Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Supporting Information

Cell Membrane-Specific Self-assembly of Peptide Nanomedicine Induces Tumor Immunogenic Death to Enhance Cancer Therapy

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Methods and materials

Synthesis of Acryl-PVA

PVA (0.8 g, MW = 9000~10000) was first dissolved in H_2O (3 mL) and heated to 80 ^oC under stirring for 30 min. Then solution temperature was cooled to 40 ^oC, and HCl (37%, 1.5 mL) and acrylic acid (3.6 mL) was added into the solution. The reaction mixture was kept at 40 ^oC in dark for 48 h, followed by dialysis against deionized water (MWCO = 2000) for 48 h. Finally, the polymer solution was lyophilized to obtain a colorless sticky solid.[1] The chemical structures and modification degree (MD) of copolymers were determined by ^1H NMR spectra.

Synthesis of CGG-CM11, NapFFYpC

CGG-WKLFKKILKVL-NH_2 peptide and NapFFYpC were prepared by standard solid-phase peptide synthesis techniques using Fmoc-coupling chemistry.[2] The peptides were dissolved in H_2O, getting molecular weight by matrix-assisted laser desorption ionization-fime of fight (MALDI TOF) (Microflex LRF).

Synthesis of PNpC and PC

Acryl-PVA (5 mg) was first dissolved in $NaHCO_3$ solution (1 mL, pH 8.4) and heated to 60 ^oC for 1 h. Then CGG-CM11 (13.2 mg) and NapFFYpC (8 mg) were added in to the solution, and the solution was stirred for 48 h under N_2 to obtain the PNpC, followed by dialysis against deionized water (MWCO = 2000) for 48 h. The PC (Acryl-PVA 5 mg and CGG-CM11 13.2 mg) was obtained by the same method. The chemical structures of copolymers were proved by ^1H NMR spectra (ARX400MHz).

Nuclear Magnetic Resonance (NMR) Analysis

5 mg NapFFYpC was dissolved in deuterated water (D_2O), and the pH was adjusted to ~8.4 with sodium bicarbonate ($NaHCO_3$). 400 MHz NMR spectrometer (ARX400MHz) was used to characterize the phosphorus characteristic peaks of the sample. Then, ALP was added and the sample was incubated in the dark at 37°C, and its ^{31}P characteristic peaks were characterized again. 5 mg PNpC and PC were respectively dissolved in 450 μL deuterated dimethyl sulfoxide, and the ^{1}H characteristic peaks of the samples were characterized using a 400 MHz NMR spectrometer.

Dynamic Light Scattering (DLS) Analysis

2 mg PC and PNpC were respectively dissolved into 2 mL deionized water to make 1 mg/mL solutions. 1 mL PNpC solution was added to ALP (2 U/mL) and incubated at 37 °C for 24 h to generate PNC. The particle size and Zeta potential of PNC, PC, and PNpC were analyzed by DLS analyzer (Zetasizer Nano ZS).

Critical Micelle Concentration (CMC) Determination

The CMC of PNpC was determined by the fluorescent probe rhodamine. The method was as follows: rhodamine solution (5 μM) was added to PNpC solution with a series of concentration gradients in sequence with a volume ratio of 1:1. The UV absorption spectrum of the mixed solution at 500 nm-550 nm was measured. The maximum wavelength λ max of the UV absorption spectra of different PNpC was recorded.

TEM Confirmed Morphology

PNpC and PC with a concentration of 1 mg/mL (10 μL) were respectively placed dropwise onto a copper mesh and stood for 15 min. Subsequently, most of the liquid was removed through a filter paper. Then, uranyl acetate (10 μL) was used to stain the

samples for 15 min followed by drying the spare liquid with the filter. All of the samples were observed by TEM (Tecnai G2 20 S-TWIN) at an accelerating voltage of 200 kV.

Cell Viability Test

The cytotoxicity of the PNpC, PC and CM11 was evaluated by the CCK-8 assay. Hela cells were seeded on 96-well plates at 5.0×10^3 cells per well, and cultured for 10 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO_2 and then cultured at 37°C overnight (MCO-15AC). PNpC, PC and CM11 were dissolved by serum-free DMEM into various concentrations (0.625, 1.25, 2.5, 5, 10, 20, 40, 80 μM), which replaced medium, and cultured with the cells for 24 h. Subsequently, 100 μL of the CCK-8 solutions was added to each well and cultured for 3 hours. The ultraviolet-visible absorption of the sample wells (A_{sample}), A_{blank}, and control wells ($A_{control}$) was measured by a microplate reader (Enspire PE, USA) at a test wavelength of 450 nm and a reference wavelength of 690 nm. Cell viability (%) was equal to $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$. All experiments were repeated three times.

SEM Confirmed Morphology on the Cell Membrane

4T1 cells were seeded at 1.0×10^5 cells/well into a six-well plate with silicon wafers, and incubated at 37 °C in a constant temperature incubator with a CO_2 concentration of 5% for 10 h until the cells adhered. PPCs with the same concentration were added to the six-well plate and incubated in a cell incubator for 24 h. The mixed solution was aspirated with a micropipette, rinsed twice with sterile PBS, and fixed with 4% paraformaldehyde for 30 min. Then paraformaldehyde was aspirated, and the silicon wafers with attached cancer cells were dehydrated using ethanol solutions of 0%, 10%, 30%, 50%, 70%, 90%,

and 100% (v/v). The silicon wafer after dehydration was then sprayed with gold and observed by scanning electron microscope (SEM).

Cellular Colocalization

PPCs were dissolved in $NaHCO_3$ solution (1mL, pH 8.4). Then 10 μL FITC-NHS (1 mg/mL) was added to the solution (5 μM) under stirring. The reaction mixture was kept in dark for 10 h, followed by dialysis against deionized water (MWCO = 2000) for 48 h. Finally, the polymer solution was lyophilized to obtain a solid. Hela cells were seeded at a density of 4.0×10^4 cells per Laser confocal petri dish, respectively culturing 12 h with FITC-PPCs and then 30min with Dil.

Exploration of Tumor Cell Death Pathways

HeLa and 4T1 cells were respectively seeded at 5.0×10^5 cells/well in a six-well plate at 37 °C, and incubated in a constant temperature incubator with a CO_2 concentration of 5% for 10 h until the cells adhered. PPCs (5 μM) were added to the six-well plate, culturing for 24 h. The cancer cells after incubation were digested with EDTA-containing trypsin for 3 min. The solution was centrifuged at 1000 rpm for 3 min, the supernatant was discarded, and the cells were resuspended with 1 mL of sterile PBS and centrifuged again, repeated 3 times. Finally, cells were resuspended by 1 mL of 1× Binding Buffer to make a cell suspension of 5.0×10^5 cells/mL. Except for the negative control, cells in each experimental group were added with 5 μL Annexin V-FITC and 5 μL PI, and then incubated on ice for 1 h after gently pipetting evenly. After the incubation, the cells were centrifuged at 1000 rpm for 3 min, and then resuspended by centrifugation for 3 times. Finally, the cells were resuspended in 100 μL of PBS, and the pathway of tumor cell death was analyzed by Flow Cytometry (FACS).

Detection of CALR *in Vitro*

4T1 cells were seeded in Laser confocal petri dish for 1.0×10^4 cells and further cultured for 10 h. The medium was replaced with PPCs (5 μM) culturing for 24 h. Cancer cells were incubated with individual primary antibodies against CALR for 2 h. Then cells were rinsed by PBS and were incubated with individual second antibodies FITC for 30 min. Last, cells were incubated with Hoechst for 20 min. The exposure of CALR was measured by FACS, and Multi-beam laser confocal imaging system (UltraVIEW VoX, PE, Germany).

Detection of ATP *in Vitro*

Hela/4T1 cells were respectively seeded at 3.0×10^5 cells per well in 24-well plates and cultured for 10 h. The medium was replaced with PPCs (5 μM) culturing for 24 h. Then, extracellular ATP in the conditioned media that secreted from treated cells was measured via an ATP bioluminescent assay kit by Multimode Microplate Detection System (Enspire, PE, USA).

Measurement of Released HMGB1 *in Vitro*

4T1 cells were seeded at a density of 1.0×10^4 cells per Laser confocal petri dish and further cultured 10 h. The medium was replaced with PPCs (5 μM) culturing for 24 h. Following a further incubation of 24 h, cells were incubated with individual primary antibodies against HMGB1 for 2 h. Then cells were rinsed by PBS and were incubated with individual second antibodies FITC for 30 min. Last, cells were incubated with Hoechst for 20 min. The exposure of HMGB1 was measured by Multi-beam laser confocal imaging system (UltraVIEW VoX, PE, Germany).

DCs Maturation *in Vitro*

4T1 cells were seeded in six-well plates at 1.0×10^5 cells/well, treated with PPCs (5 μM) for 24 h, The supernatant and cancer cell debris from each well were collected as "curatives" that stimulate DC 2.4 maturation. DC2.4 cells were seeded into six-well plates at 5.0×10^5 cells/well for 10 h, and then incubated with tumor cell debris solutions treated with PNpC, CM11 and PC. After incubation for 24 h, DCs were digested with trypsin containing EDTA for 1 min, then resuspended in 1 mL of PBS, and the supernatant was discarded after centrifugation for 30 min with specific fluorescent antibodies CD80-PE and CD86-APC on ice. The maturation of DCs was analyzed by FACS to analyze the percentage of mature DCs.

Detection of CALR and Released HMGB1 *in vivo*

When the tumor volume of the tumor-bearing mice reached 100 mm^3, PPCs solution and normal saline were injected intravenously every other day at a concentration of 100 μM. At the same time, the Dox treatment group was added. After the eighth administration, tumor tissue from tumor-bearing mice was isolated. Calreticulin was labeled with anti-CALR on tumor tissue sections, and anti-CALR was labeled with fluorescent molecules to detect the migration of calreticulin to the cell membrane in the tumor environment. Anti-HMGB1 was used to label calreticulin in cancer cell nuclei in tumor tissue sections, and anti-HMGB1 was labeled with fluorescent molecules to detect the release of HMGB1 in the tumor environment.

DCs Maturation in the TME

After the tumor tissues of the tumor-bearing mice were isolated, and anti-CD11c was used to label DC cells on tumor tissue sections, while anti-CD86 was used to label mature DC cells in DC cells. After the treatment, the mice were euthanized. The tumor tissue was mechanically divided into small pieces and then digested with collagenase to make the tumor tissue into a single-cell suspension. After passing through a 70-micron sterile mesh, centrifuge at 200 g for 5 min, the pellet was resuspended in sterile PBS to obtain a single cell suspension again, and the whole process was carried out on ice. Then, the tumor tissue suspension was incubated with anti-CD86 and anti-CD11c with fluorescent molecules, and the conditions were dark, 4 °C, and 30 min. After labeling, wash 3 times with PBS, and resuspend the cells for detection.

Animal Experiments

All animal experiments comply with the requirements of the National Institutes of Health Laboratory Animal Protection Regulations and the National Center for Nanoscience and Technology Laboratory Animal Care Committee, and have passed the National Center for Nanoscience and Technology Laboratory Animal Welfare Ethics Review. Female BALB/c mice (6–8 weeks, 20-25 g) were purchased from Vital River laboratory animal technology Co., Ltd (Beijing, China). 5.0×10^6 cells were resuspended in 100 μL sterile PBS and were injected subcutaneously at the mouse mammary gland site.

***In vivo* Fluorescence Imaging Experiments**

When the mouse tumor volume reached 150 mm^3, the mouse fluorescence imaging experiment was performed. PPCs labeled with Cy5 fluorescent molecule were injected through the tail vein (200 μL at a concentration of 100 μM). After mice were anesthetized, small animal fluorescence imaging experiments were performed to observe

the distribution of the PPCs in tumor-bearing mice. After 24 h, the mice were euthanized, and the main organs (heart, liver, spleen, lung, kidney) and tumor tissue were observed by fluorescence imaging.

Treatment Experiment

The treatment was started when the tumor volume of the mice reached about 80 mm^3. The mice were divided into 5 groups: PBS group (G0), CM11 group (G1), PC group (G2), DOX group (G3), and PNpC group (G4). The drug was injected through the tail vein every other day, and each mouse was injected with 200 μL. The concentration was 100 μM except the G3 group, and the administration concentration in the G3 group was 5 mg/kg.

Detection of T Lymphocytes in the TME

After the treatment, the mice were euthanized, and the tumor tissues of each group were dissected. A part was fixed with 4% paraformaldehyde for immunohistochemistry, and the infiltration of $CD4^+$ T cells and $CD8^+$ T cells in the tumor tissue microenvironment was analyzed. Another part used flow cytometry to analyze the infiltration of T cells in the tumor tissue microenvironment. The tumor tissue was mechanically divided into small pieces and then digested with collagenase to make the tumor tissue into a single-cell suspension. The tumor tissue suspension was passed through a 70 μM sterile mesh and centrifuged at 200 g for 5 min. Then it was resuspended by sterile PBS to obtain a single-cell suspension on ice. Then, the tumor tissue suspension was incubated with anti-CD8, anti-CD4 and fluorescent molecules in the dark for 30min. After labeling, the tumor tissue suspension was washed for 3 times with PBS.

Interferon (IFN)-γ and Tumor Necrosis Factor (TFN)-α ELISPOT Assay

After the treatment of tumor-bearing mice, blood was collected from the eyes of the mice, and the serum was collected after centrifugation. The levels of TNF-α and IFN-γ in blood were detected using ELISA kits.

Safety Evaluation

The hemocompatibility of polypeptide nanomedicines was evaluated by hemolysis experiment. 4 mL of fresh isolated rabbit blood was added into the anticoagulation tube, and 5 mL of sterile saline was added to dilute the rabbit blood to 9 mL, and the blood mixture solution was incubated at 37 °C for 1 h. PPCs solutions (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL) were also placed at 37 °C and incubated for 1 h. After the incubation, 20 μL of blood solution was added to 980 μL of different polypeptide nanomedicines, and the mixed solution was gently pipetted with a micropipette to make the solution evenly mixed, followed by incubating at 37°C for 1 h with shaking. After the incubation, the mixed solution was centrifuged at 200 g for 5 min, and 200 μL of the supernatant was carefully drawn into a 96-well plate using a micropipette, and the absorbance at 540 nm of the supernatant was measured using a functional microplate reader. PBS-treated rabbit blood was used as a negative control, and deionized water-treated rabbit blood was used as a positive control. Hemolysis rate (Hemolysis rate, HR %) = $[(As - A_1)/(A_0 - A_1)]\times 100\%$, where As, A_0 and A_1 represent the absorbance values of the sample, positive control and negative control at 540 nm, respectively.

During the treatment, the weight changes of the mice were recorded every two days, and the tumor-bearing mice were euthanized after the treatment. The main tumor tissues and main organs, including heart, liver, spleen, lung and kidney, were dissected out of

each group of mice. The tumors and organs were fixed with 4% paraformaldehyde. Embedding, sectioning, and H&E staining were performed.

Supporting Figures

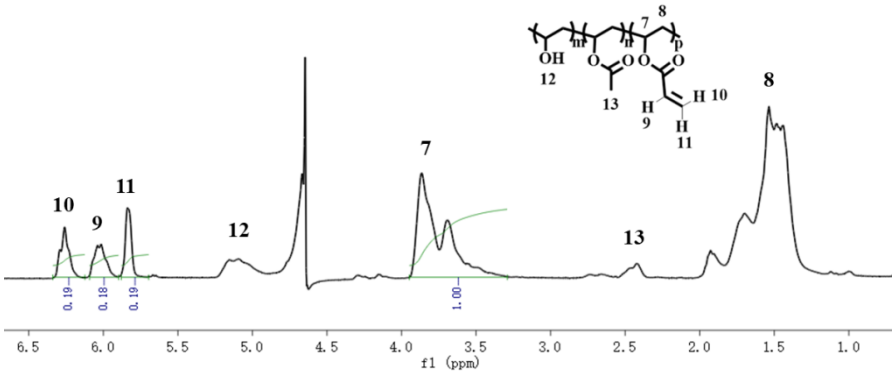

Fig. S1. NMR ^{1}H spectrum of Actyl-PVA in D_2O. The structure of Acryl-PVA was analyzed by ^{1}H NMR spectra, and the modification ratio of the acrylate was 18%. For polymer Acryl-PVA, the area of peak at 3.5~4.0 ppm and peaks at 5.7~6.4 ppm belonging to the acrylate end group were used for calculating the modification ratio of polymers.

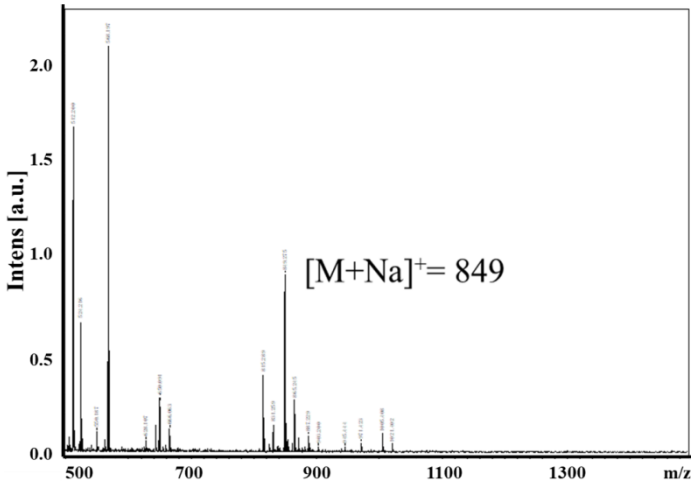

Fig. S2. MALDI-TOF-MS characterization of peptide NapFFYpC.

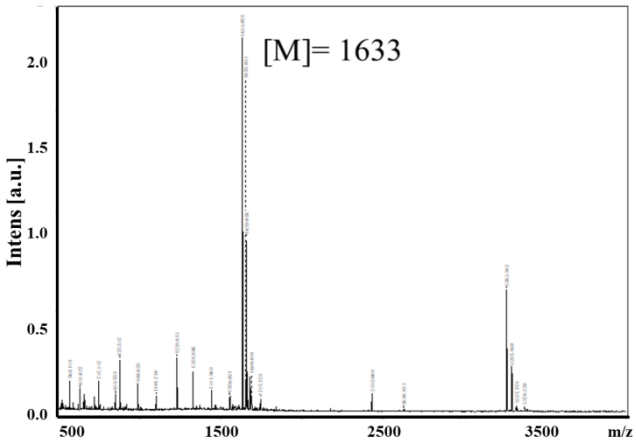

Fig. S3. MALDI-TOF-MS characterization of peptide CM11.

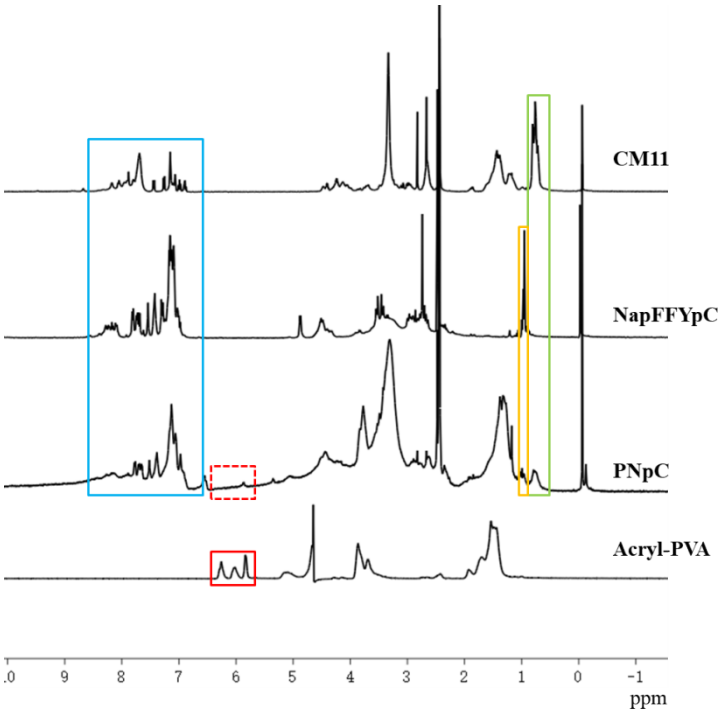

Fig. S4. ^{1}H NMR spectra of PNpC, CM11, NapFFYpC, and Acryl-PVA, dissolved as DMSO-d_6. The ^{1}H NMR spectra of PNpC showed that the characteristic peaks of the acrylate double bond at δ=6.0 ppm disappear, and the characteristic peaks of the peptides at δ=8.0-7.0 ppm appeared, which proved that PNpC was successfully synthesized.

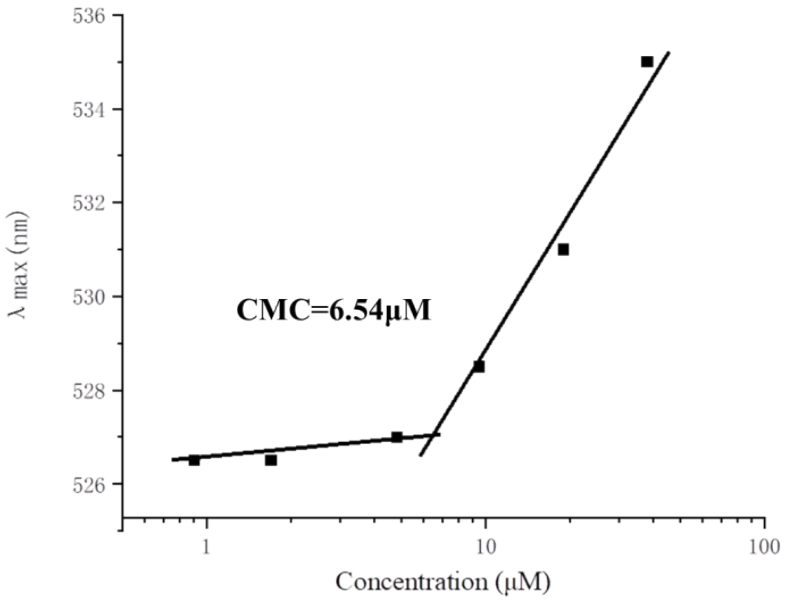

Fig. S5. Critical micellar concentration (CMC) of PNpC using pyrene as a fluorescent probe. CMC=6.54 μM.

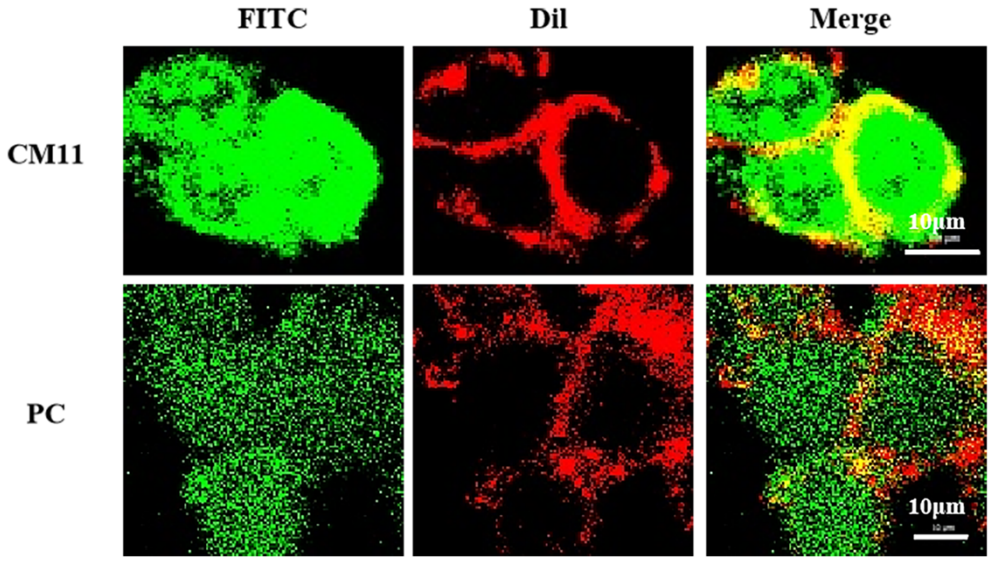

Fig. S6. Localization of nanomedicine in tumor cells. FITC-labeled CM11 and PC exhibit green fluorescence, while Dil-labeled cell membranes exhibit red fluorescence.

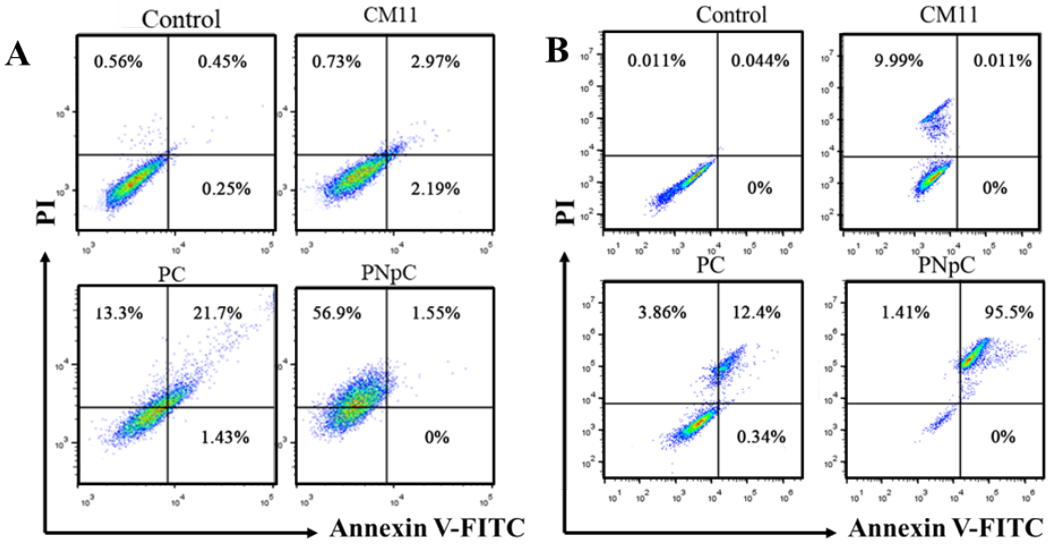

Fig. S7. Flow cytometric analysis results of apoptosis in HeLa (A) and 4T1 cells (B) treated with PPCs. PPCs (5 μM) were added to the cells, culturing at 37 °C for 24 h.

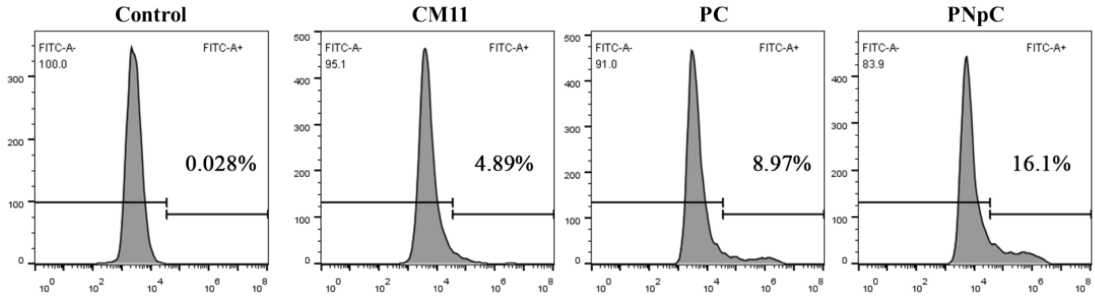

Fig. S8. Analysis of calreticulin in 4T1 cell membrane by flow cytometry. PPCs (5 μM) were added to the cells, culturing at 37 °C for 24 h.

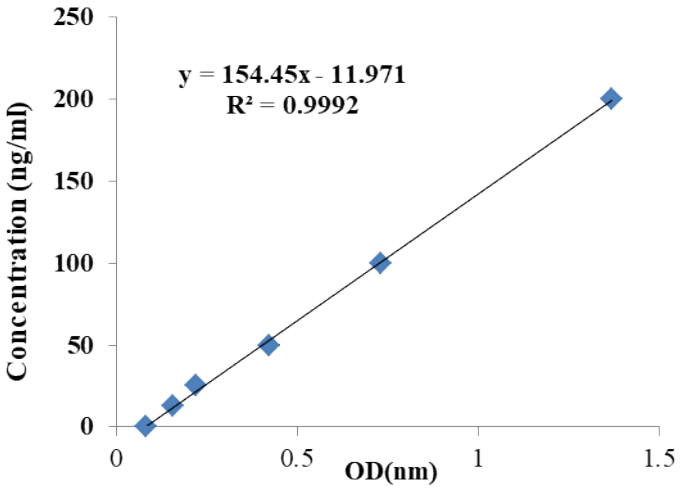

Fig. S9. Standard curve for HMGB1 concentration.

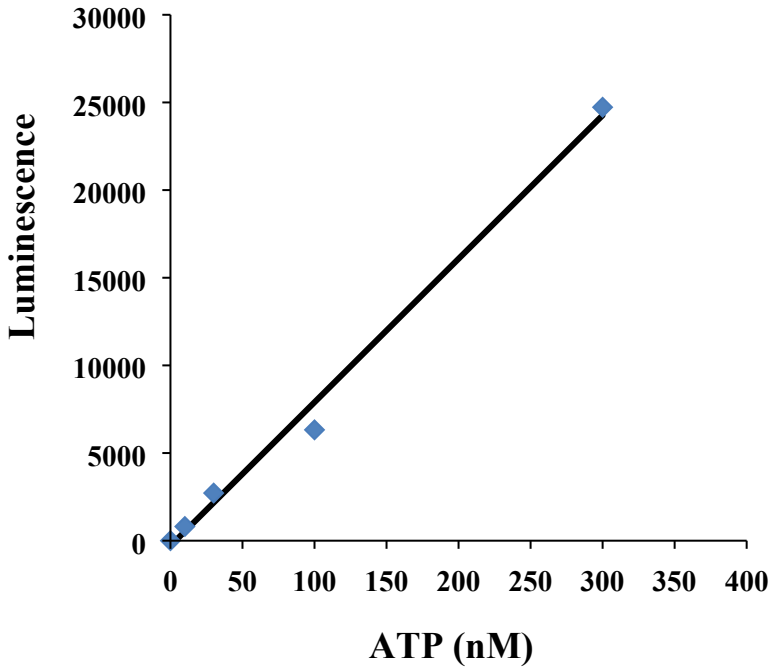

Fig. S10. Standard curve for ATP concentration.

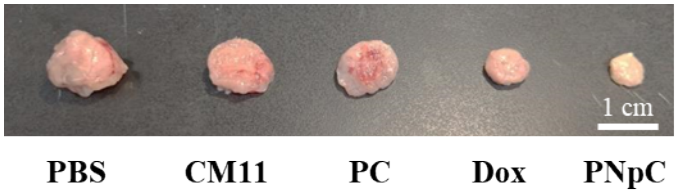

Fig. S11. Tumor pictures of mice treated with PPCs, PBS and Dox group.

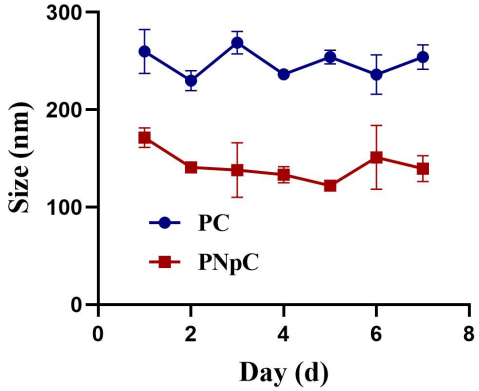

Fig. S12. Particle size of PC and PNpC in PBS solution at different time points. Data are presented as the mean ± SD.

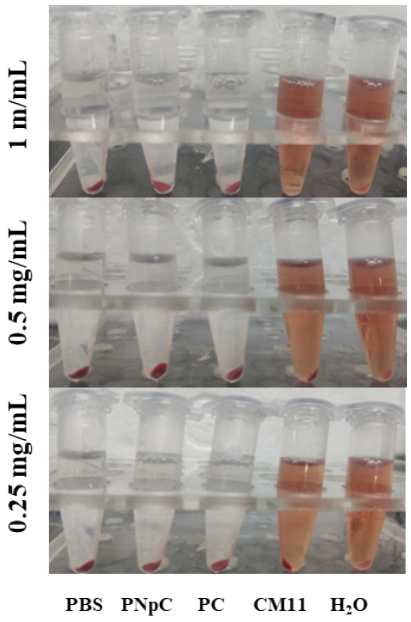

Fig. S13. Hemolysis assay of PPCs at concentrations of 0.25-1 mg/ml in PBS, using water as a positive control and PBS as a negative control.

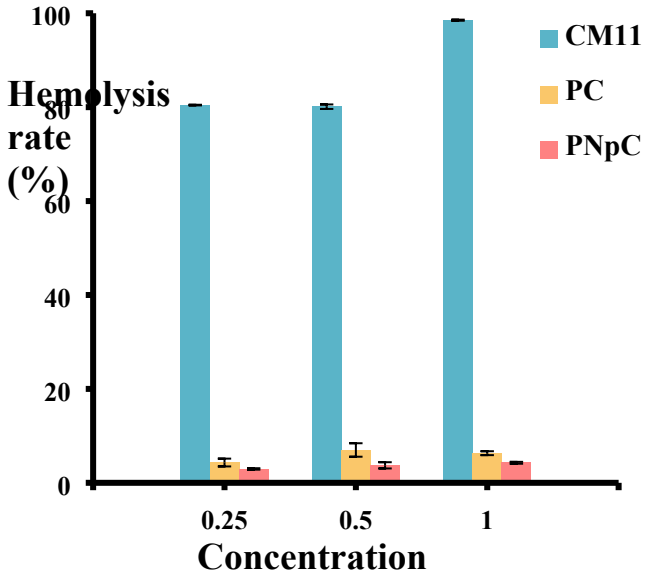

Fig. S14. Hemolysis rate of PPCs at concentrations of 0.25-2 mg/mL.

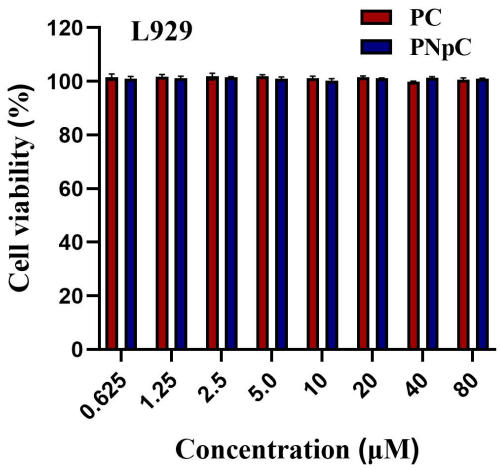

Fig. S15. Cytotoxic effect of L929 cells treated by PC and PNpC. Data are presented as the mean ± SD.

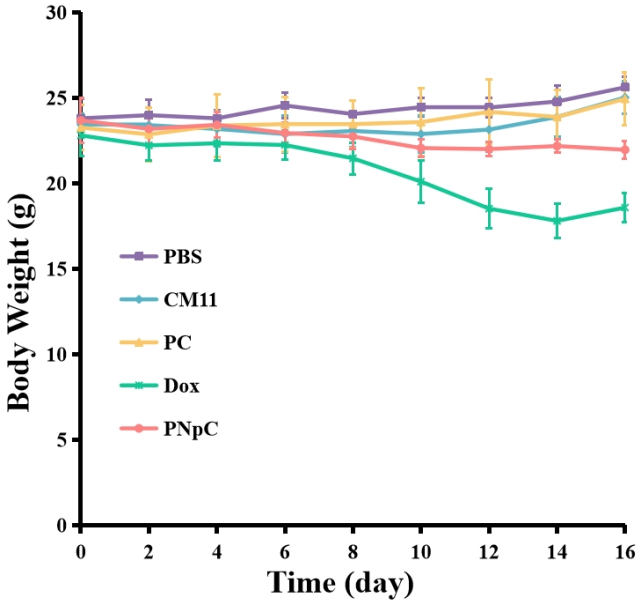

Fig. S16. Body weight curve of mice treated with different drugs.

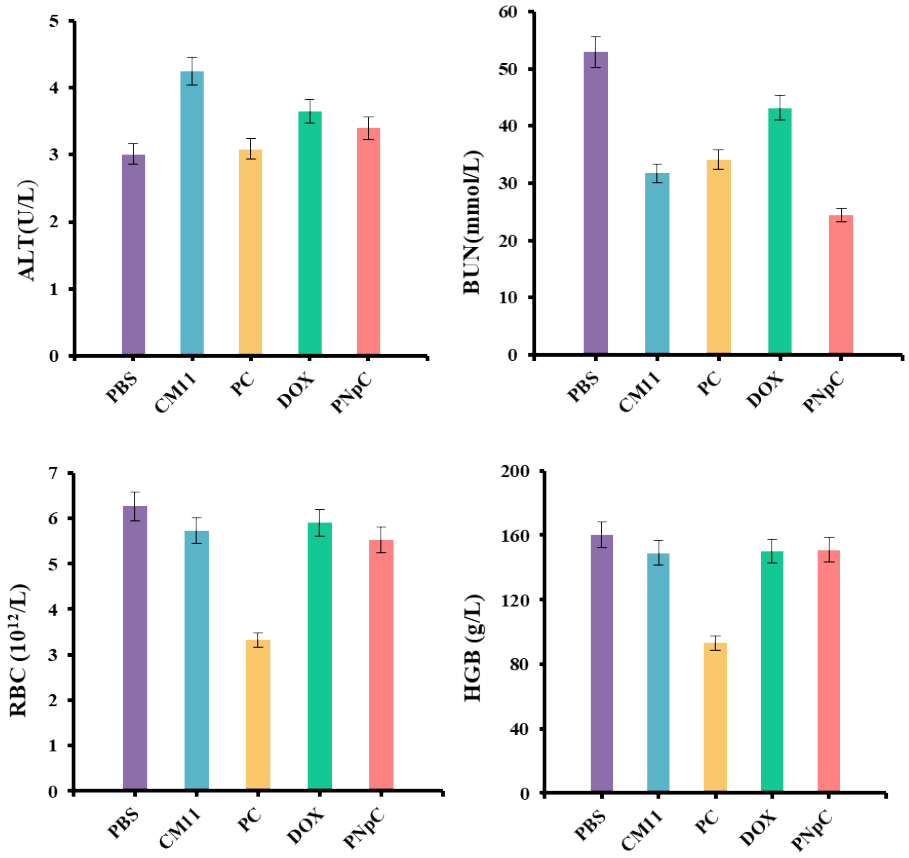

Fig. S17. The amount of glutamic-pyruvic transaminase (ALT), blood urea nitrogen (BUN), red blood cell (RBC) and hemoglobin (HGB) in the blood of mice.

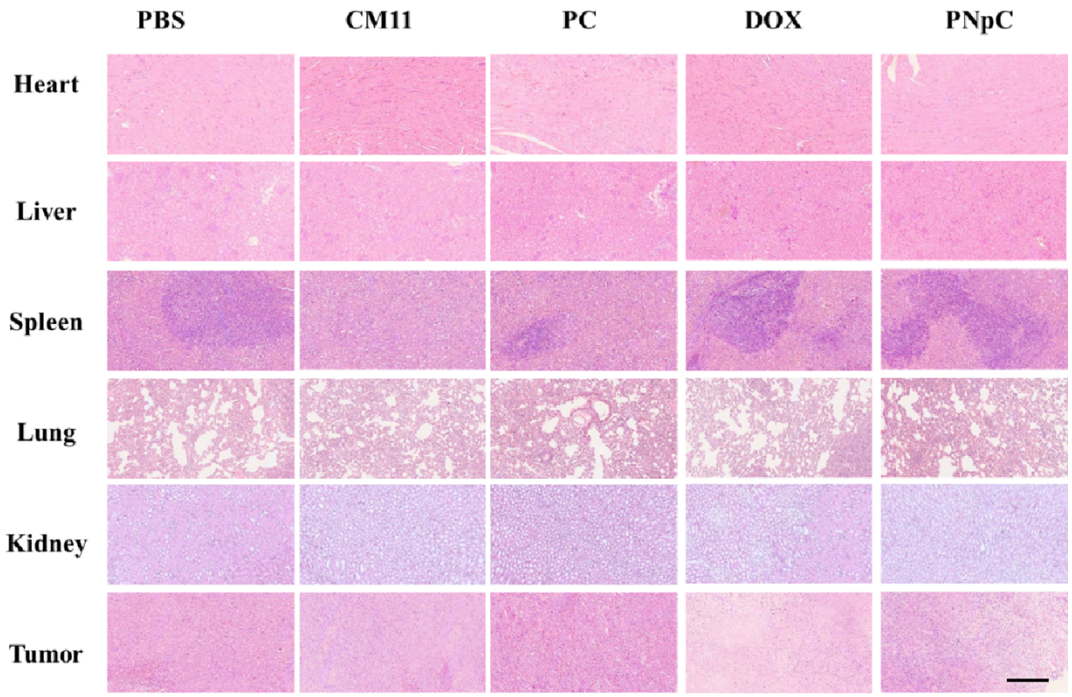

Fig. S18. Representative photomicrographs of the tumor, heart, liver, spleen, lung and kidney sections (H&E staining) of mice treated with PBS, CM11, PC, Dox and PNpC group. Scale bar: 400 μm.

Reference

1. Z. Y. Qiao, Y. X. Lin, W. J. Lai, C. Y. Hou, Y. Wang, S. L. Qiao, D. Zhang, Q. J. Fang and H. Wang, Adv Mater, 2016, 28, 1859-1867.

2. B. L. Nilsson, M. B. Soellner and R. T. Raines, Annu Rev Biophys Biomol Struct, 2005, 34, 91-118.