Supporting Information

pH-Responsive Nanodrug Encapsulated by Tannic Acid Complex for Controlled Drug Delivery

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Detailed Experimental Materials and Methods

Materials: TA, $FeCl_3 \cdot 6H_2O$,acetonitrile, ethanol, Rhodamine B and quercetinwere purchased from Sigma-Aldrich (St. Louis, MO, USA).All cell culture media were purchased from Gibco (Grand Island, New York). PTX was purchased from J&K Scientific Ltd (Beijing, China). ABI-007 was purchased from Celgene Co. (Summit, NJ, USA). Cell counting kit-8 was purchased from Dojindo Molecular Technologies (Tokyo, Japan). LysoTracker®GreenDND-26 was purchased from Invitrogen (Carlsbad, California, USA).All the reagents were of analytical reagent grade.

Preparation of PTX-C: PTX-C was synthesized by coating aerosol sprayed PTX-NPs with complexes of polyphenols and Fe^{III}. The commercially available paclitaxel powder was dissolved in ethanol at a concentration of 200 μg/mL. The solution was subsequently poured into an atomizer aerosol generator machine (ATM 221) as the

raw material. Nitrogen flowed into the generator with the pressure of 1.0 Pa at room temperature and fabricated a mass of aerosol with a special spray nozzle equipped inside the machine. The obtained aerosol was led into deionized water by impulse of continuously flowed nitrogen. In this process, dissolved paclitaxel molecules in aerosol precipitated into particles due to its poor solubility in water. After that, the suspension of paclitaxel nanoparticles was obtained. In this process, 100 mL PTX (200 μg/mL)were used to be sprayed into 500 mL deionized water to make paclitaxel nanoparticles. In order to encapsulate PTX-NPs, $FeCl_3 \cdot 6H_2O$ and TA were separately added into 10 mL PTX-NPs suspension (1.2 mg/mL),the final concentrations of TA and $FeCl_3 \cdot 6H_2O$ were 0.1 mg/mL and0.08 mg/mL, respectively. The suspension was vigorously mixed by a vortex mixer for 20s immediately after the individual additions of$FeCl_3 \cdot 6H_2O$ and TA. The pH of this suspension was subsequently raised by adding 10 ml of MOPS buffer (20 mM, pH 7.4).After all the chemicals were added, the color of the suspension immediately turned blue and the coated paclitaxel nanoparticles were successfully obtained. The coated paclitaxel particles were centrifuged (2,000 g, 3 min) and the supernatant was removed. Then the particles were washed with Milli-Q water three times to remove excess TA and $FeCl_3$. The remaining pellet was lyophilized and re-dispersed in the desired solvents.

The concentration of PTX was measured by high performance liquid chromatography (HPLC, Waters 2478, Milford, MA, USA) in triplicate with UV detection at 227 nm. A C18-column (Nova-Pak 3.9 × 250 mm, Waters, Milford, MA) was used with a mobile phase consisting of water and acetonitrile (20:80 v/v) and a flow rate of 1

mL/min. The lyophilized PTX-NPs were dissolved in ethanol for this measurement.

Characterization of PTX-C: The hydrodynamic size and zeta potential were determined by dynamic light scattering (DLS) (Zetasizer Nano ZS90, Malvern, UK). Determinations were performed at 633 nm with a constant angle of 90° at 25℃ after samples were appropriately diluted in distilled water. Scanning electron microscopy (SEM) tests were performed on HitachiS-4800. PTX-NPs and PTX-C were first dried on a silicon substrate and then sputtered with platinum for 15 seconds. Transmission electron microscopy (TEM) was performed with a FEI Tecnai 20 TEM (Tecnai G2 F20 U-TWIN, FEI, USA) at 20 KV. X-ray photoelectron spectroscopy (XPS) experiments were carried out in a Kratos AXIS Ultra DLD fitted with a monochromated Al Kα X-ray source and a charge neutralizer. C1s at 284.8 eV was used for binding energy calibration and the CasaXPS software for fitting and quantification. Ultraviolet to visible (UV-Vis) absorption spectra were performed on UV-6100 double beam spectrophotometer.

In vitro release kinetics: 100 μg freeze-dried PTX-C were suspended in 1 mL phosphate buffer saline (PBS, pH 7.4 and pH 5.2) and then transferred into mini dialysis units (8-10 kD MWCO, 1 mL), respectively. Then, the samples were dialyzed against 400 mL PBS (pH 7.4 and 5.2 respectively) at 37℃ with gentle stirring. Sink conditions were maintained throughout the experiment. At pre-determined time points, 5 μL solution was taken out from each dialysis unit and the remaining drugs in the dialysis bag was measured by high performance liquid chromatography (HPLC). The cumulative released drug could be easily obtained by subtraction of the measured

drug amount from the initial drug amount in the dialysis bag.

*Subcellular localization of fluorescent PTX-C:*MCF-7 cells were seeded on glass coverslips of 14 mm^2 in culture dishes (Nunc, USA) at a density of 1×10^5 cells per well at 37℃ for 24 hours. Before imaging experiments, the growth media was replaced with 2 mL fresh medium containing 0.5 ug/mL PTX-C labeled by Rhodamine B. After incubation at 37℃ for 7.5 hours, cells were further incubated with 50 nMLysoTracker® GreenDND-26 for 30 min to label lysosomes. Then the cells were washed three times with PBS before image acquisition and were observed with a LSM 710 laser scanning confocal microscope (LSCM, Carl Zeiss LSM710, USA) at 60× magnification. The excitation/emission wavelengths were 550 nm /627 nm for fluorescent PTX-C and 504 nm /511 nm for LysoTracker®GreenDND-26.

Demonstrations of the success marking of PTX-C by Rhodamine B: 50 μg PTX-C were incubated with 50 μg Rhodamine B overnight. After incubation, the PTX-C was centrifuged and thoroughly washed with deionized water for several times until no detectible Rhodamine B left in the supernatant. To demonstrate that the adsorbed Rhodamine B can be attached on PTX-C stably during the cellular level experiments, the PTX-C with adsorbed Rhodamine B were incubated with medium at 37℃ for 8 hours (the same conditions in subcellular localization test) for the following fluorescent test. The fluorescent PTX-C is centrifuged for 15 min at 13000 rpm to obtain fluorescent PTX-C pellet and supernatant respectively. Then the fluorescent intensity of fluorescent PTX-C pellet and supernatant are detected with the excitation/emission wavelengths at 550 nm /627 nm. The fluorescence values of PTX-

C pellet before and after 8 hours incubation with medium were almost the same, so were the culture medium before incubation and supernatant after incubation, which suggests that Rhodamine B can be stably adsorbed on PTX-C for the subcellular localization test.

Preparation of quercetin nanoparticle: The commercially available quercetin powder was dissolved in ethanol at a concentration of 5 mg/mL. The solution was subsequently poured into an atomizer aerosol generator machine (ATM 221) as the raw material. Nitrogen flowed into the generator with the pressure of 1.0 Pa at room temperature and fabricated a mass of aerosol with a special spray nozzle equipped inside the machine. The obtained aerosol was led into deionized water by impulse of continuously flowed nitrogen. After that, the suspension of quercetin nanoparticles was obtained. In this process, 100 mL quercetin (5 mg/mL) was used to be sprayed into 500 mL deionized water to make quercetin nanoparticles.

Cytotoxicity assay: All cells were seeded at a density of 6×10^3 cells per well in 96-well plates in respective media and incubated for 24 hours. Media were then replaced with 200 μL of respective media containing various equivalent concentrations of PTX, ABI-007, PTX-NPs and PTX-C. The cells were incubated for 72 hours and cytotoxicity was assayed using CCK-8 kits (Dojindo Molecular Technologies, Tokyo, Japan). Absorbance was detected at 450 nm with a Tecan Infinite M200 microplate reader (Tecan, Durham, USA). Each experiment was performed in triplicate.

In vivo anti-tumor activity: Nude Bal/bc mice (female, six weeks age) were purchased from Vital River Laboratory Animal Technology Co. Ltd. All animal protocols were

approved by the Institutional Animal Care and Use Committee. Nude Bal/bc mouse tumor models were developed by subcutaneous injection on the right flanks with 0.1 ml of 5×10^6 MCF-7 cancer cells suspended in mixture of PBS and Matrigel (BD, USA) (1:1, v/v). The day when tumor volume reached about 50 mm3 was designated Day 1 and the animals were randomized into four groups (n = 6-8 per group): 0.9% saline control, PTX, PTX-C and ABI-007. At Days 1, 2, 3, 4, 5, 6 and 7, PTX, PTX-C, ABI-007 and saline were administered intravenously through the tail vein. The dosage was 10 mg/kg/day (100 μl per injection). Tumor size was estimated by measuring its orthogonal diameter using a caliper. The tumor volume was calculated using the following formula: ($V = L\times W^2/2$), where L is the length and W is the width. At Day 15 after treatment, the mice were sacrificed, the organs were excised and fixed in 4% phosphate-buffered formalin and embedded in paraffin for histological pathology analysis.

Statistical analysis: Results were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was applied to evaluate the significance among groups according to the Bonferroni's post-test. A resulting p-value less than 0.05 is considered significant (*), and a p-value less than 0.01 is considered very significant (**).

Figure S1-S7:

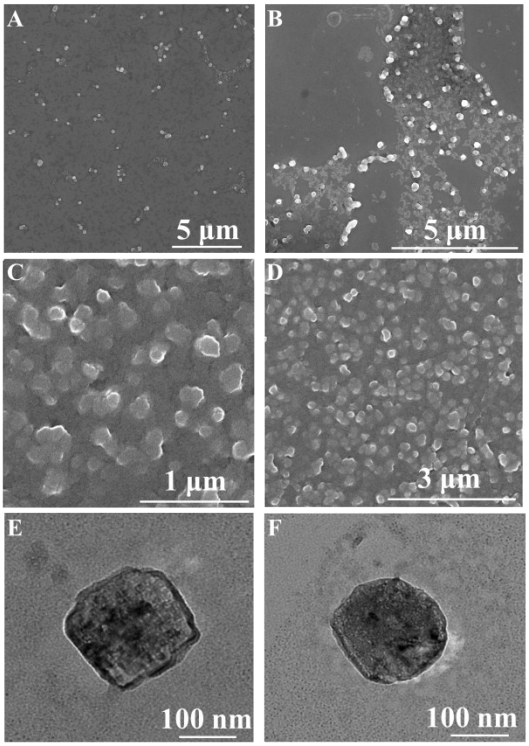

Fig.S1.(A) and (B) SEM images of PTX-C with different concentration of complex ((A) 0.12 mg/mL$FeCl_3{\cdot}6H_2O$ and 0.15 mg/mL TA, (B) 0.2 mg/mL$FeCl_3{\cdot}6H_2O$ and 0.25 mg/mL TA).(C) and (D) SEM images of PTX-NPs. (E) and (F) TEM images of PTX-NPs.

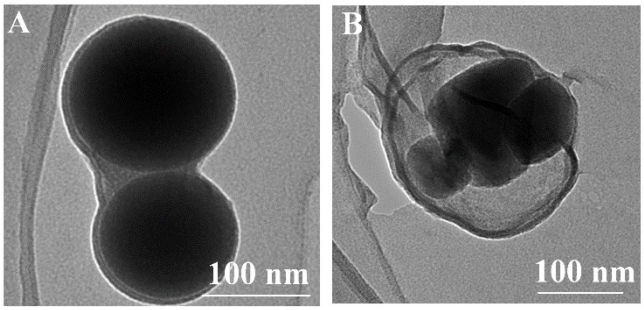

Fig. S2. (A) and (B) TEM images of multi-particle-encapsulated PTX-C.

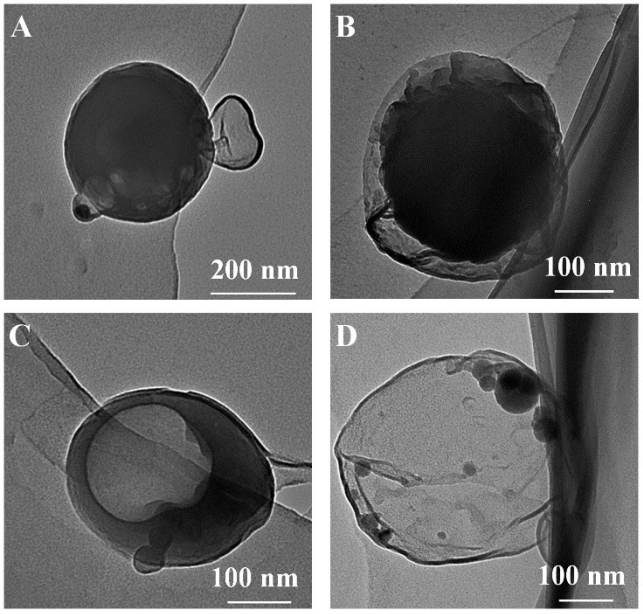

Fig.S3. (A) and (B) TEM images of PTX-C at pH 7.4. (C) and (D) TEM images of PTX-C at pH 5.2.

Table S1. IC_{50} values of different PTX formulations after 72 hours incubation with different cells (n = 3, mean ± SD).

PTX formulation	Cell lines	PTX	ABI-007	PTX-NPs	PTX-C
IC_{50}(ng/mL)	HepG2	13.8 ± 1.8	21.0 ± 2.2	32.5 ± 0.5	6.6 ± 0.1
	A549	5.5 ± 1.1	8.6 ± 0.2	31.3 ± 1.6	3.1 ± 0.5
	HeLa	7.3 ± 0.6	16.9 ± 2.4	27.4 ± 3.1	9.0 ± 1.5
	MCF-7	7.9 ± 0.4	16.7 ± 0.2	32.7 ± 1.8	7.7 ± 0.7
	SKBR-3	17.5 ± 0.3	14.0 ± 3.7	70.3 ± 6.5	9.9 ± 1.2
	PC-3	29.7 ± 1.9	19.2 ± 5.0	33.6 ± 3.2	17.1 ± 2.2

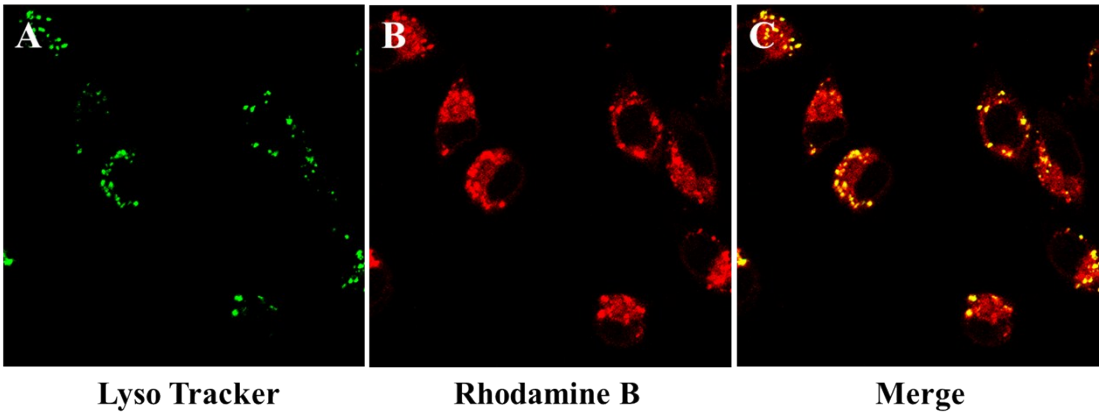

Fig. S4. Images of cells which were incubated with Rhodamine B for 8 h at 37 °C, followed by labelling with LysoTracker®Green DND-26 for 30 min and then observed by laser confocal microscopy (60X). The images from left to right show A) labeled lysosomes (green), B) Rhodamine B (red), and C) overlays of the former two images. In these images, it is obvious that the fluorescent region of Rhodamine B dye is much bigger than that of lysosome which represents the labeled lysosomes. Comparing with the result of Rhodamine B loaded PTX-C (Figure 3 (E), (F) and (G)), pure Rhodamine B spread inside the cell with no directivity.

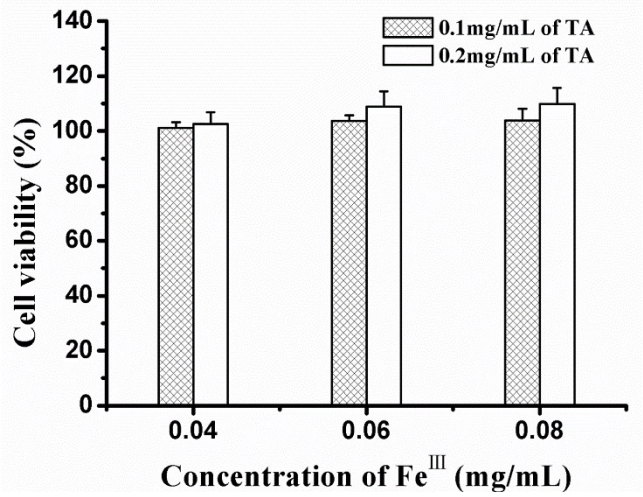

Fig. S5. CCK-8 assay of Fe^{III} and TA on SKBR3 cells. Cells were incubated

with the films formed by indicated concentration of Fe^{III} and TA for 72 h. The results are average values with standard deviations (mean ± SD, n = 4).

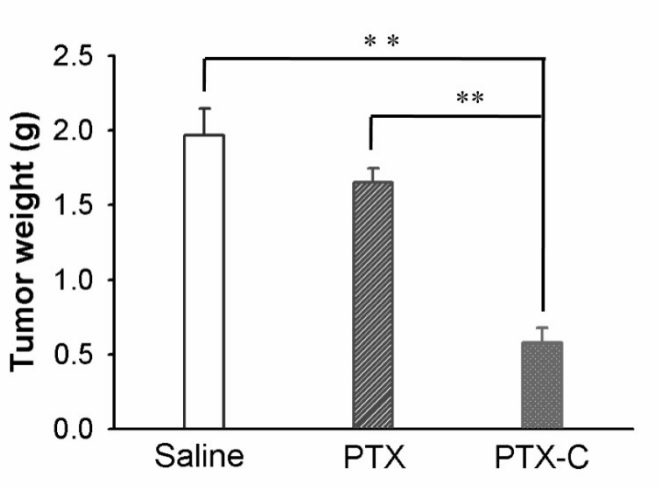

Fig. S6. The weight of tumor tissues dissected from mice after 15 days (** P < 0.01, mean ± SD, n = 6-8).

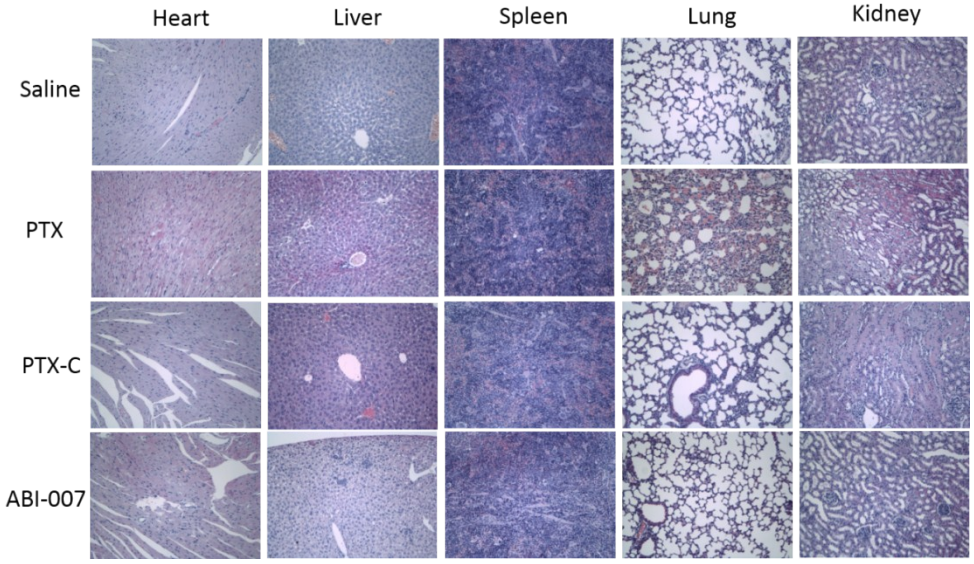

Fig.S7. H&E-stained tissue sections from xenograft mice to monitor the histological changes in, heart, liver, spleen, lung, and kidney of mice receiving intravenous injection of Saline, PTX, ABI-007, and PTX-C followed by dissections in

15 days post-injection, 40× magnification.

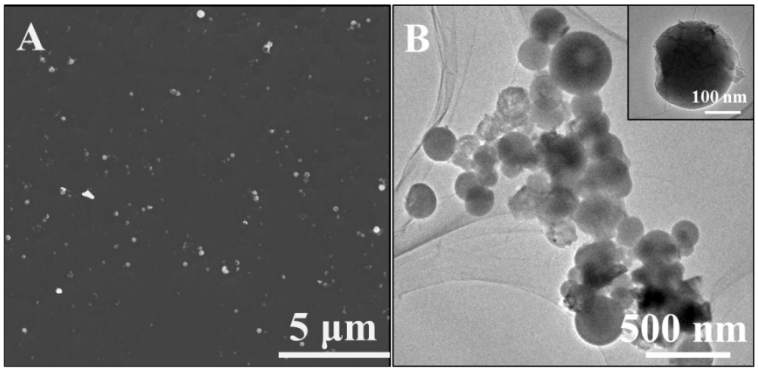

Fig. S8. SEM (A) and TEM (B) images of TA/Fe^{III} complexes coated quercetin nanoparticles. The inset in (B) is corresponding magnified TEM images.