Supplementary Information for

Self-Assembled Nanoscale Coordination Polymers Carrying Oxaliplatin and Gemcitabine for Synergistic Combination Therapy of Pancreatic Cancer

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1. Materials and Methods.

All starting materials, unless otherwise noted, were purchased from Sigma Aldrich or Fisher Scientific, and used without further purification. Oxaliplatin was purchased from AK Scientific (USA). The platinum complex, (dach)Pt(BP) complex (dach=R,R-diaminocyclohexane) was synthesized from *cis*, *trans*-[Pt(dach)Cl_2($OH)_2$] according to a previously published method [30, 31]. GMP was synthesized from gem following the previous literature report [36]. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphate sodium salt (DOPA), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000] (DSPE-PEG_{2k}) were purchased from Avanti Polar Lipids (USA).

Cell culture supplies including fetal bovine serum (FBS, Hyclone, USA), RPMI-1640 growth medium (Hyclone, USA), penicillin-streptomycin (Gibco, USA), and phosphate buffered saline (Hyclone, USA) were purchased from Fisher Scientific. AsPC-1 (ATCC# CRL-1682) and BxPc-3 (ATCC# CRL-1687) human pancreatic adenocarcinoma cells and CT26 (ATCC# CRL-2638) murine colon adenocarcinoma were purchased from the Developmental Therapeutics Core of Northwestern University. All cell lines were maintained in RPMI-1640 growth medium supplemented with 10% FBS and 2% penicillin-streptomycin. All cells were maintained at 37 °C with 5 % CO_2 and were cultured according to ATCC recommendations.

Mice (female nu/nu, 6-8 weeks old) were purchased from Harlan Laboratories, Inc. (USA). All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago and University of North Carolina-Chapel Hill.

2. Preparation of NCP-3 particles

Two separate reverse microemulsions (W=7.4) were first prepared by the addition of 0.2 mL of an aqueous 25 mg/mL GMP sodium salt solution (15 μmol) and 0.2 mL of an aqueous 100 mg/mL $Zn(NO_3)_2$ solution (67 μmol) to two separate 5 mL aliquots of 0.3 M Triton X-100/1.5 M 1-hexanol in cyclohexane. The two mixtures were stirred vigorously for 5 minutes. Forty microliters of 1,2-dioleoyl-sn-glycero-3-phosphate sodium salt (DOPA, 11 μmol in $CHCl_3$) was added to the solution containing the GMP and the resulting mixture was stirred for 15 min to obtain a clear solution. The two microemulsions were combined and stirred for an additional 30 min to form DOPA-capped NCP-3. The nanoparticles were washed with 20 mL of ethanol once, 50% (v/v) ethanol/cyclohexane mixture once, and 50% (v/v) ethanol/tetrahydrofuran (THF) twice, and redispersed in THF. The resulting particles were purified and filtered through a 200 nm syringe filter..

Table S1. Sizes and polydispersities of DOPA-NCP and DOPA-Zn control particles in THF (Data are expressed as means±SD).

NCPs	Drug	Number-Ave diameter (nm)	PDI
DOPA-NCP-**1**	Oxaliplatin & gem	39.7±0.8	0.032
DOPA-NCP-**2**	Oxaliplatin	28.7±9.2	0.151
DOPA-NCP-**3**	gem	64.4±0.8	0.058
DOPA-Zn Control	none	25.4±5.2	0.116

3. Characterization of NCP particles

Particle sizes and zeta potentials were determined using dynamic light scattering (DLS) with a Malvern Instruments Zetasizer Nano-ZS. Morphologies of the nanoparticles were observed by transmission electron microscope (TEM, JEM 100CX-II). Dry nanoparticles were weighed and digested in concentrated nitric acid overnight and then diluted with water for determining the oxaliplatin drug loading by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700x ICP-MS). Samples were introduced via a concentric glass nebulizer with a free aspiration rate of 0.4 mL/min, a Peltier-cooled double pass glass spray chamber, and a quartz torch. A peristaltic pump carried samples from an ASX-520 autosampler (Agilent) to the nebulizer. All standards and samples were prepared by digesting a known amount of sample in concentrated nitric acid overnight and then diluting with water to 2% HNO_3 by volume.

GMP loading was determined by UV-Vis spectroscopy and thermogravimetric analyses (TGA). UV-Vis absorption spectra were obtained using a Shimadzu UV-2401PC UV-Vis Spectrophotometer. TGA was performed using a Shimadzu TGA-50 equipped with a platinum pan and heated at 3 ^{o}C/min in air. Different concentrations of GMP and (dach)Pt(BP) solution in 6 M HCl were prepared as standards. A baseline spectrum was recorded using 6 M hydrochloric acid. The absorbance of GMP at 275 nm was recorded. Particles were digested overnight in 6 M hydrochloric acid. The concentration of GMP in the solution was determined by the corresponding absorbance at 275 nm. Using the Pt-drug loading from ICP-MS and corresponding to the standards of oxaliplatin, the absorbance from Pt was subtracted from the total absorbance to determine the GMP-drug loading.

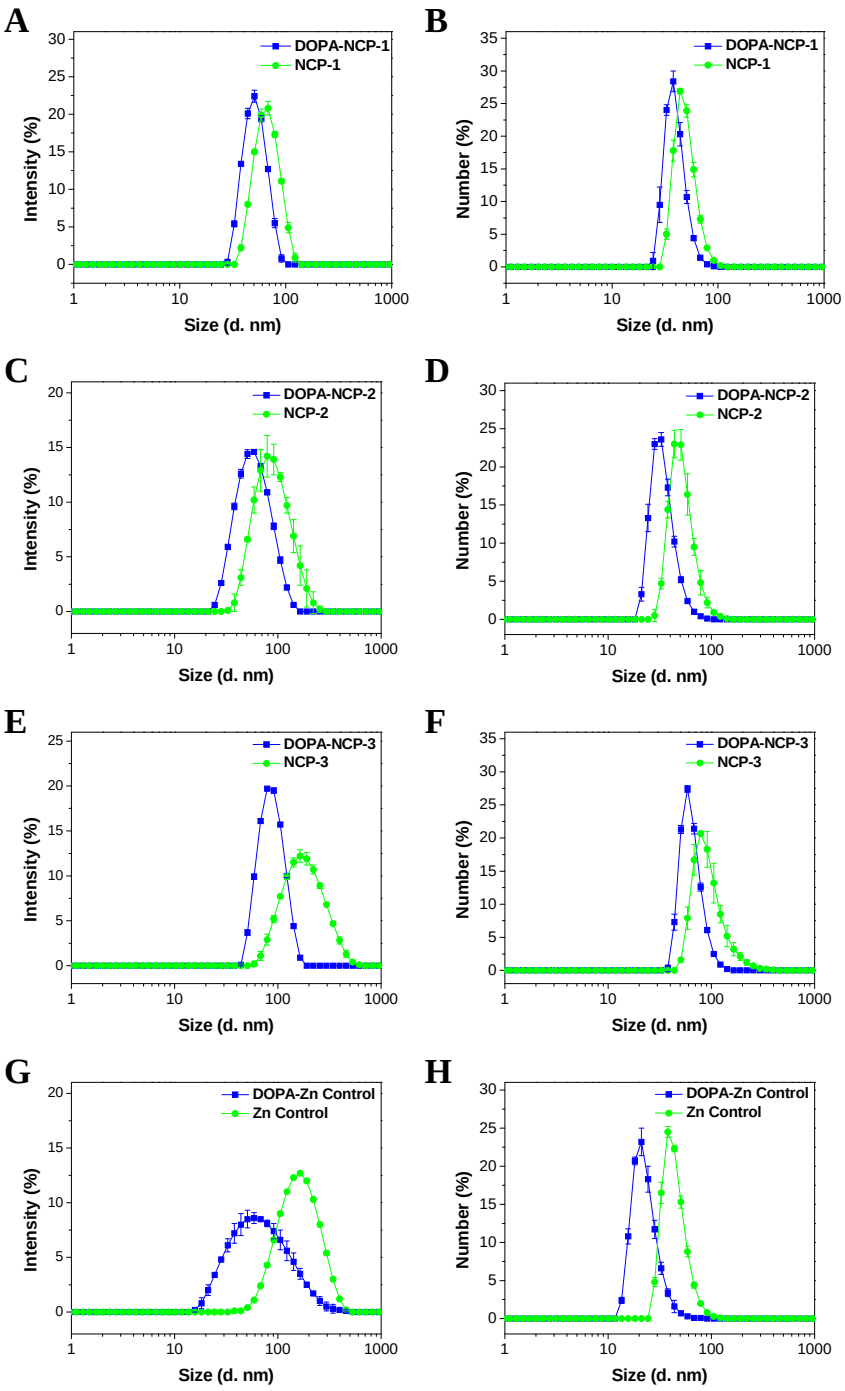

Fig. S1. Intensity-average and Number-average size distribution of NCP-1 (A, B), NCP-2 (C, D), NCP-3 (E, F), and Zn Control (G, H) particles. Bare and PEG particles were measured in THF and PBS buffer, respectively.

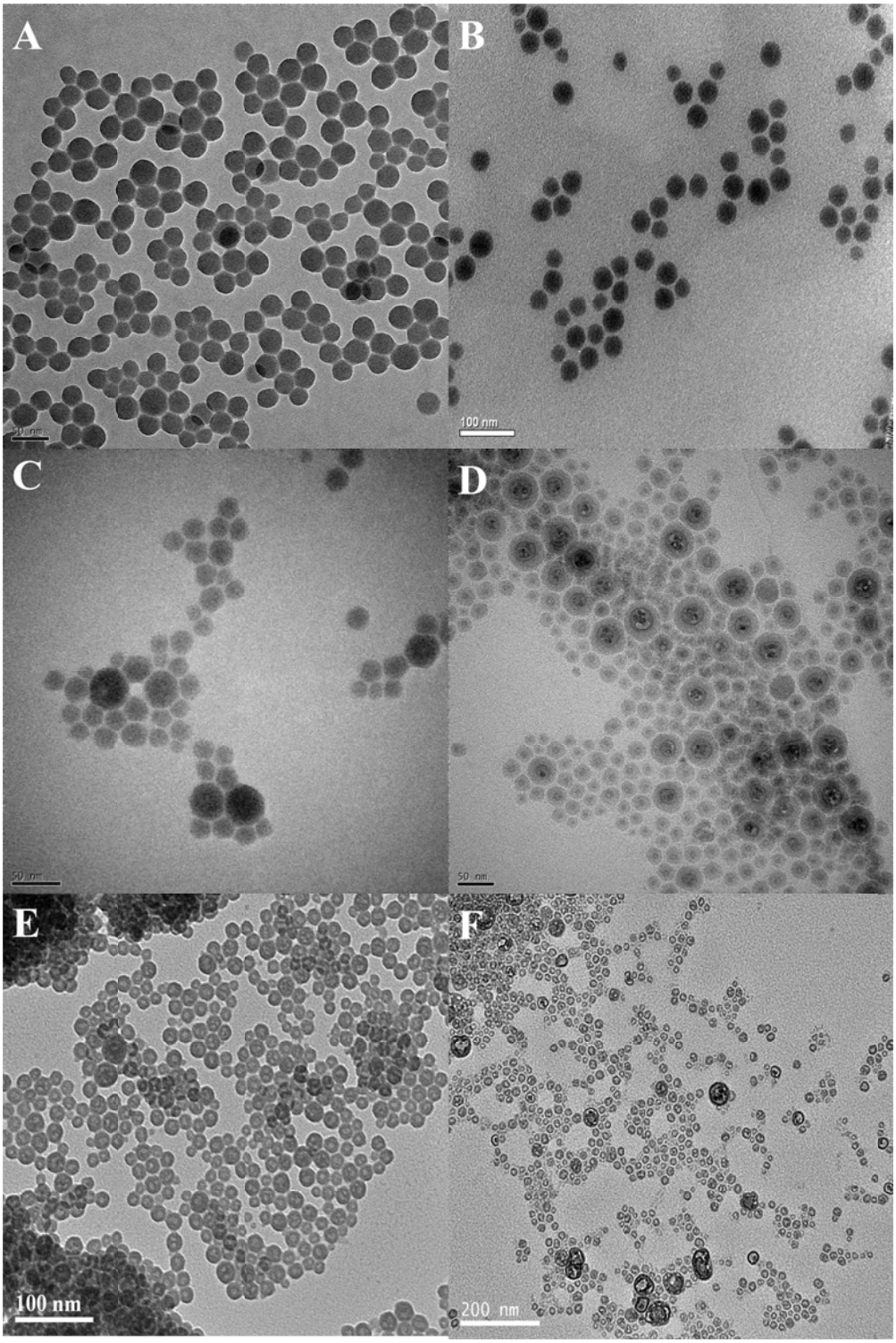

Fig. S2. TEM micrographs of DOPA-NCP-2 (A), NCP-2 (B), DOPA-NCP-3 (C), NCP-3 (D), DOPA-Zn Control (E), and Zn Control (F)

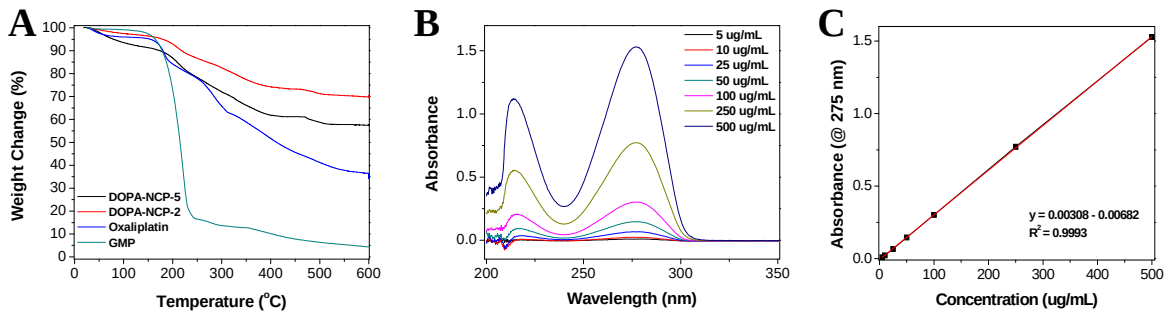

Fig. S3. TGA analysis (A) and UV-Vis analysis (B, C) of DOPA-NCP-1 to determine GMP wt% loading.

4. In vitro stability studies

In vitro stability of NCP particles was evaluated by bovine serum albumin (BSA) binding and time-dependent drug release. In the BSA binding analysis, 0.5 mg of NCP-1 was dispersed in 1 mL of PBS, and the particle diameter was measured by DLS. BSA (2 mg) was then added to the nanoparticle suspension. DLS measurements were obtained every 10 min for 10 hours to determine the size of nanoparticles in the suspension over time. Differences in the size and count rate of the particles were used to determine extent of protein binding and sedimentation.

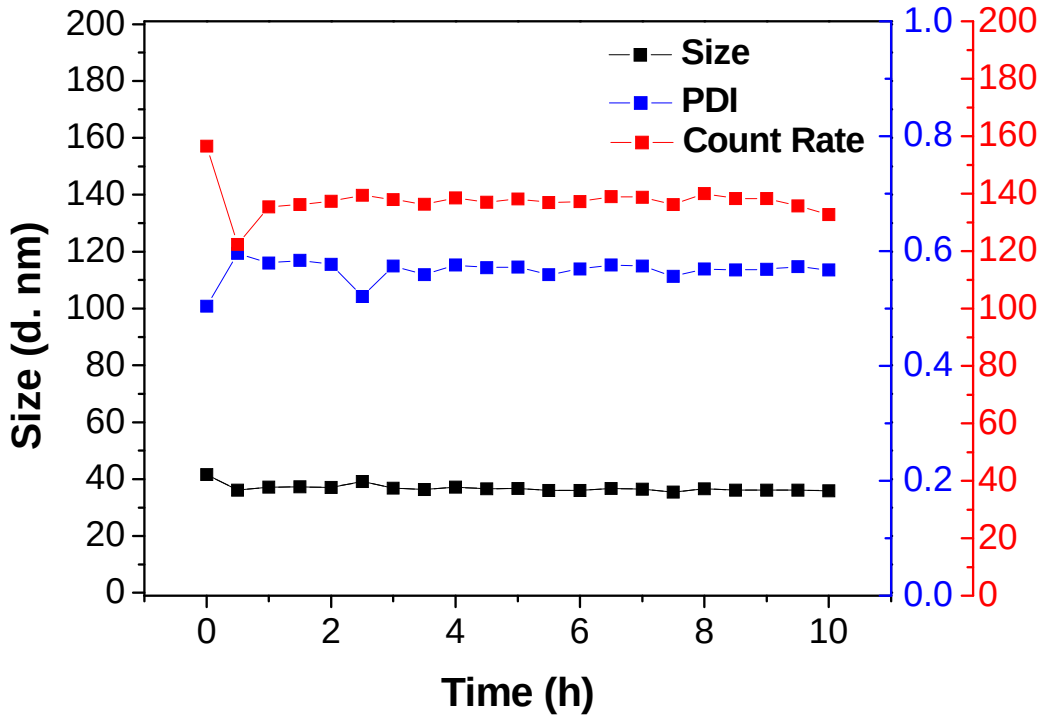

Fig. S4. Stability test of NCP-1 after PEGylation in PBS buffer with BSA at 37 °C

5. *In Vitro* Drug Release

The release profiles were performed in 400 mL of 5 mM PBS buffer (pH=7.4) in a beaker under stirring and kept at 37°C. DOPA-NCP-1 or NCP-1 (3 mg) were suspended in 4 mL of the buffer solution in a 10,000 MWCO pleated dialysis bag. The dialysis bag containing the nanoparticle suspension was then added to the beaker, and the system was incubated at 37 °C. Periodically, 1 mL aliquots of the solution were removed, and 1 mL of fresh buffer solution was added to the beaker. The removed aliquots were collected and analyzed by ICP-MS for Pt content and by UV-Vis for GMP content. For the release experiment under reducing environments, 5 mM cysteine was added to 5 mM PBS to simulate the intracellular reductant concentration.

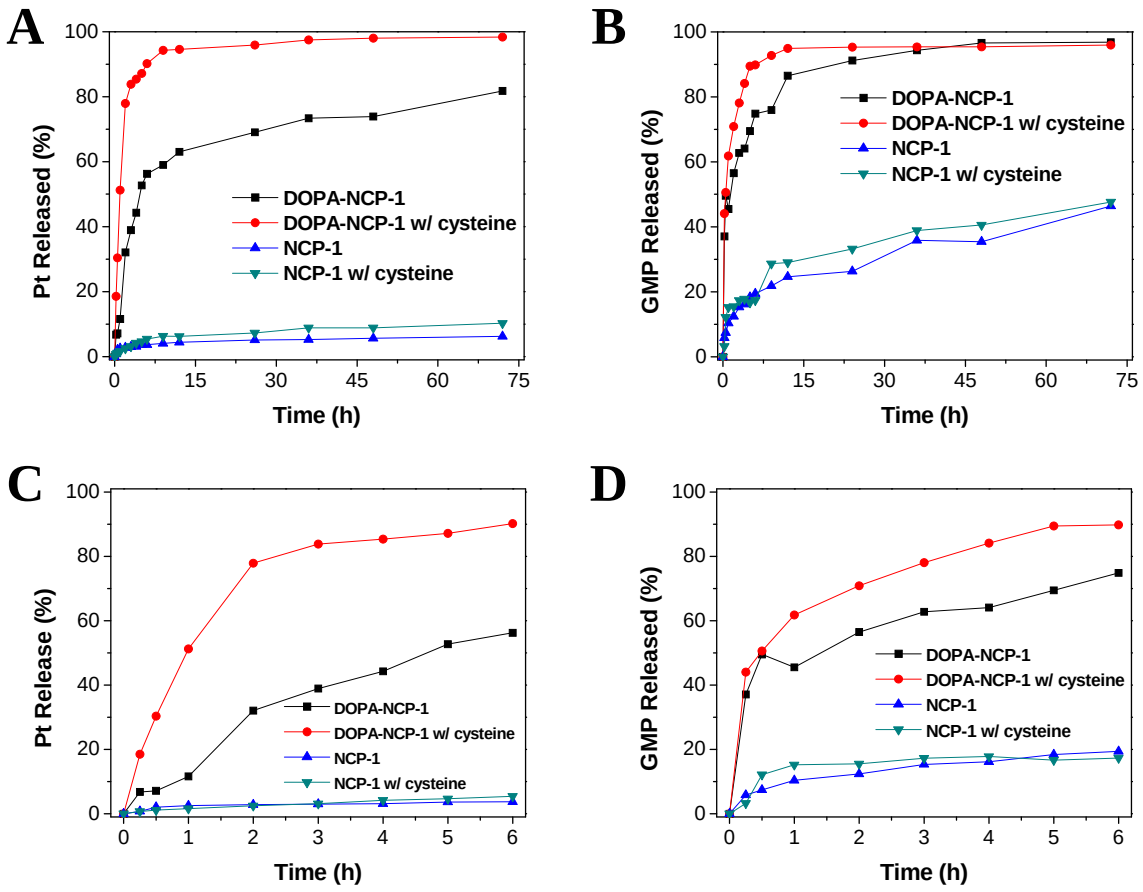

Fig. S5. Pt (A) and GMP (B) release profiles of DOPA-NCP-1 and NCP-1 in 5 mM PBS buffer at 37 °C. Zoom-in view highlighting the first six hours Pt (C) and GMP (D) release profiles of DOPA-NCP-1 and NCP-1 in 5 mM PBS buffer at 37 °C.

6. Synthesis of Chlorin e6-Doped NCPs

A *W*=7.4 microemulsion was prepared with the addition of 25 mg/mL (dach)Pt(BP) sodium salt solution (7.6 μmol), 15 mg/mL GMP sodium salt solution (1.3 μmol), 23 mg/mL chlorin e-6 sodium salt solution (0.8 μmol), and DOPA (22 μmol) to a 5 mL aliquot of Triton-X-100 (0.3 M in 1.5 M hexanol/cyclohexane) solution. Another microemulsion of 5 mL Triton-X-100 (0.3 M,

1.5 M hexanol/cyclohexane) containing $Zn(NO_3)_2 \cdot 6H_2O$ aq. (131 mmol) was also prepared. The two microemulsions were stirred vigorously for 15 min at room temperature, after which they were combined. The resulting microemulsion was stirred for 30 min at room temperature. After the addition of 20 mL ethanol, Ce6-NCP-1 particles were washed once with ethanol, once with 50% (v/v) ethanol/cyclohexane, twice with 50% (v/v) ethanol/THF, and redispersed in THF. Ce6-NCP-2, Ce6-NCP-3, and Ce6-Zn controls were prepared in the same fashion.

Table S2. Particle sizes Ce6-NCP.

NCPs	Number-Ave diameter (nm)	PDI	Zeta Potential (mV)
DOPA-Ce6-NCP-**1**	58.2±1.8[#]	0.089	NA
Ce6-NCP-**1**	66.9±4.6[$]	0.107	-5.2±1.0
DOPA-Ce6-NCP-**2**	40.0±2.3[#]	0.073	NA
Ce6-NCP-**2**	50.2±0.8[$]	0.086	-1.0±0.6
DOPA-Ce6-NCP-**3**	31.4±1.2[#]	0.174	NA
Ce6-NCP-**3**	152.1±7.2[$]	0.159	-3.4±0.3

[#]Measured in THF. [$]Measured in PBS buffer. Data are expressed as means±SD.

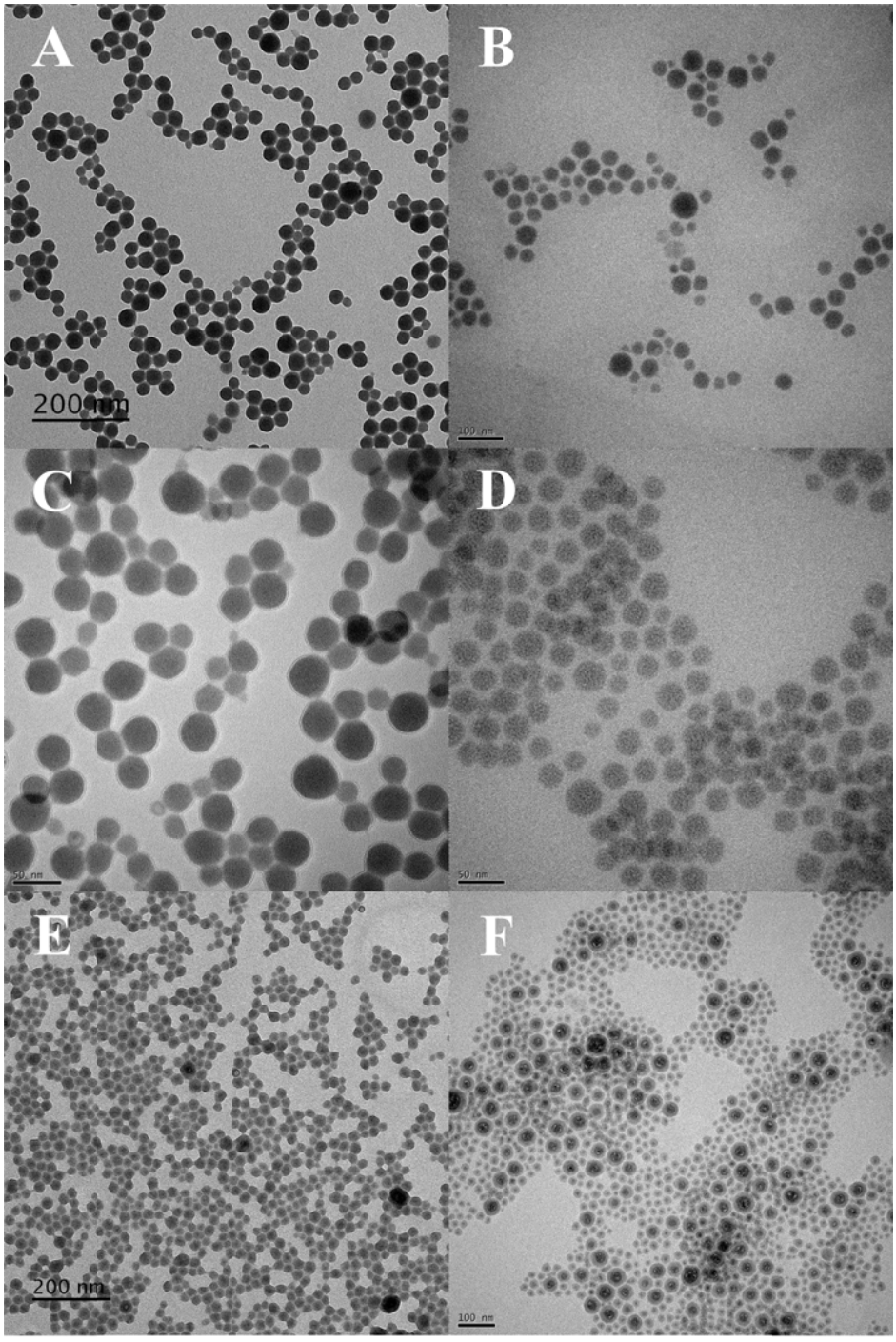

Fig. S6. TEM micrographs of DOPA-Ce6-1 (A), and Ce6-NCP-1 (B), DOPA-Ce6-NCP-2 (C), Ce6-NCP-2 (D), DOPA-Ce6-NCP-3 (E), and Ce6-NCP-3 (D)

7. Intracellular Uptake and Cell Apoptosis *In Vitro*

Wells containing coverslips in 6-well plates were seeded with AsPc-1 or BxPc-3 cells at a density of 5×10^5 cells per well in RPMI-1640 media (10% FBS, 2% penicillin-streptomycin). The cells were incubated for 24 h at 37°C and 5% CO_2 prior to drug treatment. Dispersions of PBS, oxaliplatin, GMP, free oxaliplatin/GMP, Ce6-Zn Control, Ce6-NCP-1, Ce6-NCP-2, and Ce6-NCP-3 were incubated with cells for 48 h at 37°C and 5% CO_2. The cell suspensions were washed with PBS, fixated with iced 4% paraformaldehyde, and stained with 10 μg/mL of DAPI and Alexa Fluor 488 conjugated Annexin V (Invitrogen, USA), based on manufacturer's instructions. The cells were imaged using a confocal laser scanning microscope (Olympus FV1000, Japan) at excitation wavelengths of 405 nm, 488 nm, and 546 nm to visualize nuclei (blue fluorescence), cell apoptosis (green fluorescence), and nanoparticle internalization from chlorin e6 (red florescence), respectively.

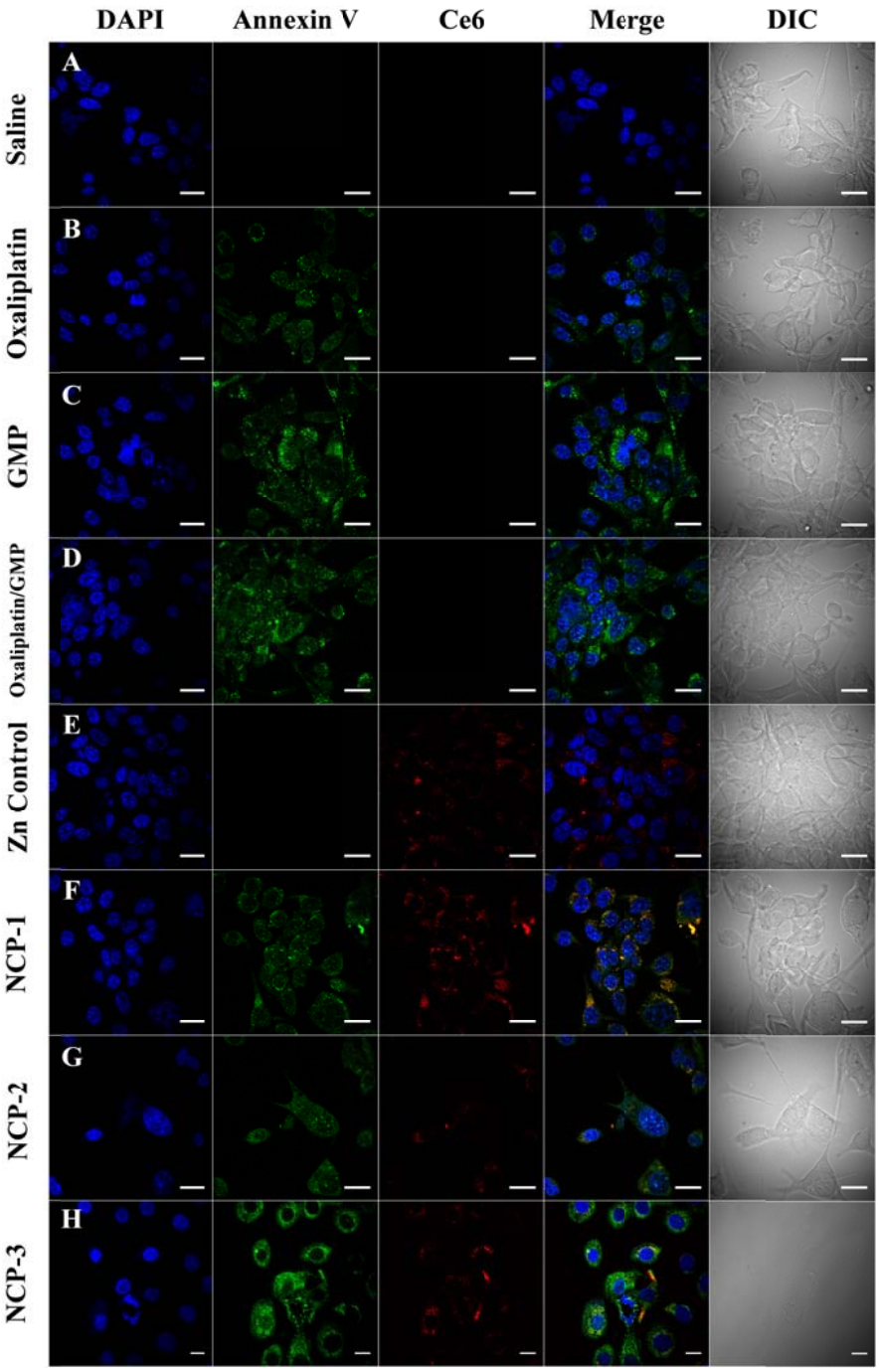

Fig. S7. CLSM images showing the apoptosis induced by saline (A), oxaliplatin (B), GMP (C), oxaliplatin/GMP (D), Ce6-Zn Control (E), Ce6-NCP-1 (F), Ce6-NCP-2 (G), and Ce6-NCP-3 (H) in AsPc-1 pancreatic cancer cells. First column represents DAPI-stained nucleus. Second column represents annenxin-5 stained cells. Third column presents Ce6 labeled particles (only presence in NCP particles). Fourth column is the merged images. Fifth column is the DIC. Bar = 20 μm.

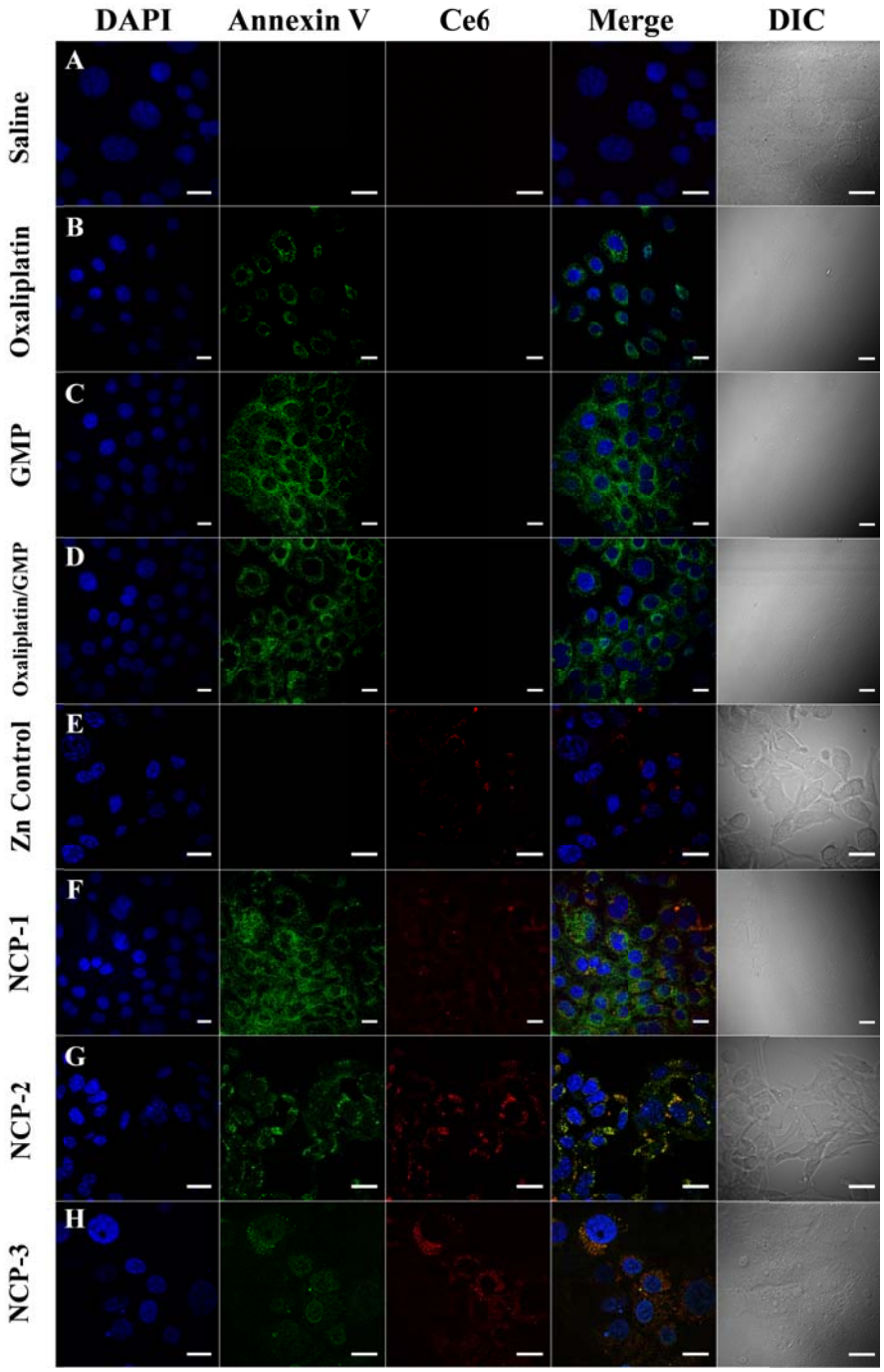

Fig. S8. CLSM images showing the apoptosis induced by saline (A) oxaliplatin (B), GMP (C), oxaliplatin/GMP (D), Ce6-Zn Control (E), Ce6-NCP-1 (F), Ce6-NCP-2 (G), and Ce6-NCP-3 (H) in BxPc-3 pancreatic cancer cells. First column represents DAPI-stained nucleus. Second column represents annenxin-5 stained cells. Third column presents Ce6 labeled particles (only presence in NCP particles). Fourth column is the merged images. Fifth column is the DIC. Bar = 20 μM.

8. Flow cytometry

AsPc-1 or BxPc-3 cells were seeded at a cell density of 5×10^5 cells per well and 2 mL total volume in a 6-wells plate and cultured for 24 h at 37 ^oC and 5% CO_2. Media were removed from the wells and aliquots of PBS, oxaliplatin, GMP, oxaliplatin/GMP, Zn Control, NCP-1, NCP-2, and NCP-3 in fresh media were added to each well at an oxaliplatin concentration of 3.5 μM for AsPc-1 or 4.8 μM for BxPc-3 and/or a GMP concentration of 1.4 μM for AsPc-1 or 1.9 μM for BxPc-3. After incubating at 37^oC and 5% CO_2 for 24 h, the floating and adherent cells were collected by a cell scraper and stained with Alexa Fluor 488 Annexin V/dead cell apoptosis kit with Alexa Fluor 488 annexin V and propidium iodide (PI, Invitrogen, USA) according to manufacturer's instructions. Cell apoptosis was analyzed on a flow cytometer (LSRII 3-8, BD, USA).

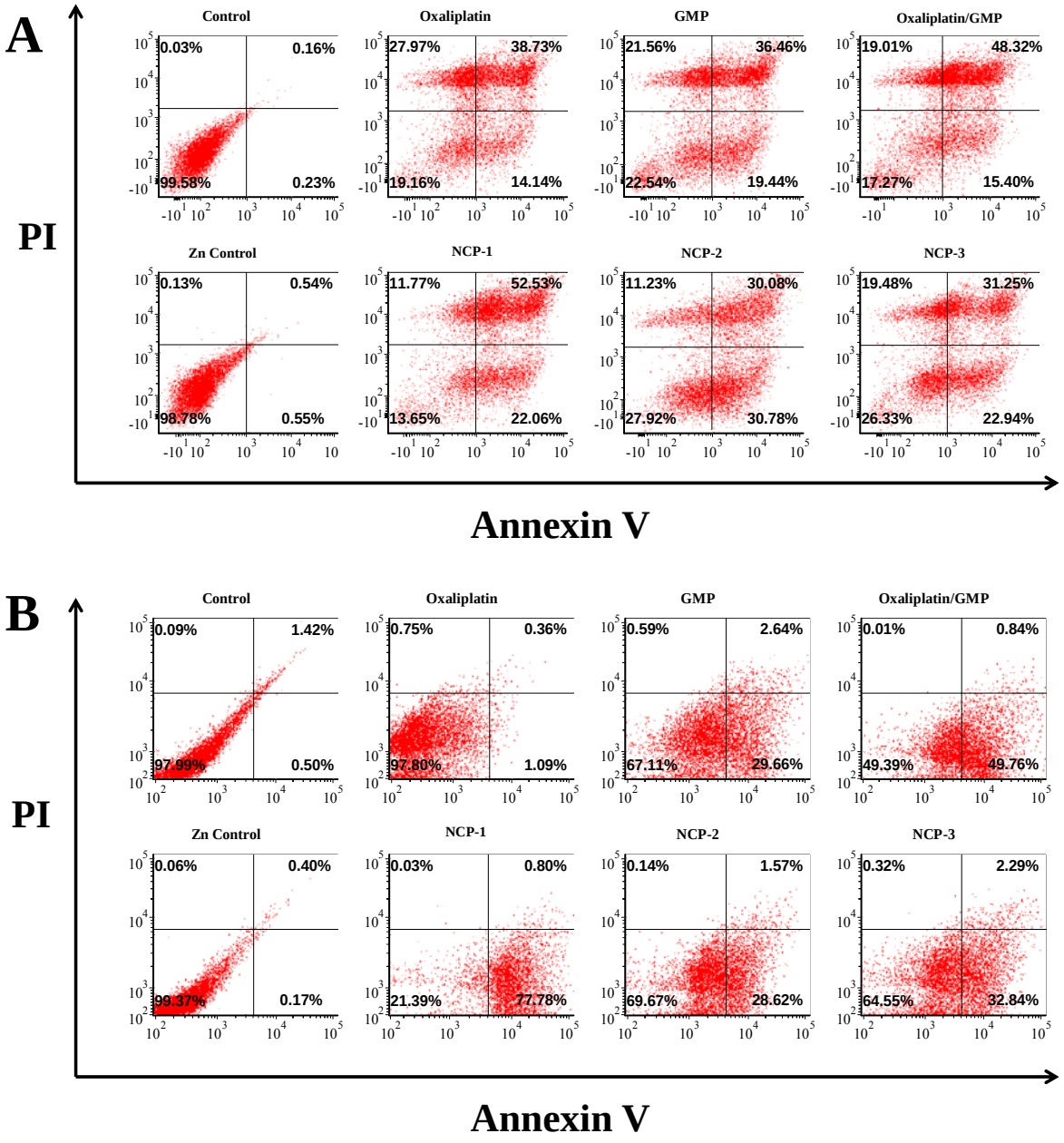

Fig. S9. Flow cytometry analysis of saline, oxaliplatin, GMP, oxaliplatin/GMP, Zn Control, NCP-1, NCP-2, and NCP-3 in AsPc-1 (A) and BxPc-3 (B) pancreatic cancer cells.

9. Pharmacokinetic Studies

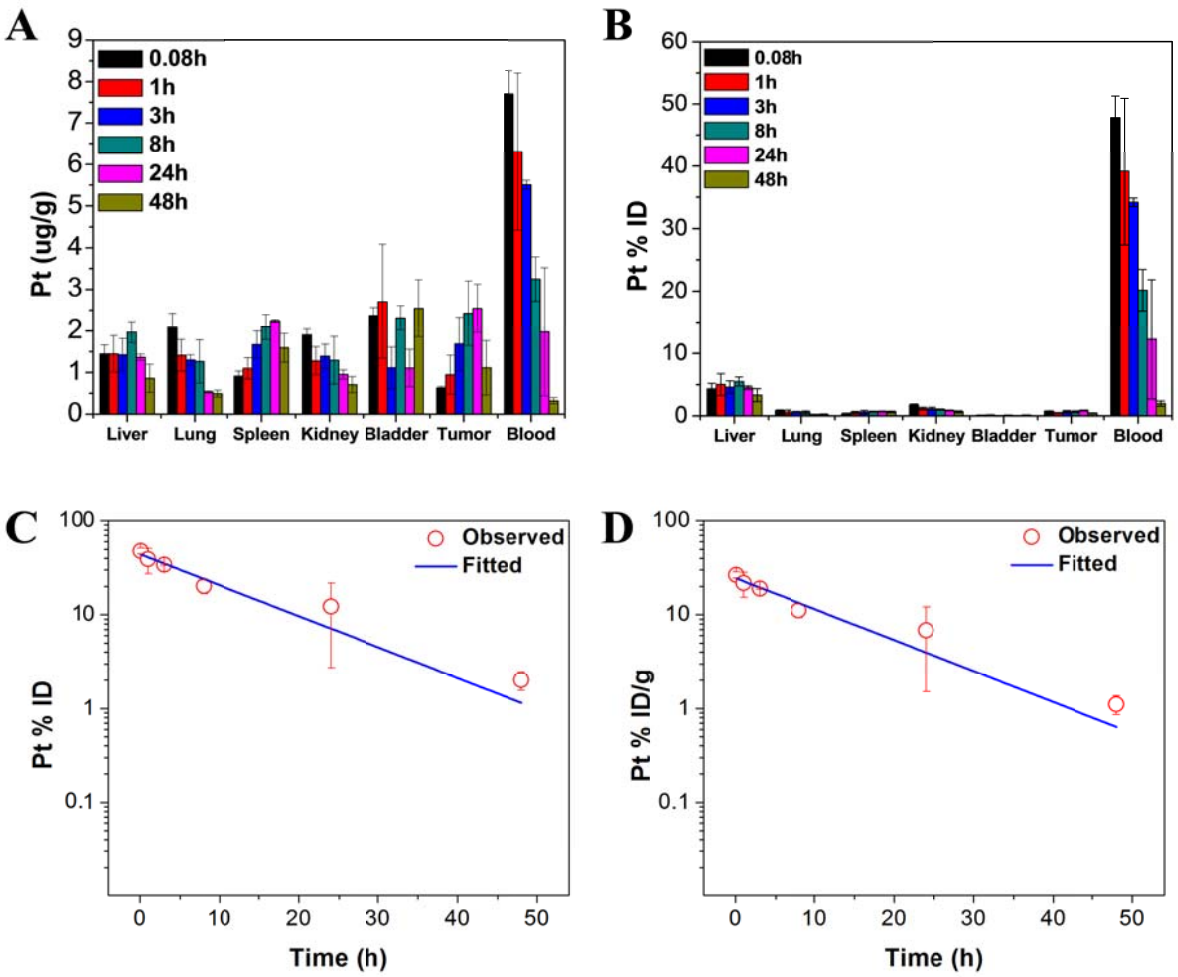

Fig. S10. Concentration (μg/g) (A), and percentage injected dose (% ID) (B) of Pt distribution in tissues and blood after intravenous administration of PEG-NCP-1 in CT26 tumor-bearing mice at time points 5 min, 1 h, 3 h, 8 h, 24 h, and 48 h. Observed and fitted time-dependent Pt % ID (C) and percentage injected dose per gram (% ID/g) (D) in blood. Data are mean ± S.D. (n=3).

Table S3. Pharmacokinetic parameters of Pt distribution for NCP-1 in CT26 bearing nude mice*.

C_0 (μg/mL)	k_0 (1/h)	V_{ss} (mg/kg/(μg/mL))	CL ((mg/kg)/(μg/mL)/h)	AUC (μg/mL×h)	MRT (h)	$t_{1/2}$ (h)
7.1±1.0	0.075±0.028	0.428±0.061	0.031±0.008	96.1±23.9	14.5±4.8	10.1±3.3

*C_o, blood concentration at time=0; k_o, elimination rate constant; V_{ss}, volume of distribution at steady state; CL, systemic clearance; AUC, total area under curve; MRT, mean resident time; $t_{1/2}$, the time required to reduce the plasma concentration to one half its initial value

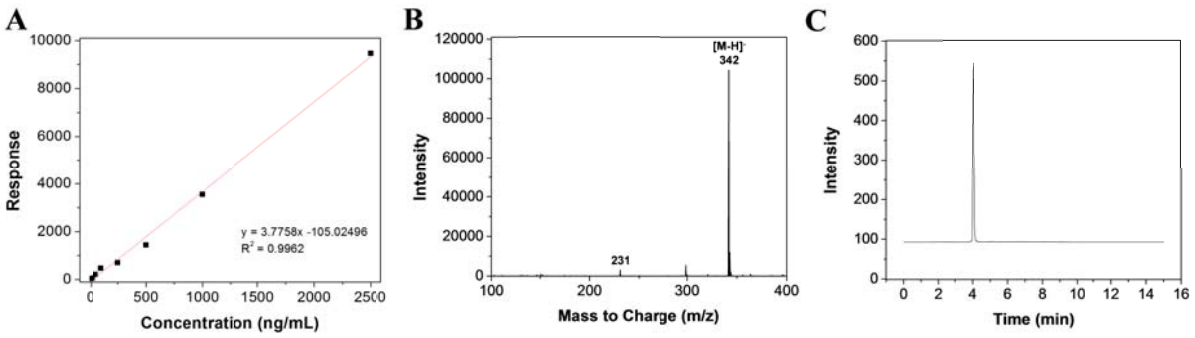

Fig. S11. (A) Standard curve for the determination of GMP in blood using LC-MS. (B) Product ion and deprotonated parent ion MS/MS spectrum of GMP at m/z 341.9. (C) Chromatograms of GMP at 5 min in blood (retention time = 4.1 min)(m/z transition from 342 to 231)

10. Tumor Growth Inhibition Studies

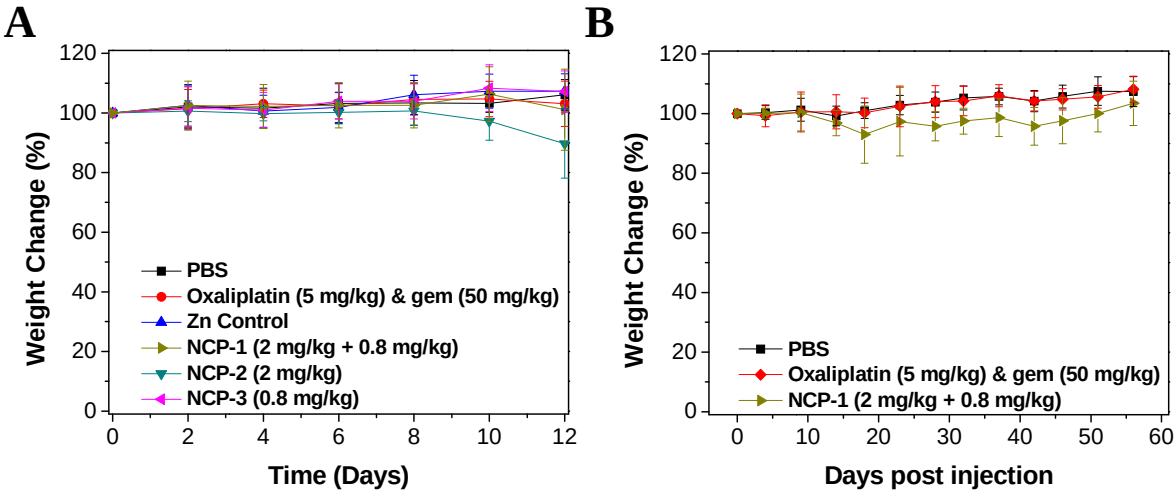

Fig. S12. Body weights of mice bearing BxPc-3 (A) and AsPc-1 (B) tumors over the post injection period

11. In vivo immunogenic response, hypersensitivity, and general toxicity evaluation of NCP-1

At the endpoint of the in vivo tumor growth inhibition experiment, blood samples were taken from the mice. Serum was separated for immunogenic response analysis. The serum concentrations of TNF-α, IFN-γ, and IL-6 were evaluated by ELISA (R&D Systems, USA). The plasma concentration of IgE was detected by ELISA (R&D Systems, USA) as indications of hypersensitivity triggered by NCP-1. Blood from the control group was also analyzed under the same treatment above for comparisons. Organs (liver, lung, spleen, and kidney) of mice treated with NCP-1 were fixed with formalin. Paraffin-embedded 5 μm issue sections were stained with hematoxylin and erosin (H&E) and observed for general toxicity.

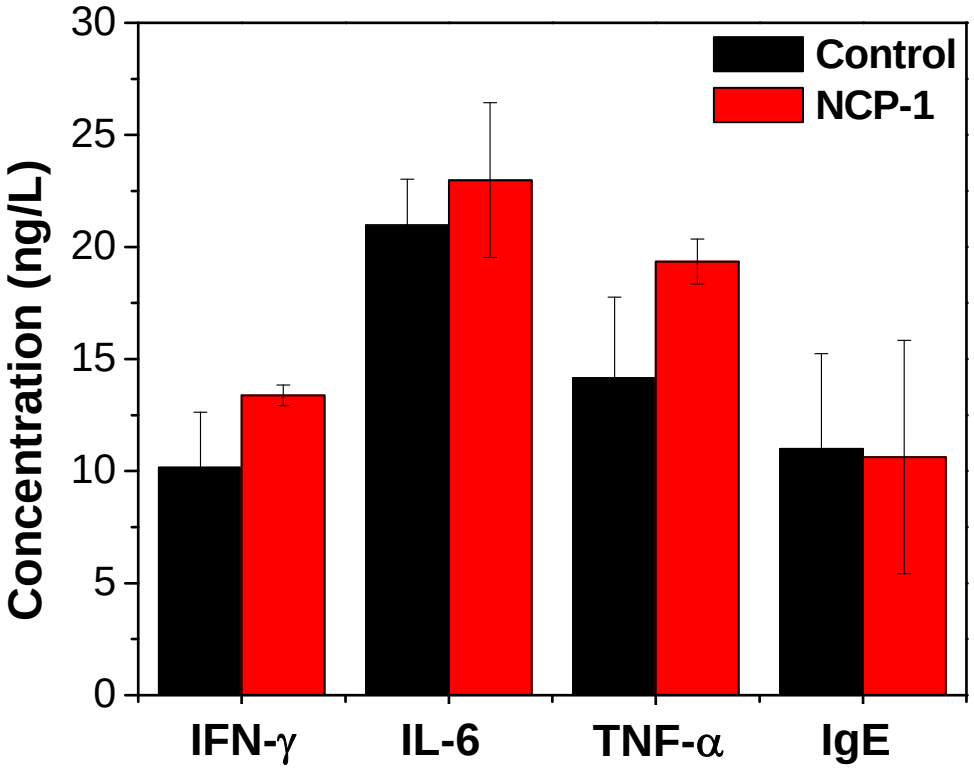

Fig. S13. Immunogenic response and hypersensitivity of BxPc-3 tumor bearing mice treated with saline and NCP-1.

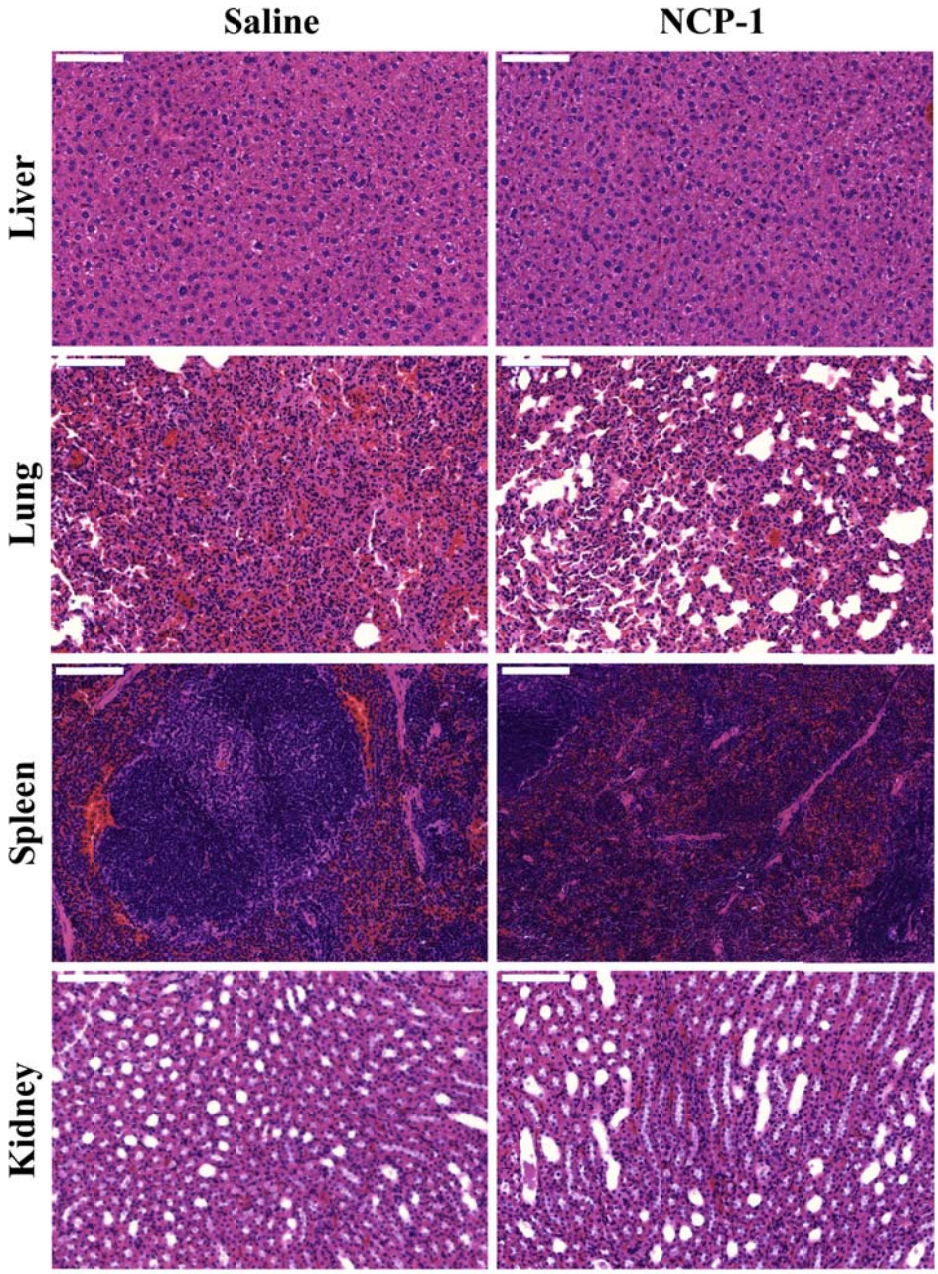

Fig. S14. Histology images of resected organs (with H&E staining) of BxPc-3 tumor bearing mice treated with saline and NCP-1. Bar = 100 μm.

12. In Vivo Tumor Cell Apoptosis

The BxPc-3 tumors were collected at the endpoint of the in vivo tumor growth inhibition experiment, embedded in OCT medium and sectioned at 5 μm thickness. TdT-mediated dUTP nick end labeling (TUNEL) staining was performed using DNA Fragmentation Detection Kit (Life Technology, USA) recommended by the manufacturer and observed under CLSM. The nuclei were stained with DAPI (10 μg/mL). DNA fragments in apoptotic cells were stained with fluorescein-conjugated deoxynucleotides (green). The percentage of apoptotic cells in the

samples was determined by dividing the amount of TUNEL-positive cells by the total numbers of cells observed in each microscopic field. Three tumors were randomly selected in each treatment group for analysis.

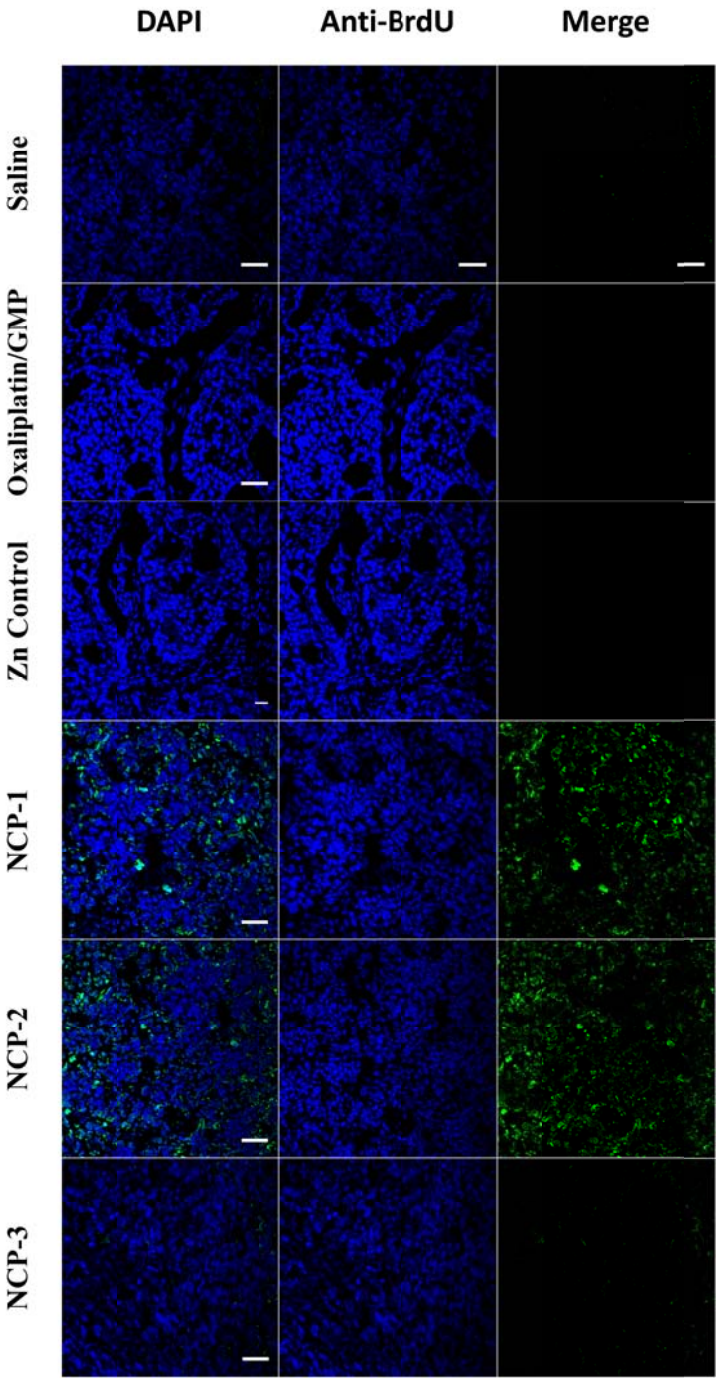

Fig. S15. TUNEL analysis of different treatments on the apoptosis in BxPc-3 tumors. First column represents DAPI-stained nucleus. Second column represents Anti-BrdU stained cells. Third column is the merged images. Bar = 40 μm.