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Supporting Information

Paclitaxel-induced Formation of 3D Nanocrystal Superlattices within Injectable Protein-based Hybrid Nanoparticles

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Experimental Section

Materials. HSA (purity ≥ 96 %), iron chloride ($FeCl_3 \cdot 6H_2O$), indium (III) acetate ($In(Ac)_3$), oleic acid, 1-octadecene, 9,10-dimethylanthracene, deoxycholic acid, pyrene and PBS were purchased from Sigma–Aldrich (St. Louis, MO, USA). *N*-hydroxysuccinimide-functionalized six-arm-branched PEG (6-arm PEG-NHS, M_w = 15 kDa) was obtained from Sunbio Inc. (Walnut Creek, CA, USA). Sodium oleate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Paclitaxel (> 97 %) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). FBS and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Gibco BRL (Grand Island, NY). All other chemicals and reagents were of reagent grade and used without further purification.

Synthesis of IONCs. Briefly, 10.8 g of iron chloride ($FeCl_3 \cdot 6H_2O$, 40 mmol, 98 %) and 36.5 g of sodium oleate (120 mmol, 95 %) were dissolved in a mixture of 80 mL ethanol, 60 mL deionized water, and 140 mL hexane. The resulting solution was heated to and kept at 70 °C for 4 h. When the reaction was completed, the upper organic layer containing the iron–oleate complex was washed three times with 30 mL water in a separatory funnel. After washing, hexane was removed by evaporation to obtain the iron–oleate complex in a waxy solid form.

The following is a typical synthetic procedure for monodisperse iron oxide (magnetite) nanocrystals with a particle size of 12 nm. 36 g (40 mmol) of the iron-oleate complex synthesized as described above and 5.7 g of oleic acid (20 mmol, 90%) were dissolved in 200 g of 1-octadecene (90%) at room temperature. The reaction mixture was heated to 320 °C with a constant heating rate of 3.3 °C min–1, and then kept at that temperature for 30 min. When the reaction temperature reached 320 °C, a severe reaction occurred and the initial transparent solution became turbid and brownish black. The resulting solution containing the nanocrystals was then cooled to room temperature, and 500 ml of ethanol was added to the solution to precipitate the nanocrystals. The nanocrystals were separated by centrifugation.

Preparation of IONCs-loaded HSA/PEG NCs. The IONCs-loaded HSA/PEG (IONC-HSA/PEG) NCs were synthesized using a modified emulsification/solvent evaporation method.[31] In brief, 10 mg or 20 mg of IONCs and 22.6 mg of 6-armed PEG-NHS were first dissolved in 2 mL of dichloromethane. The mixture was added in a dropwise manner to 10 mL HSA solution (1 mg mL^{-1}, pH 9) saturated with dichloromethane, and then sonicated at ambient temperature for 5 min using a Branson Sonifier® 450 at a frequency of 20 kHz with a duty cycle of 20 and an output control of 3.5.[22] The prepared O/W emulsion was transferred to a rotary evaporator, and the organic solvent was rapidly evaporated under reduced pressure at 30 °C. The produced HSA/PEG NCs were subjected to dialysis against deionized water (M_w cutoff of 100 kDa) to remove free residues and then stored at 4 °C until use.

Characterization of IONC-HSA/PEG NCs. The hydrodynamic diameter and zeta potential of the prepared HSA/PEG NCs was measured by dynamic light scattering (ELSZ-1000, Otsuka Electronics, Japan) at a concentration of 1 mg mL^{-1} at 20 °C. The size and shape of the NCs were determined using transmission electron microscopy (TEM). 10 µL of NCs dispersed in an aqueous solution (2 mg mL^{-1}) was deposited on a Formvar/carbon-supported copper grid. The grid was dried in air for 12 h and observed using a JEM – 2100F HR TEM (JEOL, Japan) operated at 80 kV. The loading amount of the IONCs was analyzed by using ICP-AES.

To determine the loading amount of paclitaxel within the IONC-PTX-HSA/PEG, we freeze-dried and re-dispersed the nanocapsules in acetonitrile with sonication for shaking for 24 h to extract paclitaxel following 10 second of sonication. After filtration through a 0.22 µm cellulose filter, the amount of paclitaxel in the filtrate was analyzed using reverse-phase high-performance liquid chromatography (HPLC 1100 series, Agilent Technologies, Palo Alto, CA) equipped with a Waters Spherisorb ODS2 column (4.6 mm × 250 mm). Acetonitrile was used as a mobile phase with a flow rate of 1.0 mL min^{-1}. Eluted peaks

were monitored at 227 nm. A calibration curve was obtained using a series of paclitaxel solutions at different concentrations.

Magnetic Resonance Measurements. The T_2 relaxation time and relaxivity of the IONCs were measured at different field strengths with NMR minispec (mq series, Bruker, MA, USA). The T2-weighted images were measured with a clinical MRI instrument (Philips, Achieva ver. 1.2, Philips Medical Systems, Best, Netherlands). The temperature in the magnet room was 20 °C. The specific relaxivity, r_2, of the IONCs-loaded HSA/PEG NCs dispersed in deionized water was measured in the concentration range of 6.25 to 200 μM. The IONCs were placed in a 3.0 T whole-body MRI system with a gradient amplitude of 80 mT m^{-1} at a slew rate of 200 ms m^{-1}. Then, r_2 values calculated from the slope of the graph of $1/T_2$ were plotted against the iron concentration.

Evaluation of Cytotoxic Effect. The cytotoxicity of the paclitaxel or IONC in the HSA/PEG NCs was evaluated by examining the inhibition of cancer cell growth. The HSA/PEG NC samples incorporating paclitaxel and/or IONC were diluted with PBS to reach a final concentration of paclitaxel from 0.1 to 100 μM and IONC from 0.33 to 330 μM. MCF-7 (human breast cancer) and HeLa (human cervical adenocarcinoma) cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and grown in DMEM at 37 °C for 24 h. The culture medium was replaced with DMEM containing different paclitaxel and/or IONC formulations, and further incubated at 37 °C for 24 h and 48 h. The number of viable cells was determined using the CCK-8 assay.

Statistical Analysis. Statistical analysis was performed using a standard Student's *t*-test with a minimum confidence level of 0.05 for significant statistical difference.

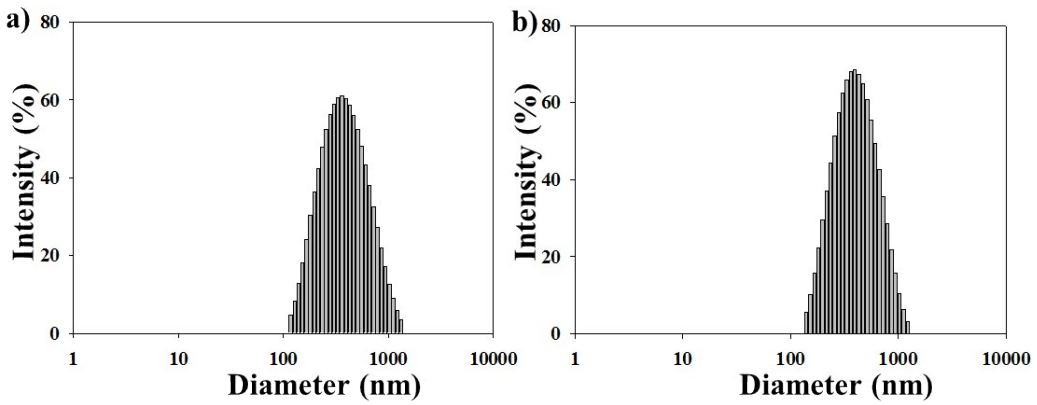

Figure S1. Size distribution graphs of the IONC-HSA/PEG (a) and IONC-PTX(5mg)-HSA/PEG nanoparticles (b).

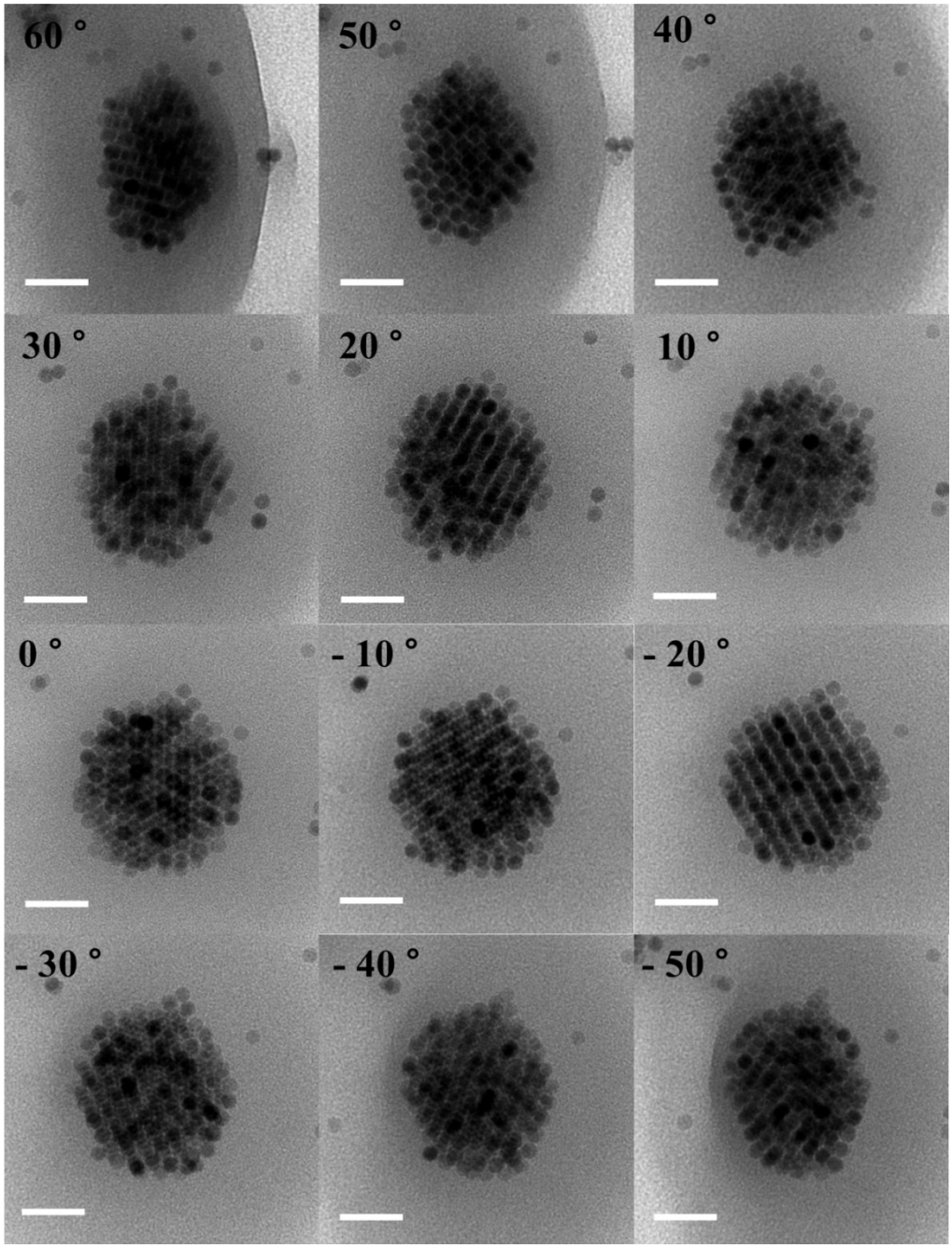

Figure S2. Electron tomography images of IONC-PTX(5mg)-HSA/PEG nanoparticles. An identical particle from a tilt series was taken in bright-field TEM.

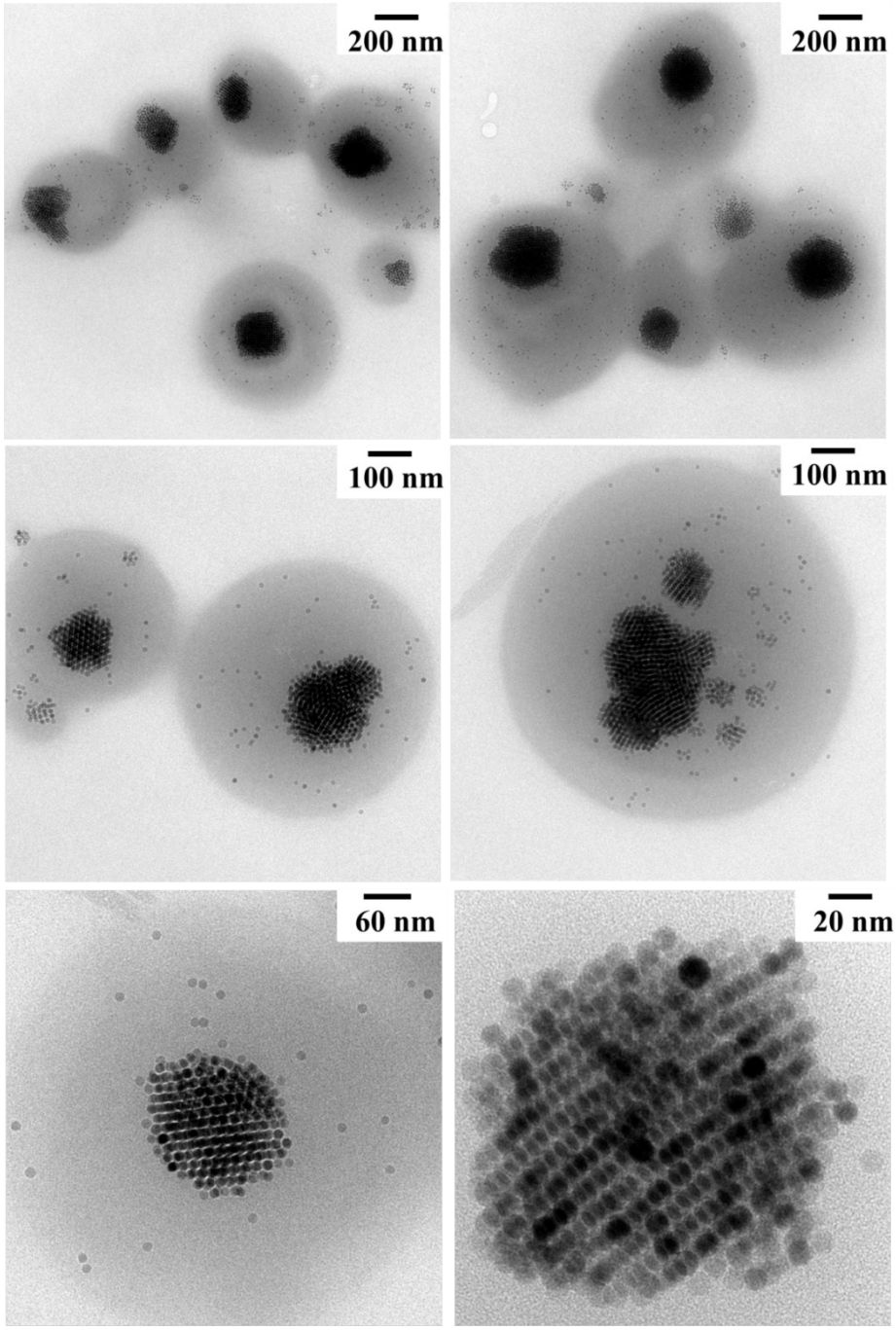

Figure S3. TEM images of IONC-PTX(10mg)-HSA/PEG nanoparticles.

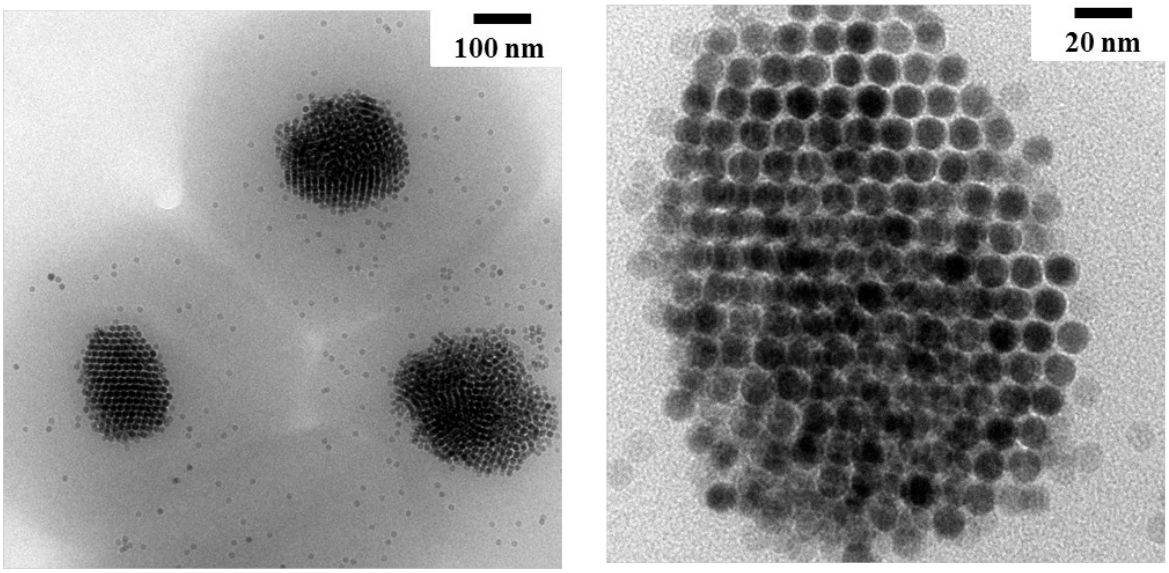

Figure S4. TEM images of IONC-DMA-HSA/PEG nanoparticles.

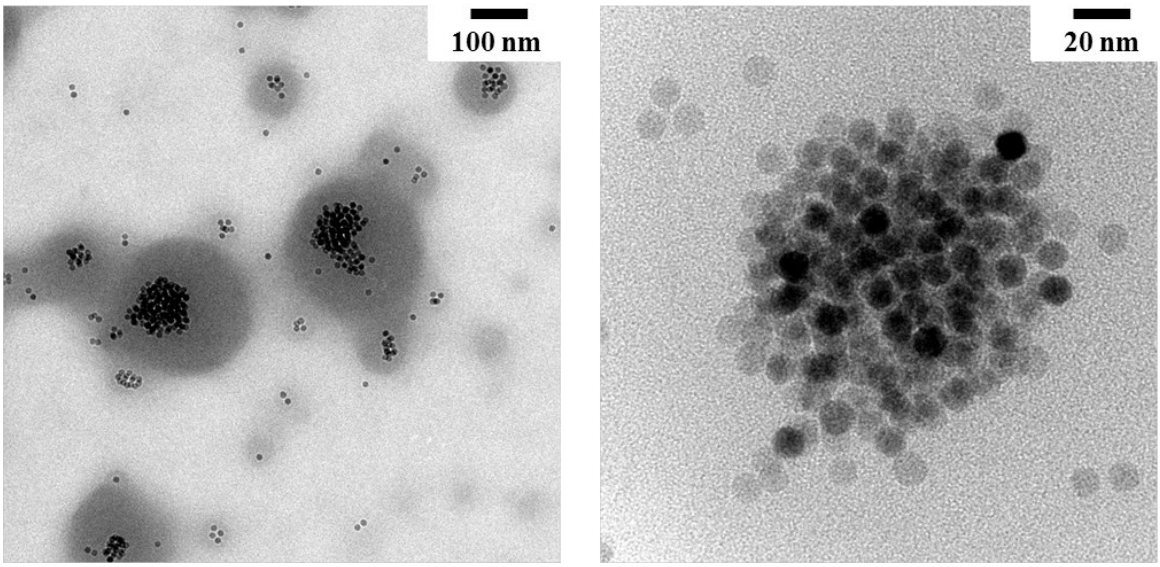

Figure S5. TEM images of IONC-pyrene-HSA/PEG nanoparticles.

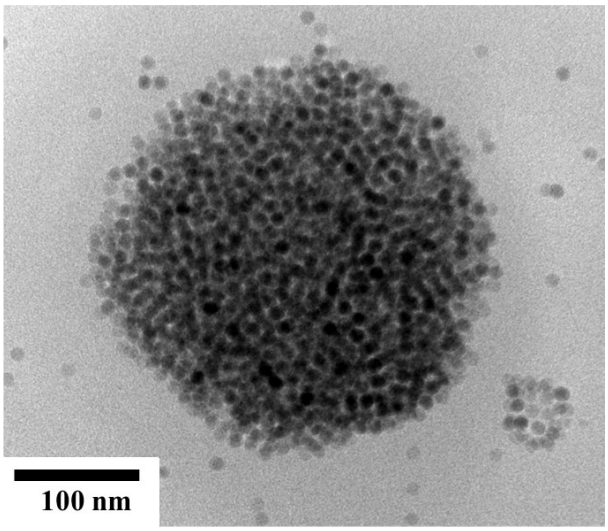

Figure S6. TEM images of IONC-deoxycholic acid-HSA/PEG nanoparticles.

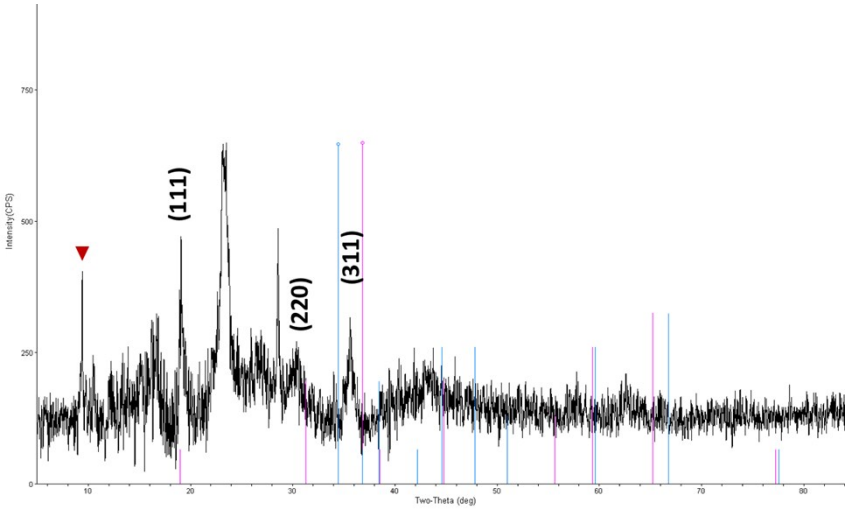

Figure S7. XRD pattern of IONC-PTX-HSA/PEG

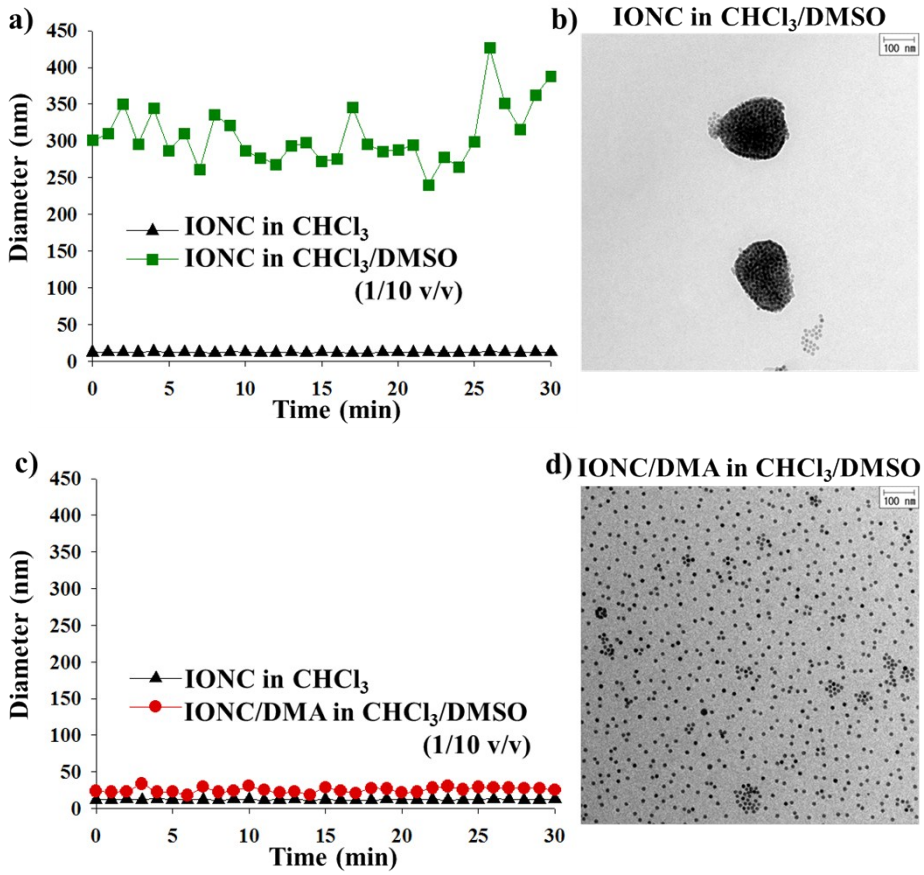

Figure S8. a) Average diameter of IONC in chloroform ($CHCl_3$) and mixture of chloroform and DMSO. b) TEM images of IONC in mixture of chloroform and DMSO. c) Average diameter of IONC in chloroform ($CHCl_3$) and IONC/DMA in mixture of chloroform and DMSO. d) TEM images of IONC/DMA in mixture of chloroform and DMSO.

To examine the existence of an interaction between the IONCs and the multi-ring aromatic additives, we determined the size changes in the IONCs in dimethyl sulfoxide (DMSO) using dynamic light scattering (DLS) (**Figure S8a and c**). We incubated the IONCs with DMA (IONC-DMA), washed them with an excess of ethanol twice, and transferred them to DMSO. The IONCs aggregated immediately when transferred to DMSO, while the IONC-DMA did not exhibit any significant changes in DLS measurements. TEM analysis of the IONC-DMA also showed individually re-dispersed particles in DMSO (**Figure S8b and d**). During the vigorous washing, the DMA might not have been removed from the surface of the IONCs, due to van der Waals and hydrophobic interactions, and it subsequently became a part of the ligand shell of the IONCs. During the formation of the IONC superlattice, unremoved aromatic molecules could occupy the interstitial spaces of the superlattice.

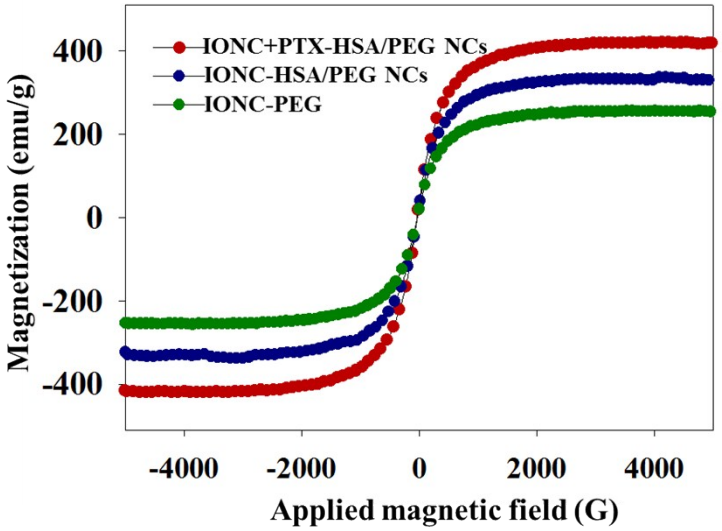

Figure S9. Magnetization curves of IONC-PTX(10mg)-HSA/PEG, IONC-HSA/PEG nanoparticles and IONC-PEG mixtures recorded at room temperature.

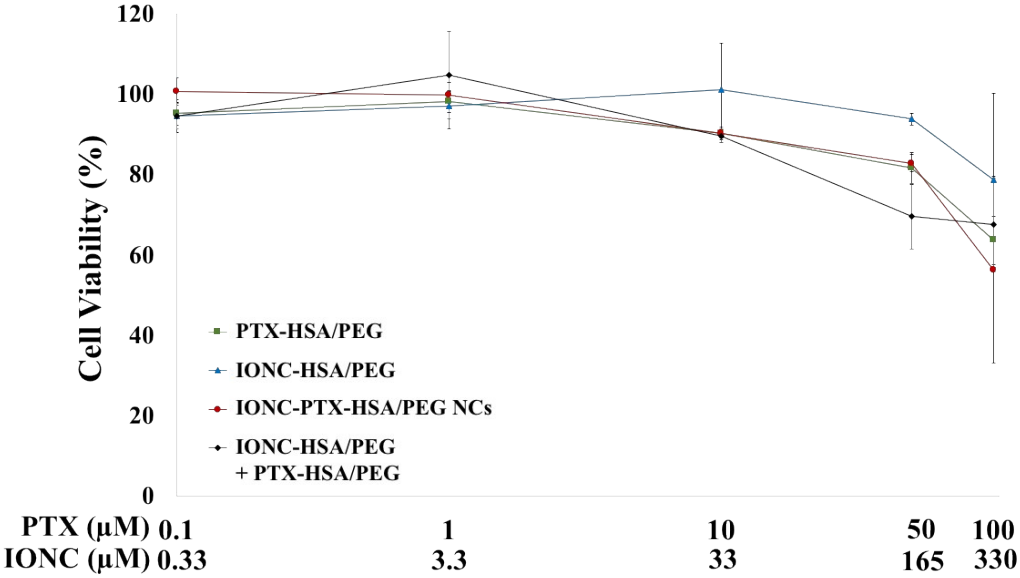

Figure S10. Viability of human fibroblast cells exposed to increasing concentration of paclitaxel and IONC at 48 h after treatment of PTX-HSA/PEG, IONC-HSA/PEG, and IONC-PTX-HSA/PEG, and co-treatment of IONC-HSA/PEG and PTX-HSA/PEG.