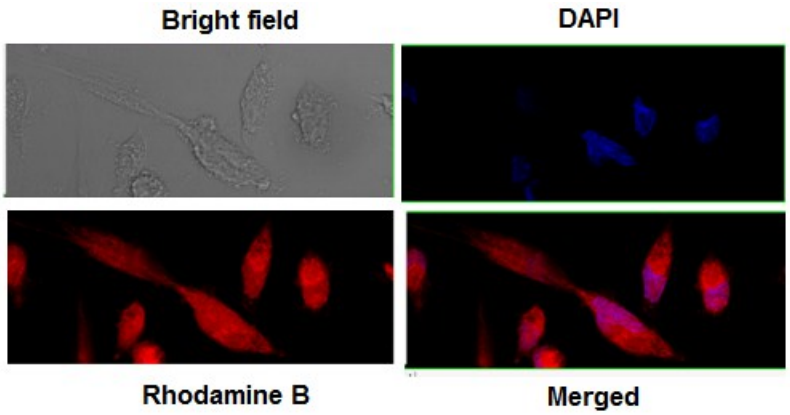


Figure 10. Confocal laser scanning of PyPt-NP labeled with Rhodamine B(5 μg/ml) at 4 h incubation with A2780DDP cells. The red fluorescence comes from Rhodamine B, indicating the intracellular distribution of PyPt-NP in the cells everywhere. DAPI at 1 μg/ml (2-(4-amidinophenyl)-1H -indole-6-carboxamidine) was used to stain the cell nucleus.

Reference:

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