Supporting Information

of

Polyprodrug-based nanoplatform for cisplatin prodrug delivery and combination cancer therapy

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1. Materials

Dimethylformamide (DMF), diethyl ether, *cis*-diammineplatinum(II) dichloride ($Pt(NH_3)_2Cl_2$, cisplatin), mercaptoethanol, acetic acid, 4-dimethylaminopyridine (DMAP), potassium fluoride (KF), potassium hydroxide (KOH), triethylamine (TEA), trifluoroacetic acid (TFA), *p*-toluenesulfonic acid (PTSA), 1,6-hexanediol (HDO), dichloromethane (DCM), and sebacic anhydride were purchased from Sigma-Aldrich and used directly. Mitoxantrone dihydrochloride (MTO.2HCl) was obtained TSZCHEM^{TM}. Potassium superoxide (KO_2) was provided by Alfa Aesar and used as received. 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Thermo Fisher Scientific. RPMI 1640 medium, fetal bovine serum (FBS), and trypsin were purchased from Gibco BRL. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine- *N*-(polyethylene glycol)-3000 (ammonium salt) (DSPE-PEG_{3k}) was acquired from NANOCS. All other reagents and solvents are of analytical grade and used without further purification.

2. Synthesis of acetyl protected mercaptoethanol (1)

The acetyl protected mercaptoethanol was synthesized according to previous report [1]. In brief, mercaptoethanol (4.84 g, 62 mmol) and KF (4.4 g, 76 mmol) were suspended in 100 mL of acetic acid and the mixture was stirred at 80 ^{o}C for 16 h. Subsequently, the mixture was diluted with deionized water, and the crude product was extracted with ethyl acetate. After multiple washings with saturated sodium bicarbonate and brine, the organic phase was dried over Na_2SO_4. The purified product was obtained as liquid after passing flash silica gel column. The synthesis route is shown in Scheme S1. The ^{1}H-NMR spectrum is shown in Fig. S1.

3. Synthesis of acetyl protected thioketal containing monomer (2)

2,2-Dimethoxypropane (0.42 g, 4 mmol), acetyl protected mercaptoethanol (1.2 g, 10 mmol), and PTSA (0.57 g, 5 mmol) were dissolved in 25 mL of benzene. After addition of 5Å molecular sieves

(10 g), the mixture was stirred at room temperature for 24 h. Subsequently, the sieves were removed and the solvent was evaporated to give the crude product, which was then purified via column chromatography using a gradient mobile phase from 100% hexane to 50% hexane and 50% ethyl acetate [2]. The synthesis route is shown in Scheme S1. The ^{1}H-NMR spectrum is shown in Fig. S2.

4. Synthesis of thioketal containing monomer (3)

Acetyl protected thioketal containing monomer (1.17 g, 4 mmol) and KOH (1.0 g, 18 mmol) were suspended in 15 mL methanol, and the mixture was stirred at room temperature for 16 h. Thereafter, the solvent was removed by rotary evaporator, and the residues were dissolved in deionized water. After adding hydrochloric acid solution to neutralize the solution pH, ethyl acetate was added to extract the product. After drying over Na_2SO_4 and removal of the solvent, the thioketal containing monomer was collected as liquid. The synthesis route is shown in Scheme S1. The ^{1}H-NMR spectrum is shown in Fig. S3.

5. Synthesis of BOC protected MTO (4)

The BOC protected MTO was synthesized according to previous report [3]. In brief, MTO.2HCl (0.52 g, 1 mmol) and TEA (5 mL) were dissolved in 100 mL of methanol. After stirring in ice-salt bath for 1 h, BOC_2O (0.87 g, 4 mmol) dissolved in 10 mL of THF was added dropwise. The mixture was then allowed to stir at room temperature for 10 h. After removal of the solvent, the residue was dissolved in 50 mL of ethyl acetate and washed with saturated K_2CO_3 solution. After drying over Na_2SO_4 and then removal of solvent, the BOC protected MTO was purified by silica gel column chromatography using a mixture of hexane, ethyl acetate and methanol in the volume ratio of 10:5:1. The synthesis route is shown in Scheme S2. The ^{1}H-NMR spectrum is shown in Fig. S4.

6. Synthesis of MTO-based polyprodrug

The MTO-based polyprodrug (polyMTO) was synthesized according to our previous report [4]. Thioketal containing monomer (78.5 mg, 0.4 mmol), triphosgene (472 mg, 1.6 mmol), and DMAP (196 mg, 1.6 mmol) are dissolved in 10 mL of DCM and the mixture was stirred for 1 h under a nitrogen atmosphere. Subsequently, BOC-protected MTO (258 mg, 0.4 mmol) dissolved in 5 mL of DCM was added and the mixture was stirred for another 48 h. Thereafter, the mixture was concentrated and cold ethyl acetate was added. After placing at 4 ^{o}C for 24 h, the precipitation was collected through centrifugation and then washed with ethyl acetate thrice. After drying in vacuum for 24 h, the product was dissolved in DMF and then transferred to dialysis tube (MWCO 3000). After dialyzing against DMF for 48 h followed by deionized water for 24 h, the BOC protected MTO-based polyprodrug was obtained after freeze-drying under vacuum. To remove the BOC protected group, the BOC protected polyprodrug was dissolved in 15% TFA/DCM (V/V). After stirring at room temperature for 30 min, excess TEA was added to neutralize TFA and the mixture was concentrated by rotary evaporator. The residue was dissolved in DMF and then transferred to dialysis tube (MWCO 3000). After dialyzing against DMF for 48 h followed by deionized water for 24 h, the final MTO-based polyprodrug (denoted as polyMTO) was collected after freeze-drying under vacuum. The synthesis route is shown in Scheme S2. The ^{1}H-NMR spectrum of polyMTO is shown in Fig. S5. The synthesis of the control polyMTO without thioketal group was similar as the method described above by changing the thioketal containing monomer into HDO. The structure and ^{1}H-NMR spectrum of the control polyMTO is shown in Fig. S6.

7. Gel permeation chromatography (GPC)

Number- and weight-average molecular weights (M_n and M_w, respectively) of the polyprodrug were determined by Agilent Technologies 1260 Infinity. DMF containing 10 mM LiBr was used as the eluent at a flow rate of 0.5 mL/min. Waters millennium module software was used to calculate molecular weight on the basis of a universal calibration curve generated by linear PMMA standard of

narrow molecular weight distribution.

8. High-performance liquid chromatography (HPLC)

HPLC analysis was performed on an Agilent Technologies 1200 Series system. The isocratic mobile phase consisted of acetonitrile and sodium phosphate (10 mM, pH 2.3) in volume ratio of 19:81. Triethylamine (0.1%) was added to the mobile phase to prevent peak tailing. The detector was set at 610 nm for data collection and analysis.

9. Investigation of ROS-responsive behavior of the polyprodrug

GPC and HPLC analysis were used to study the ROS-responsive behavior of the polyprodrug. The polyprodrug (1 mg) was dissolved in 1 mL of DMF/H_2O (9:1, V/V) and then KO_2 (3.6 mg, 0.05 mmol) was added to obtain a solution with KO_2 concentration of 50 mM. At predetermined intervals, 100 μL of the solution was taken for GPC and HPLC analysis.

10. Synthesis of *cis,trans,cis*-[$PtCl_2(OH)_2(NH_3)_2$]

Cisplatin (2 mmol, 0.6 g) was suspended in 10 mL of DMF and 4 mL of 30% H_2O_2 was added dropwise to the suspension. After dissolving in dark for 24 h, the mixture was placed at 4 ^oC overnight. The precipitation was collected and washed with ice-cold water, ethanol, and diethyl ether, respectively. The product was finally obtained through drying under vacuum.

11. Synthesis of cisplatin prodrug

The cisplatin prodrug was synthesized according to previous report through the reaction between *cis,trans,cis*-[$PtCl_2(OH)_2(NH_3)_2$] and sebacic anhydride [5]. In brief, sebacic anhydride (2.4 mmol, 0.44 g) was dissolved in 2 mL of DMF and then added to a 20 mL DMF suspension of *cis,trans,cis*-[$PtCl_2(OH)_2(NH_3)_2$] (0.6 mmol, 0.2 g). The mixture was then stirred at 60 ^oC for 16 h. Thereafter, the

solution was filtered and the filtration was concentrated using a rotary evaporator. The obtained residue was added dropwise to cold ether (50 mL) and the precipitation was collected. After washing with cold ether thrice, the cisplatin prodrug was finally collected through drying under vacuum. The synthesis scheme of the cisplatin prodrug is shown in Fig. S8. The ^{1}H-NMR, ^{13}C-NMR and Pt^{195}-NMR spectra of this prodrug are shown in Fig. S9-S11, respectively.

12. Preparation and characterizations of nanoparticles (NPs)

The polyprodrug NPs were prepared using the classic nanoprecipitation method [6]. The MTO-based polyprodrug, cisplatin prodrug, and DSPE-PEG_{3K} were respectively dissolved in DMF to form a homogenous solution at a concentration of 10 mg/mL. Subsequently, 20 μL of the cisplatin prodrug solution was taken and mixed with the polyHCPT and DSPE-PEG_{3K} solution. Under vigorous stirring (1000 rpm), the mixture was added dropwise to 5 mL of deionized water. The formed NP dispersion was transferred to an ultrafiltration device (EMD Millipore, MWCO 100 K) and centrifuged to remove the organic solvent and free compounds. After washing with PBS solution (pH 7.4) (3 × 5 mL), the final NPs were dispersed in 1 mL of PBS solution (pH 7.4).

Size and zeta potential of the polyprodrug NPs were determined by using dynamic light scattering (DLS, Malvern Zetasizer). The morphology of NPs was visualized on a Tecnai G^2 Spirit BioTWIN transmission electron microscope (TEM). To determine encapsulation efficiency (EE%) of MTO, 5 μL of the NP solution was taken and mixed with a pre-mixed 20-fold DMSO/H_2O solution (9/1, v/v) containing 50 mM KO_2 and the fluorescence intensity of MTO was measured using Synergy HT multi-mode microplate reader and the EE% of MTO is calculated according to the standard curve. The EE% of cisplatin prodrug was determined using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS).

Through overall consideration of the NP size and drug encapsulation efficiency, the polyprodrug NPs with small size (~90 nm, denoted NP90-Pt) but relatively high cisplatin encapsulation efficacy

(~50%) were chosen for the following experiments.

13. *In vitro* drug release

The polyprodrug NPs were prepared according to the method described above and then dispersed in 1 mL of PBS solution. Subsequently, the NP90-Pt suspension was transferred to a Float-a-lyzer G2 dialysis device (MWCO 100 kDa, Spectrum) that was immersed in PBS (pH 7.4) at 37 ^{o}C containing different concentration of KO_2 (0, 50, and 100 μM). At a predetermined interval, 5 μL of the NP solution was withdrawn and dissolved in 95 μL of DMSO/H_2O (9:1, V/V) containing 50 mM KO_2. After placing at room temperature for 24 h, the fluorescence intensity of MTO was examined by Synergy HT multi-mode microplate reader and the amount of released cisplatin prodrug was examined by ICP-MS. The cumulative drug release was calculated as follows:

$$\text{Cumulative release (\%)} = (M_t / M_\infty) \times 100$$

where M_t is the amount of MTO or cisplatin prodrug released from NPs at time t and M_∞ is the amount of MTO or cisplatin prodrug loaded in the NPs.

14. Cell culture

PCa cell lines (LNCaP, PC3, DU145, 22RV1) were incubated in RPMI 1640 medium with 10% FBS at 37 ^{o}C in a humidified atmosphere containing 5% CO_2.

15. Evaluation of ROS production

PCa cell lines (LNCaP, PC3, DU145) were seeded in 6-well plates (50,000 cells per well) and incubated in 2 mL of RPMI 1640 medium containing 10% FBS for 24 h. Subsequently, the cells were incubated with DCFDA according to the manufactory's protocol. After washing with free medium and digesting using trypsin, the ROS production was analyzed using flow cytometry. To observe the ROS production in the PCa cells, 50,000 cells were seeded in round disc and then incubated with

DCFDA according to the manufactory's protocol. Subsequently, the cells were washed with PBS and then fixed by 4% paraformaldehyde. After staining the nuclei with Hoechst 33342, the cells were viewed under a ZEISS 800 confocal laser scanning microscope (CLSM).

16. Cellular uptake

LNCaP cells were seeded in 6-well plates (50,000 cells per well) and then the NP90-Pt was added. After 24 h incubation, the cells were trypsinized and collected for PE Texas Red and FITC Annexin V staining using PI PE Apoptosis Detection Kit I. The apoptosis analysis was performed using flow cytometry.

17. *In vitro* cytotoxicity

LNCaP cells were seeded in a 96-well plate with a density of 5000 cells/well. After the incubation in 50 μL of 1640 medium containing 10% FBS for 24 h, a fixed amount of NP90-Pt dispersed 50 μL of fresh medium was added and the cells were allowed to incubate for another 48 h. After replacing the medium with 100 μL of fresh medium without FBS, 10 μL of AlamarBlue solution was added to each well. After incubating for another 1 h, the fluorescence intensity (E_x = 530 nm, E_m = 590 nm) was measured by Synergy HT multi-mode microplate reader. The average value of five independent experiments was collected and the cell viability was calculated as follows:

$$\text{Cell viability (\%)} = (FI_{treated} / FI_{control}) \times 100$$

where $FI_{control}$ is the fluorescence intensity obtained in the absence of the NPs and $FI_{treated}$ is fluorescence intensity obtained in the presence of the NPs.

18. Animals

Healthy male BALB/c normal mice and nude mice (4-5 weeks old) were purchased from the Sun Yat-Sen University Experimental Animal Center (Guangzhou, China). All *in vivo* studies were

performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Sun Yat-Sen University.

19. Pharmacokinetics

Healthy male BALB/c normal mice were randomly divided into two groups (n =3) and given an intravenous injection of either (i) free MTO at a 3 mg/kg dose or (ii) NP90-Pt at a 3 mg/kg MTO-equivalent dose and 0.3 mg/kg Pt-equivalent dose. At predetermined time intervals, 20 μL of blood was withdrawn and mixed with 80 μL of 35 wt% H_2O_2. The fluorescence intensity of MTO in the blood was determined by Synergy HT multi-mode microplate reader

20. LNCaP xenograft tumor model

The tumor model was constructed by subcutaneous injection with 200 μL of LNCaP cell suspension (1:1 mixture of medium and Matrigel) with a density of 1×10^6 cells/mL into the back region of healthy male nude mice. When the volume of the tumor xenograft reached 70~100 mm^3, the mice were used for the *in vivo* experiments.

21. Biodistribution

LNCaP tumor-bearing nude mice were randomly divided into two groups (n = 3) and received an intravenous injection of either (i) free MTO at a 3 mg/kg dose, or (ii) NP90-Pt at a 3 mg/kg MTO-equivalent dose and 0.3 mg/kg Pt-equivalent dose. The mice were viewed under a Maestro 2 In-Vivo Imaging System (Cri Inc) at 24 h post injection. Main organs and tumors were then harvested and imaged. To quantify the accumulation of NPs in tumors and organs, the fluorescence intensity of each tissue was quantified by Image-J.

22. Immune response

Healthy male BALB/c normal mice were randomly divided into four groups (n = 3) and given an intravenous injection of either (i) PBS, (ii) mixture of free MTO (3 mg/kg) and cisplatin (0.3 mg/kg), (iii) NP90 at a 3 mg/kg MTO-equivalent dose, or (iv) NP90-Pt at a 3 mg/kg MTO-equivalent dose and 0.3 mg/kg Pt-equivalent dose. Twenty-four hours after injection, blood was collected and serum isolated for measurements of representative cytokines (TNF-α, IL-6, IL-12, and IFN-γ) by enzyme-linked immunosorbent assay or ELISA (PBL Biomedical Laboratories and BD Biosciences) according to the manufacturer's instructions.

23. Histological analysis

Healthy male BALB/c normal mice were randomly divided into four groups (n = 3) and given an intravenous injection of either (i) PBS, (ii) mixture of free MTO (3 mg/kg) and cisplatin (0.3 mg/kg), (iii) NP90 at a 3 mg/kg MTO-equivalent dose, or (iv) NP90-Pt at a 3 mg/kg MTO-equivalent dose and 0.3 mg/kg Pt-equivalent dose. After three consecutive injections, the main organs were collected, fixed with 4% paraformaldehyde, and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin (H&E) and then viewed under an optical microscope.

24. Inhibition of tumor growth

LNCaP tumor-bearing nude mice were randomly divided into four groups (n = 4) and intravenously injected with (i) PBS, (ii) mixture of free MTO (3 mg/kg) and cisplatin (0.3 mg/kg), (iii) NP90 at a 3 mg/kg MTO-equivalent dose, or (iv) NP90-Pt at a 3 mg/kg MTO-equivalent dose and 0.3 mg/kg Pt-equivalent dose once every two days. All the mice were administrated three consecutive injections and the tumor growth was monitored every two days by measuring perpendicular diameters using a caliper and tumor volume was calculated as follows:

$$V = W^2 \times L/2$$

where W and L are the shortest and longest diameters, respectively.

25. Histology

After the aforementioned treatment, the mice were sacrificed at end of the evaluation period, and the tumors were collected. After fixing with 4% paraformaldehyde and then embedding in paraffin, the tissue was sectioned and stained with hematoxylin-eosin (H&E) and then viewed under an optical microscope.

26. Statistical analysis

Statistical significance was determined by a two-tailed Student's t test assuming equal variance. A p value < 0.05 is considered statistically significant.

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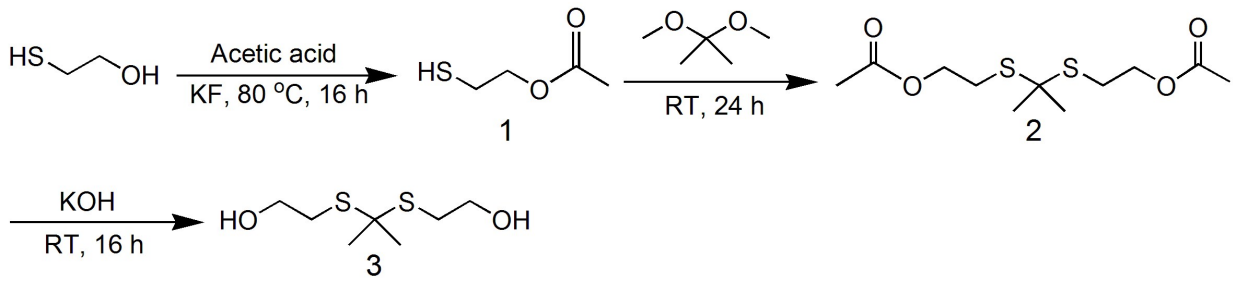
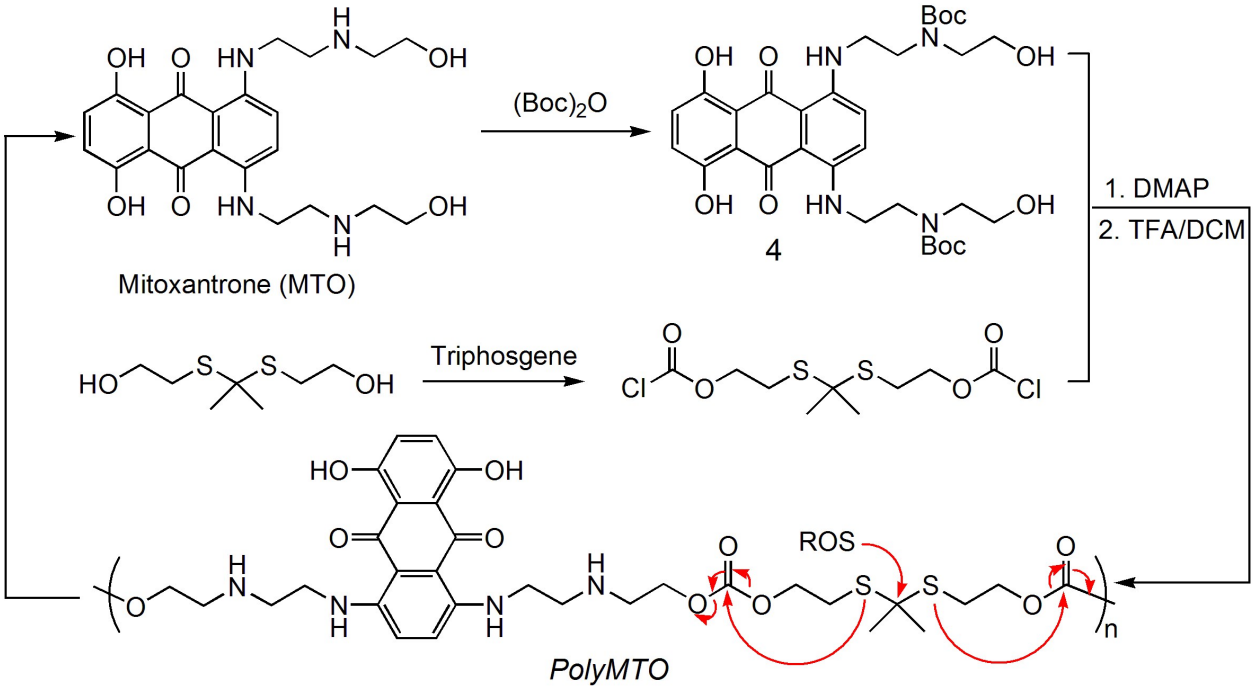


Scheme S1. Synthesis route of acetyl protected mercaptoethanol (**1**), acetyl protected thioketal containing monomer (**2**), and thioketal containing monomer (**3**).



Scheme S2. Synthesis route of BOC protected MTO (**4**) and MTO-based polyprodrug (polyMTO), and the mechanism of the ROS-triggered chain-breakage patterned release of intact MTO.

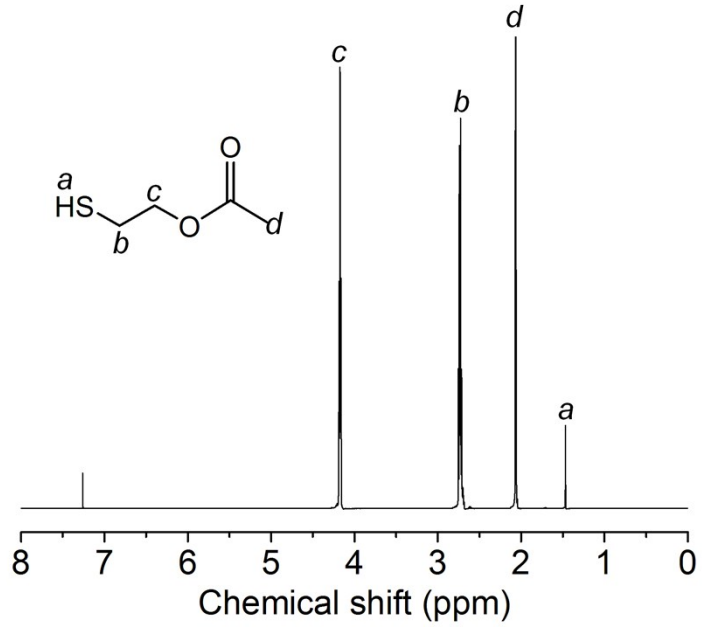


Fig. S1. ^{1}H-NMR spectrum of acetyl protected mercaptoethanol (**1**) in $CDCl_3$.

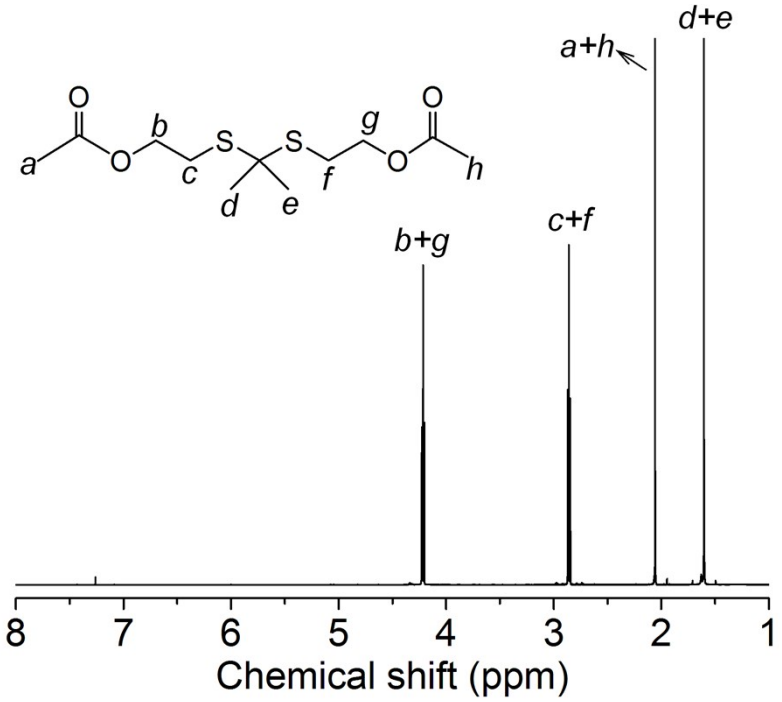


Fig. S2. ^{1}H-NMR spectrum of acetyl protected thioketal containing monomer (**2**) in $CDCl_3$.

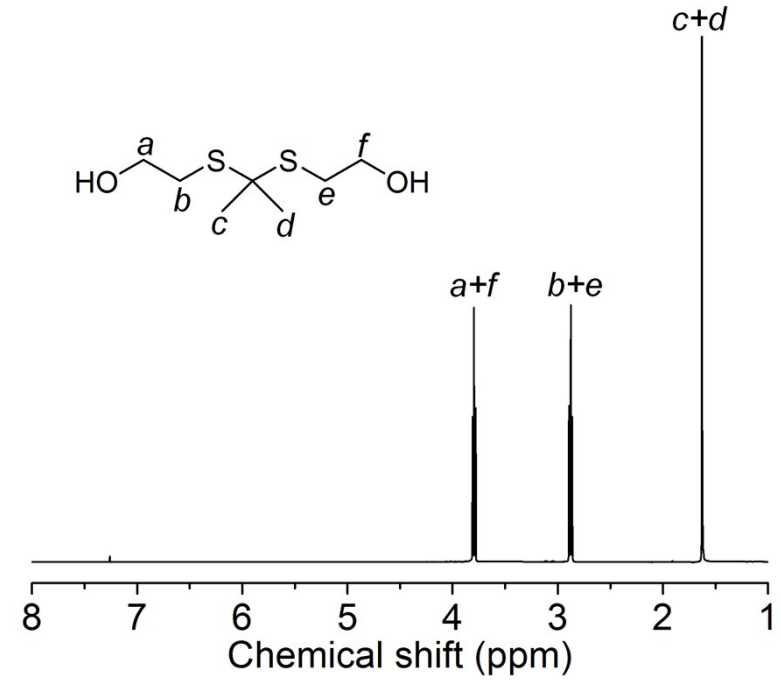


Fig. S3. ^{1}H-NMR spectrum of thioketal containing monomer (**3**) in $CDCl_3$.

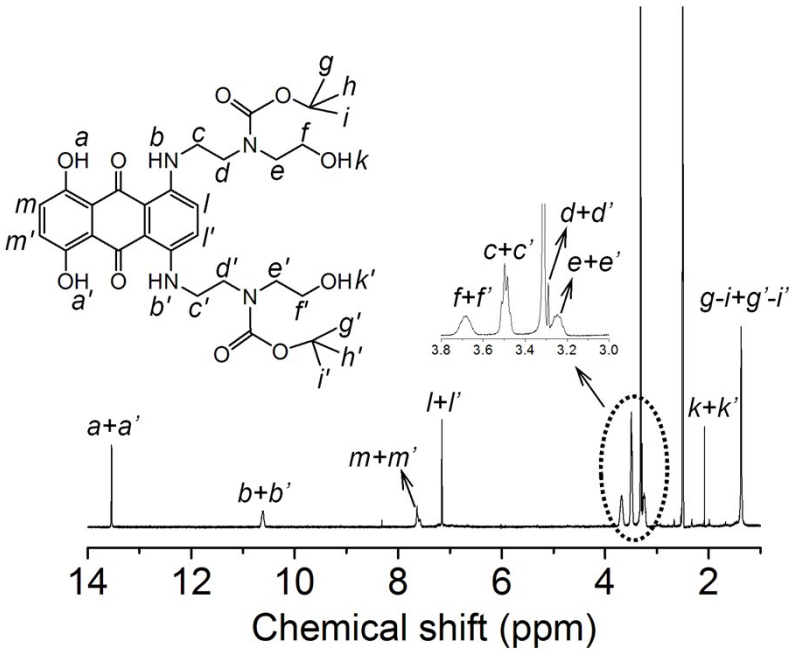


Fig. S4. ^{1}H-NMR spectrum of BOC protected MTO (**4**) in DMSO-d_6.

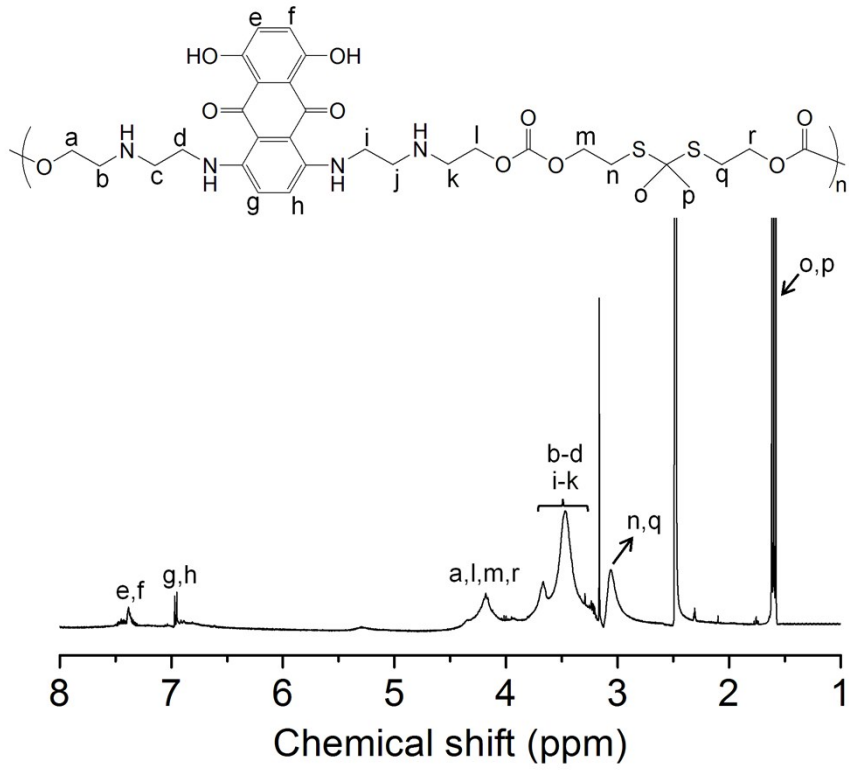


Fig. S5. ^{1}H-NMR spectrum of ROS-responsive polyMTO in DMSO-d_6.

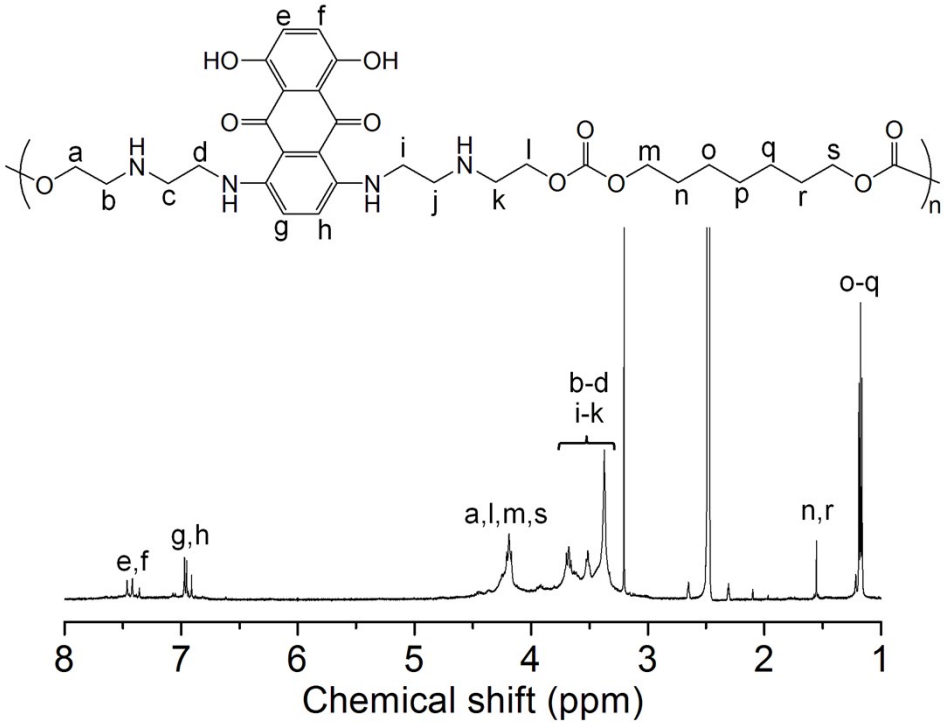


Fig. S6. ^{1}H-NMR spectrum of control polyMTO in DMSO-d_6.

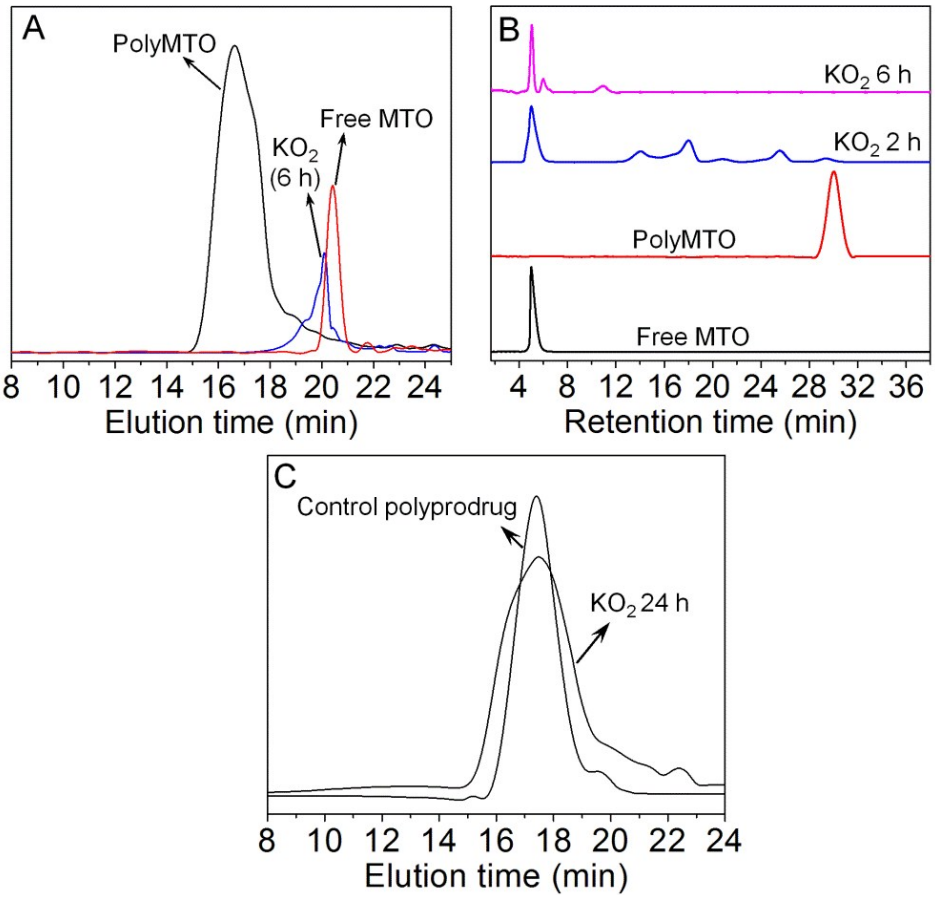


Fig. S7. (A, B) GPC (A) and (B) HPLC profiles of polyMTO incubated in the mixture of DMF and H_2O (9:1, v/v) containing 50 mM KO_2. (C) GPC profile of the control polyprodrug incubated in the mixture of DMF and H_2O (9:1, v/v) containing 50 mM KO_2.

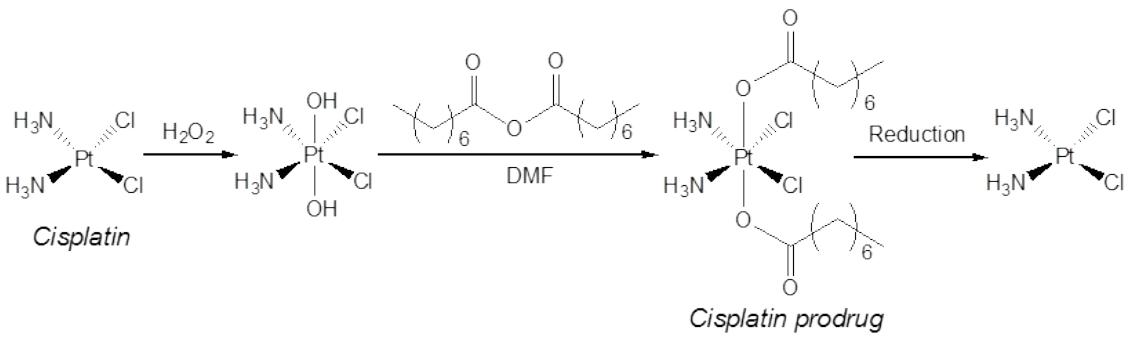


Fig. S8. Synthesis route of the cisplatin prodrug and the reduction of this prodrug into cisplatin with the presence of reductive agents.

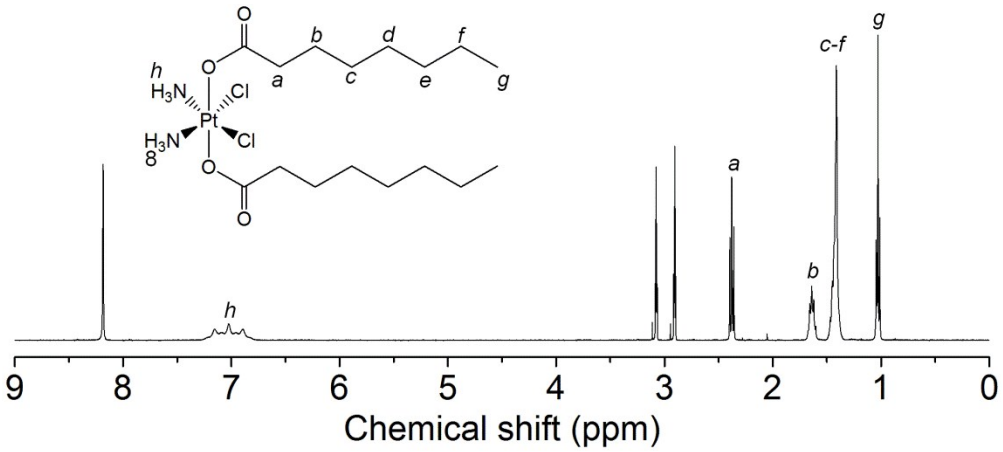


Fig. S9. ^{1}H-NMR spectrum of the cisplatin prodrug in DMF-d_7.

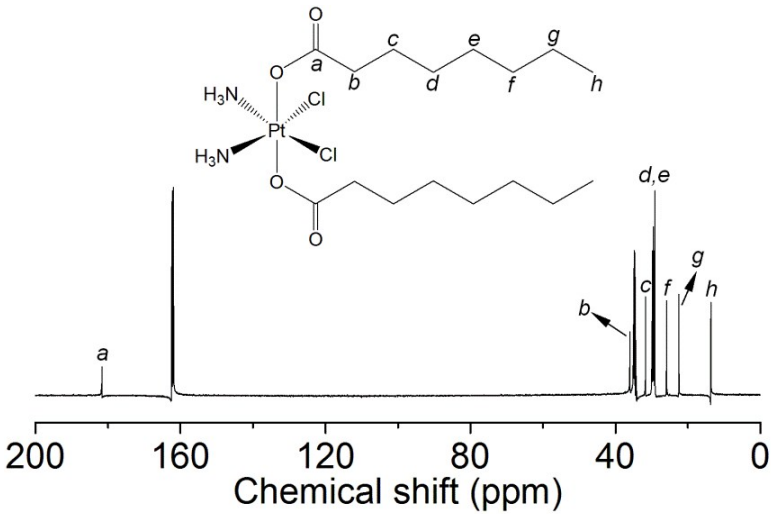


Fig. S10. ^{13}C-NMR spectrum of the cisplatin prodrug in DMF-d_7.

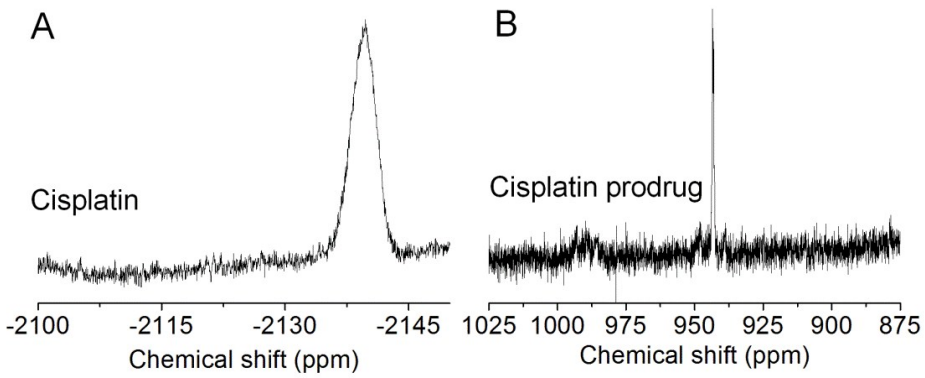


Fig. 11. ^{195}Pt-NMR spectra of cisplatin (A) in D_2O and cisplatin prodrug (B) in DMF-d_7.

No.	NP102-Pt	NP129-Pt	NP141-Pt	NP90-Pt	NP74-Pt
PolyMTO (10 mg/mL in DMF)	150 μL	180 μL	200 μL	200 μL	200 μL
DSPE-PEG (10 mg/mL in DMF)	100 μL	100 μL	100 μL	140 μL	180 μL
Cisplatin prodrug (10 mg/mL in DMF)	20 μL	20 μL	20 μL	20 μL	20 μL
Size (nm)	102.8 ± 3.7	129.3 ± 2.5	141.5 ± 4.2	89.8 ± 2.9	74.1 ± 4.6
Zeta potential (mV)	-9.3 ± 1.9	-4.8 ± 2.4	-2.9± 0.9	-7.3± 3.5	-10.6 ± 3.1
EE% (MTO)	72.2	74.6	80.2	78.5	68.3
EE% (Pt)	21.6	32.6	42.5	49.8	36.4

Fig. S12. Feed compositions and physiochemical properties of the polyprodrug NPs made with the polyMTO, DSPE-PEG, and cisplatin prodrug.

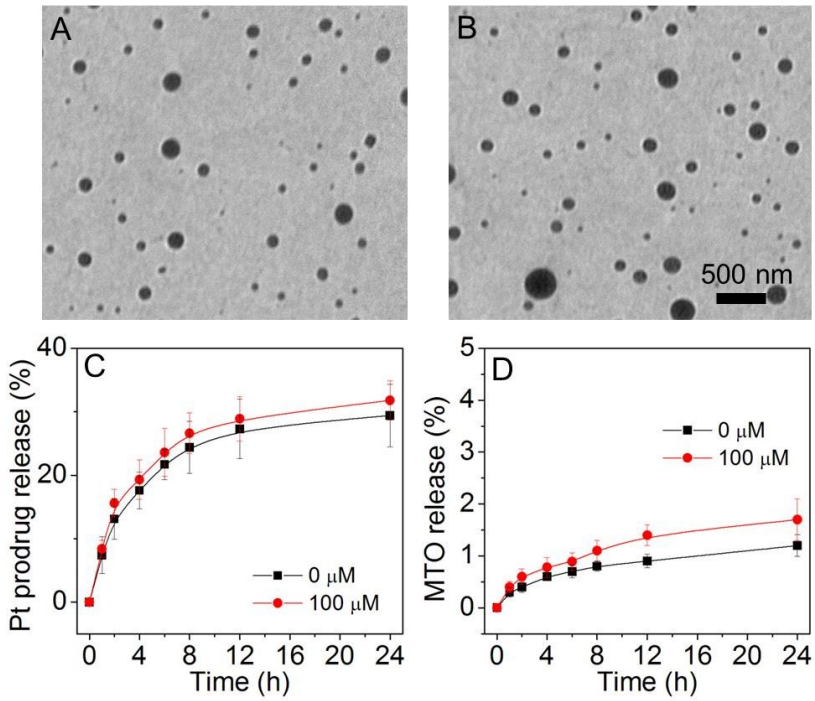


Fig. S13. (A, B) TEM images of the control polyprodrug NPs incubated in PBS solution (A) and the solution containing 100 μM KO_2 for 24 h. (C, D) Cumulative release of cisplatin prodrug (C) and MTO (D) from the control polyprodrug NPs incubated in the PBS solution containing 100 μM KO_2.

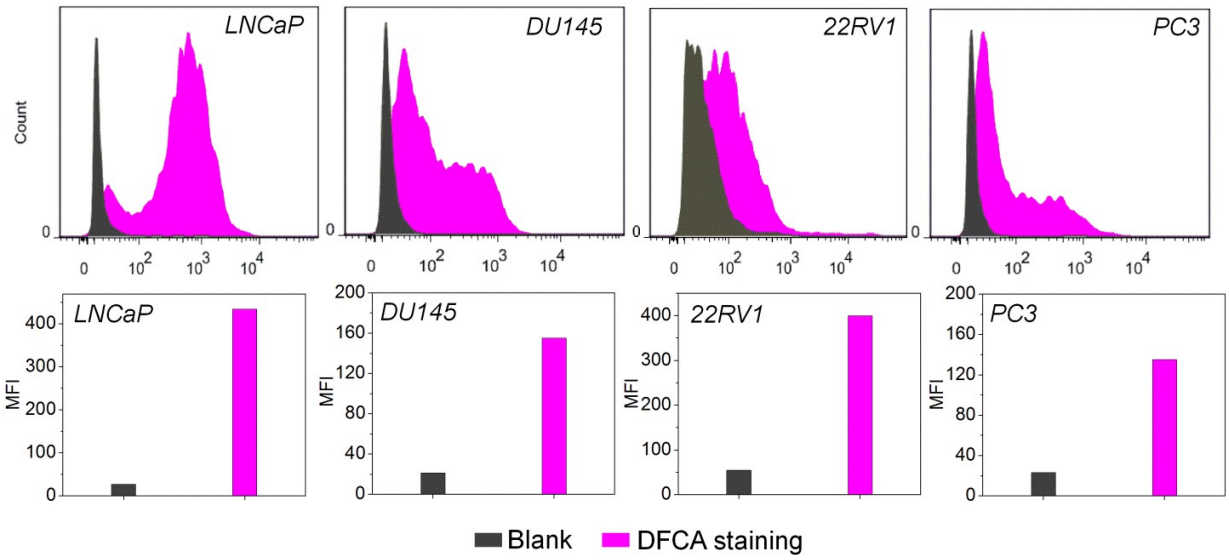


Fig. S14. Flow cytometry profiles and mean fluorescence intensity (MFI) of PCa cells stained by DCFDA for the determination of ROS production.

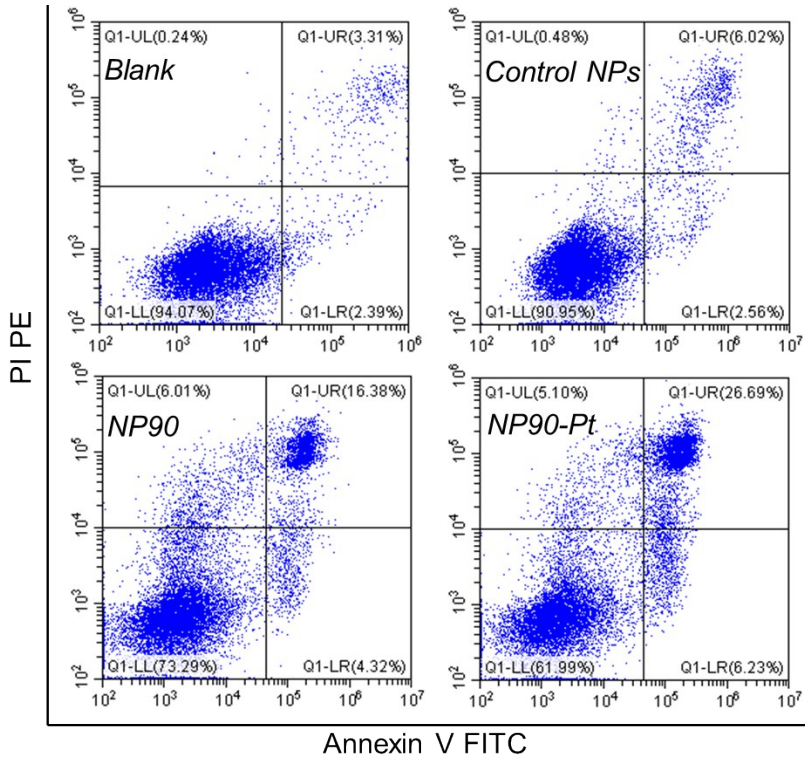


Fig. S15. Flow cytometry analysis of LNCaP cells treated with the control polyprodrug NPs loading with cisplatin prodrug (Control NPs), and the polyMTO NPs (NP90) and the NPs loading cisplatin prodrug (NP90-Pt) for 24 h. The concentration of cisplatin and MTO is 0.2 and 2 mg/L, respectively.

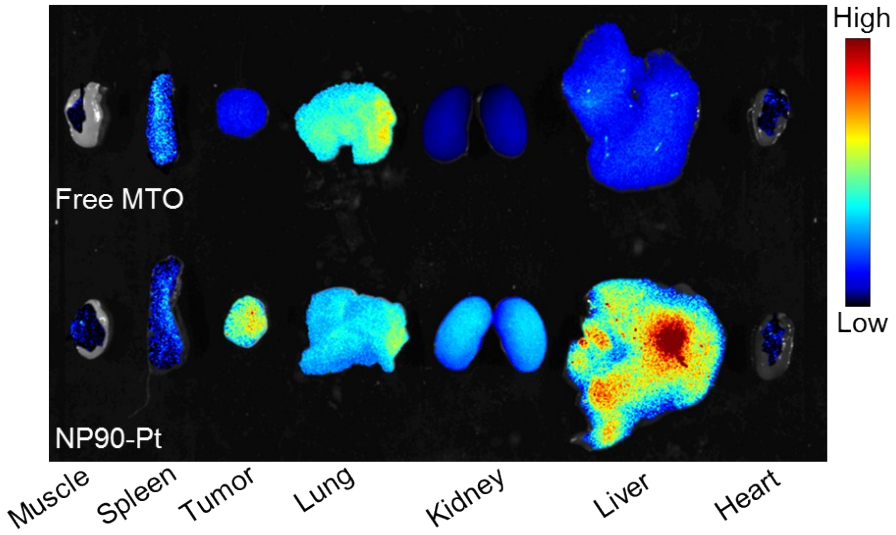


Fig. S16. Overlaid fluorescence image of the tumors and main organs of the LNCaP xenograft tumor-bearing mice at 24 h post injection of free MTO and the NP90-Pt platform.

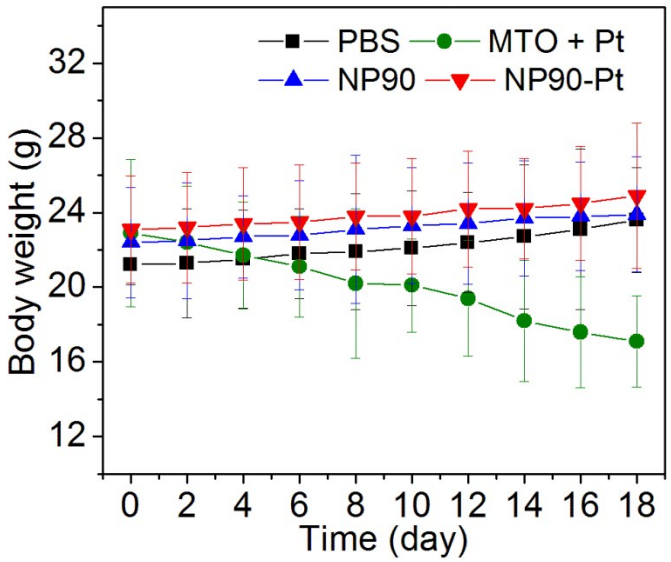


Fig. S17. Body weight of the LNCaP xenograft tumor-bearing mice treated with PBS, the mixture of free MTO and cisplatin (MTO + Pt), and the polyMTO NPs (NP90) and the NPs loading cisplatin prodrug (NP90-Pt).

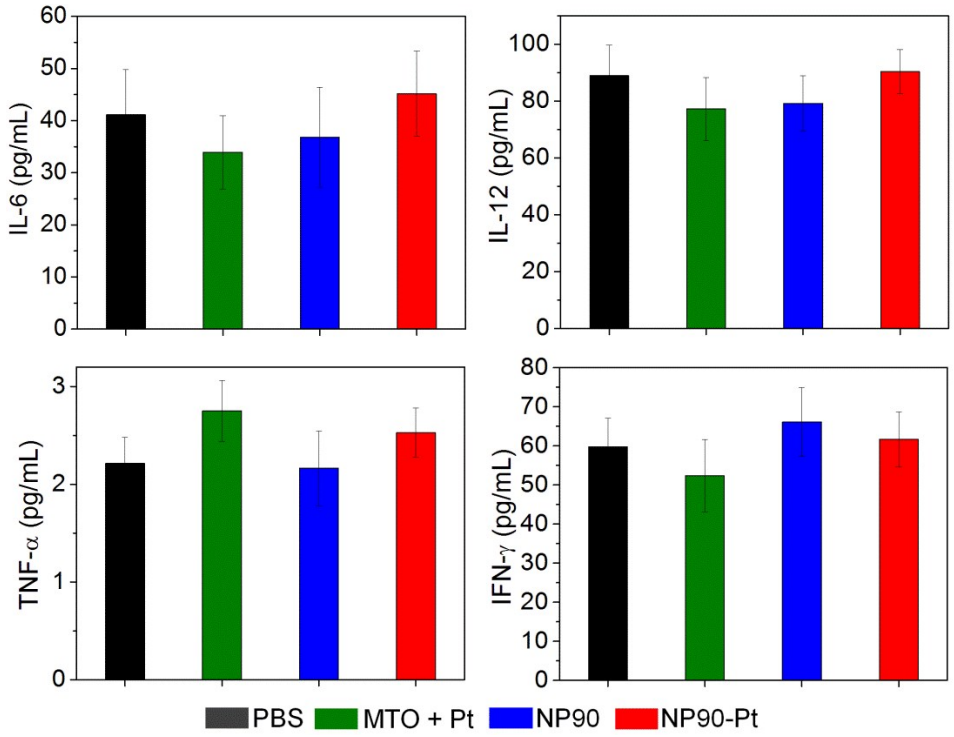


Fig. S18. Serum levels of IL-6, IL-12, TNF-α, and IFN-γ at 24 h post injection of PBS, the mixture of free MTO and cisplatin (MTO + Pt), and the polyMTO NPs (NP90) and the NPs loading cisplatin prodrug (NP90-Pt).

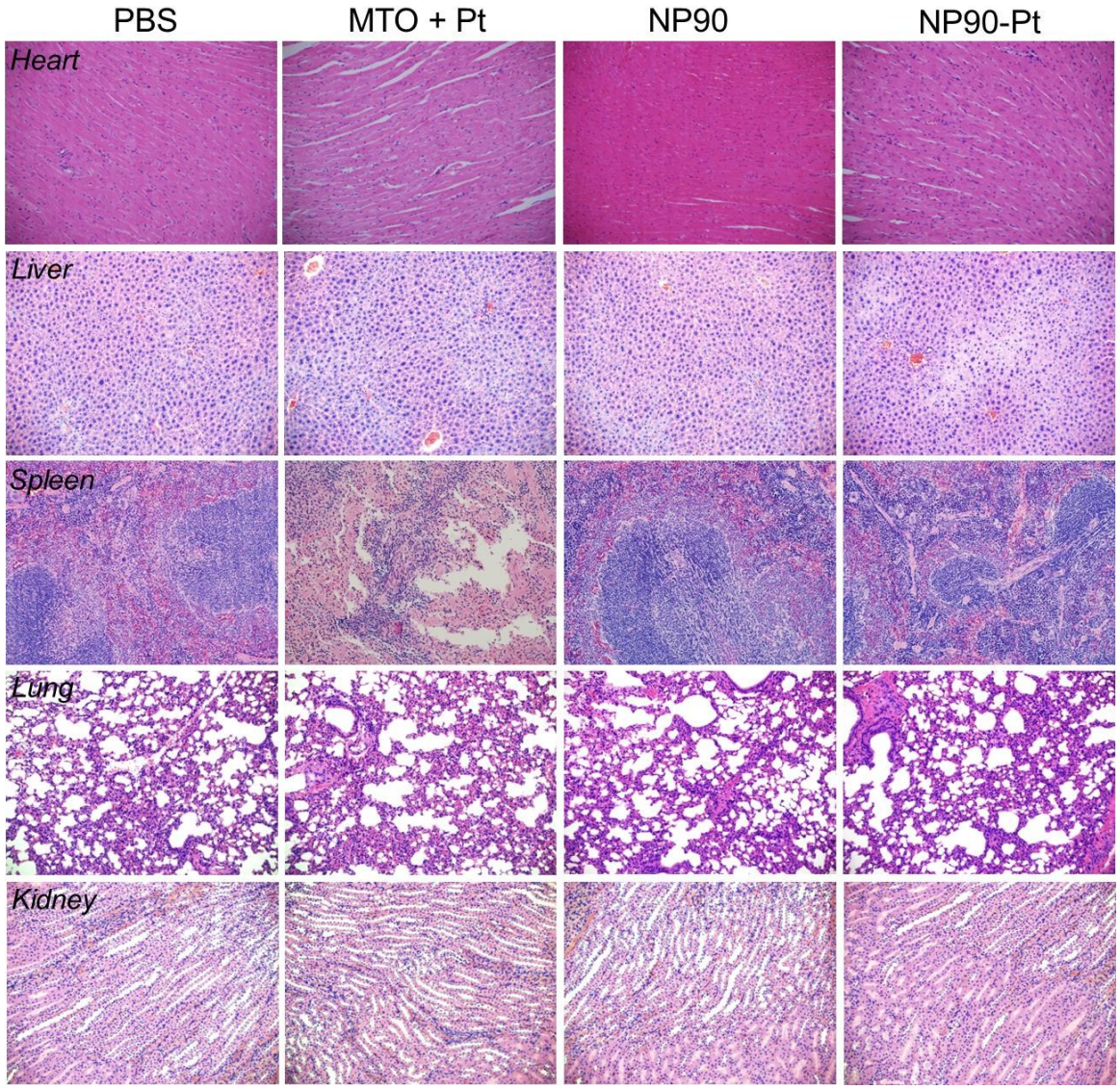


Fig. S19. Histological section of the major organs of the LNCaP xenograft tumor-bearing mice after treatment with PBS, the mixture of free MTO and cisplatin (MTO + Pt), and the polyMTO NPs (NP90) and the NPs loading cisplatin prodrug (NP90-Pt). Hematoxylin-eosin; 100 ×.