Electronic Supplementary Information

Hyaluronic acid-polypyrrole nanoparticles as pH-responsive theranostics

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Materials and methods

Materials

Pyrrole (98 %), polyvinylpyrrolidone (PVP, MW ~ 29,000), iron (III) chloride hexahydrate ($FeCl_3$•$6H_2O$), and doxorubicin hydrochloride (DOX) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Hyaluronic acids (HA, MW 16,100 Da & MW 66,300 Da) were purchased from Lifecore Biomedical (Chaska, MN, USA) and used as received. Micro dialysis tube (D-Tube^{TM} Dialyzer Mini, molecular weight cut off 12-14 kDa) was obtained from Novagen (Madison, WI, USA). All the other chemicals and solvents were analytic grade and used without further purification. Primary coronary artery smooth muscle cell (VSMC, human) and Raw264.7 (Mouse macrophage cell line) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic/antimycotic were purchased from Life Technologies (Gaithersburg, MD, USA).

Methods

Synthesis of polypyrrole nanoparticles (PPyNP)

Polypyrrole nanoparticles with narrow size distribution were synthesized as described by Mei et al [1]. Briefly, PVP (1 g) was dissolved in 25mL of deionized (DI) water with magnetic stirring for 30 min at room temperature (R.T), followed by addition of 130 μL of pyrrole. After 10 min, 1mL of iron (III) chloride hexahydrate (0.75 g/mL) was rapidly added to the reaction mixture to initiate polymerization reaction. The color of the solution was changed from bight brown to dark black in two minutes. Reaction was continued by further stirring for 3 h at R.T. The product was purified by washing the nanoparticles with ethanol-acetone mixture at least twice. Then, the solution was dialyzed using a membrane (molecular weight cut-off [MWCO] 50,000 Da) for 24 h and lyophilized to obtain the black powder.

Synthesis of HA-coated polypyrrole nanoparticles (HA-PPyNP)

HA-conjugated polypyrrole nanoparticles (HA-PPyNP) were synthesized by modifying the method of PPyNP synthesis. In brief, HA (400 mg) was dissolved in 20 mL of DI water.

PVP (0.5 g) was dissolved in 12.5 mL of DI water with magnetic stirring for 30 min at room temperature (R.T), followed by addition of 65 μL of pyrrole. After 10 min, 0.5 mL of iron (III) chloride hexahydrate (0.75 g/mL) was rapidly added to the reaction mixture. Pre-dissolved HA solution (20 mL) was added in 2 min. After 3 h of incubation, the solution was purified using a membrane dialysis (MWCO: 50,000) for 2 days. Large sized precipitate was removed by centrifugation at 1,200 rpm for 2 min. Black powder was obtained by freeze-drying.

Fluorescence quenching of DOX by HA-PPyNP

The experiment was carried out by monitoring fluorescence spectra of doxorubicin upon addition of increasing amounts of HA-PPyNP. Doxorubicin (10 μg) dissolved in 100 μL DI water were mixed with 200 μL of DI water containing various amounts of HA-PPyNP (0, 10, 20, 40, 60, 80, 100, 120, 150, and 200 μg), incubated for 30 min, and then fluorescence spectra of doxorubicin was measured (λ_{ex} = 480 nm).

Preparation and Characterization of DOX-loaded HA-PPyNP (DOX@HA-PPyNP)

HA-PPyNP (30 mg) was dissolved in 3 mL of DI water. Meanwhile, 3 mg of DOX was dissolved in 300 μL of DI water. DOX solution was slowly dropped into the HA-PPyNP solution, and the mixture was incubated for 3 h to form charge complex between DOX and HA-PPyNP. Free DOX was removed by PD-10 column (GE Healthcare, Sweden) and then the final product was lyophilized.

The surface charge and dynamic light scattering measurements were performed using a zeta potential / particle-sizer (Malvern Instrument, Malvern, UK). For comparison of optical properties, DOX@HA-PPyNPs were dispersed in DI water, whereas free DOX was dissolved in DI water containing sodium dodecyl sulfate (SDS, 5 wt/v %) to avoid

unnecessary aggregation and quenching of DOX molecules in neutral pH condition. Then, UV / Vis spectrophotometer (DU730, Beckman Coulter, Brea, CA) and multifunctional microplate reader (Safire 2; Tecan, Männedorf, Switzerland) were used to measure UV/Vis absorption and fluorescence spectra of DOX, respectively. Morphologies of HA-PPyNP and DOX@HA-PPyNP were observed using field emission scanning electron microscope (FE-SEM, JEOL-7001F, JEOL Ltd, Japan) and transmission electron microscope (TEM, JEM-2010, JEOL Ltd, Japan).

To measure amount of DOX in DOX@HA-PPyNP, DOX@HA-PPyNP was dissolved in SDS contained-(5 wt/v %) DI water to detach DOX from HA-PPyNP, then UV/Vis. Absorption spectrum of DOX@HA-PPyNP was obtained (**Fig. S3**). Absorbance of DOX in DOX@HA-PPyNP at 498 nm was separated from that of HA-PPyNP, and concentration of DOX in the solution was calculated by comparing with the standard curve obtained from SDS-contained free DOX solution.

In vitro release profile of DOX from DOX@HA-PPyNP

Release behaviors of DOX from DOX@HA-PPyNP were examined at different pH conditions. DOX@HA-PPyNP (200 μg/200 μL) were transferred into micro dialysis tube, and immersed in 25 mL of phosphate-buffered saline (PBS; 160 mM, pH 7.4, NaCl 150 mM) and acetate buffer (200 mM, pH 5.0) solution with gentle shaking. At each selected time point, 400 μL of buffer solutions were replaced with fresh one, and fluorescence spectra of the collected solution were measured λ_{ex} 480 nm λ_{em} 593 nm). The experiments were performed in triplicate.

Cell culture

Culture of VSMC was performed under vascular cell basal medium containing vascular smooth muscle cell growth kit and antimicrobials/antimycotics from ATCC. Raw264.7 cells were maintained in DMEM media supplemented with 10 % FBS and 1% antibiotic/antimycotic (Life Technologies) at 37 ℃ under 5% CO2 in a standard humidified incubator.

Live cell imaging for analysis of fluorescence quenching and subsequent recovery in macrophage cells

Raw264.7 cells were seeded into a Lab-Tek chamber cover glass at a density of 2 x 10^4 cells per well, and incubated for 24 h for cell attachment. The cells were then treated cell culture medium with lipopolysaccharide (LPS, 1 μg/mL) and interferon-gamma (INF-γ, 50 ng/mL) for 24 h. DOX@HA-PPyNP and free DOX were diluted in fresh DMEM medium supplemented with 10% FBS to obtain 2 μM DOX equivalent. The cell culture medium was replaced with phenol red-free fresh medium containing the drugs. Then, without washing the cells, fluorescence images (λ_{ex} = 470 ± 20 nm, λ_{em} = 540 ± 25 nm) of the macrophage cells were acquired every 15 min for 3 h using a Live Cell Imaging System (Axio observer Z1, 10x, NA 0.55, Carl Zeiss, Germany). All the images were acquired at the same microscope settings in order to ensure reproducibility, and analyzed with ZEN lite 2012 software (Carl Zeiss, Germany).

Cytotoxicity test of HA-PPyNP

Raw264.7 cells were seeded into 96-well plate at a density of 1 x 10^4 cells per well, and incubated overnight for cell attachment. The cell culture medium was replaced with fresh medium containing various concentrations of HA-PPyNP, and the cells were incubated for 24 h. After washing the cells with PBS solution two times, cell viability was evaluated using a

CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan). The viability of the untreated control cells were taken to be 100%. Data are expressed as the mean (SD) of 4 data samples.

Cytotoxicity test of DOX@HA-PPyNP

Macrophage cells were seeded into a 96-well plate at a density of 1 x 10^4 cells per well and incubated for 24 h for cell attachment. The macrophage cells were then treated cell culture medium with LPS (1 μg/mL) and INF-γ (50 ng/mL) for 24 h. Both free DOX and DOX@HA-PPyNP were diluted in fresh DMEM medium supplemented with 10% FBS to obtain various DOX concentration. The medium was replaced with fresh cell culture medium containing free DOX and DOX@HA-PPyNP, and the cells were incubated for 3 h. For competition assay, macrophage cells were incubated with DOX@HA-PPyNP for 3 h in the presence of free HA (10 mg/mL, 66,300 Da). Then the cells were washed two times and further incubated for an additional 48 h. Cell viability was analyzed using a cell counting kit-8 (CCK-8) assay kit. The viability of the untreated control cells were taken to be 100%.

Human vascular smooth muscle cells (VSMC) were seeded into a 96-well plate at a density of 1 x 10^4 cells per well and incubated for 24 h for cell attachment. Culture medium of VSMC was changed to starvation medium (i.e., cell culture medium containing 0.5% FBS) and incubated for 72 h to obtain quiescent state. Then the cells were treated with the medium containing free DOX and DOX@HA-PPyNP at various concentrations for 3 h, washed two times, and further incubated for an additional 48 h. Cell viability was analyzed using a cell counting kit-8 (CCK-8) assay kit. The viability of the untreated control cells were taken to be 100%.

Data are expressed as the mean (SD) of 4 data samples.

Evaluation of CD44-mediated endocytosis of DOX@HA-PPyNP by confocal microscopy

Macrophage cells were seeded in an 8-well Lab-Tek chambered coverglass at a density of 2×10^4 cells per well, and incubated for 24 h to allow cell attachment. The macrophage cells were then treated with LPS (1 μg/mL) and INF-γ (50 ng/mL) in cell culture medium for 24 h. The medium was replaced with fresh cell culture medium containing DOX@HA-PPyNP, and the cells were incubated for 3 h. For the competition assay, macrophage cells were incubated with DOX@HA-PPyNP for 3 h in the presence of free HA (10 mg/mL, 66,300 Da). Finally, the cells were washed two times in culture media, and then fluorescence images of the cells (λ_{ex} = 488 nm, λ_{em} = 588 nm) were acquired using a confocal laser scanning microscope.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Student's t-test was performed for statistical analyses.

References

1. M. Chen, X. Fang, S. Tang and N. Zheng, *Chem. Commun.*, 2012, **48**, 8934.

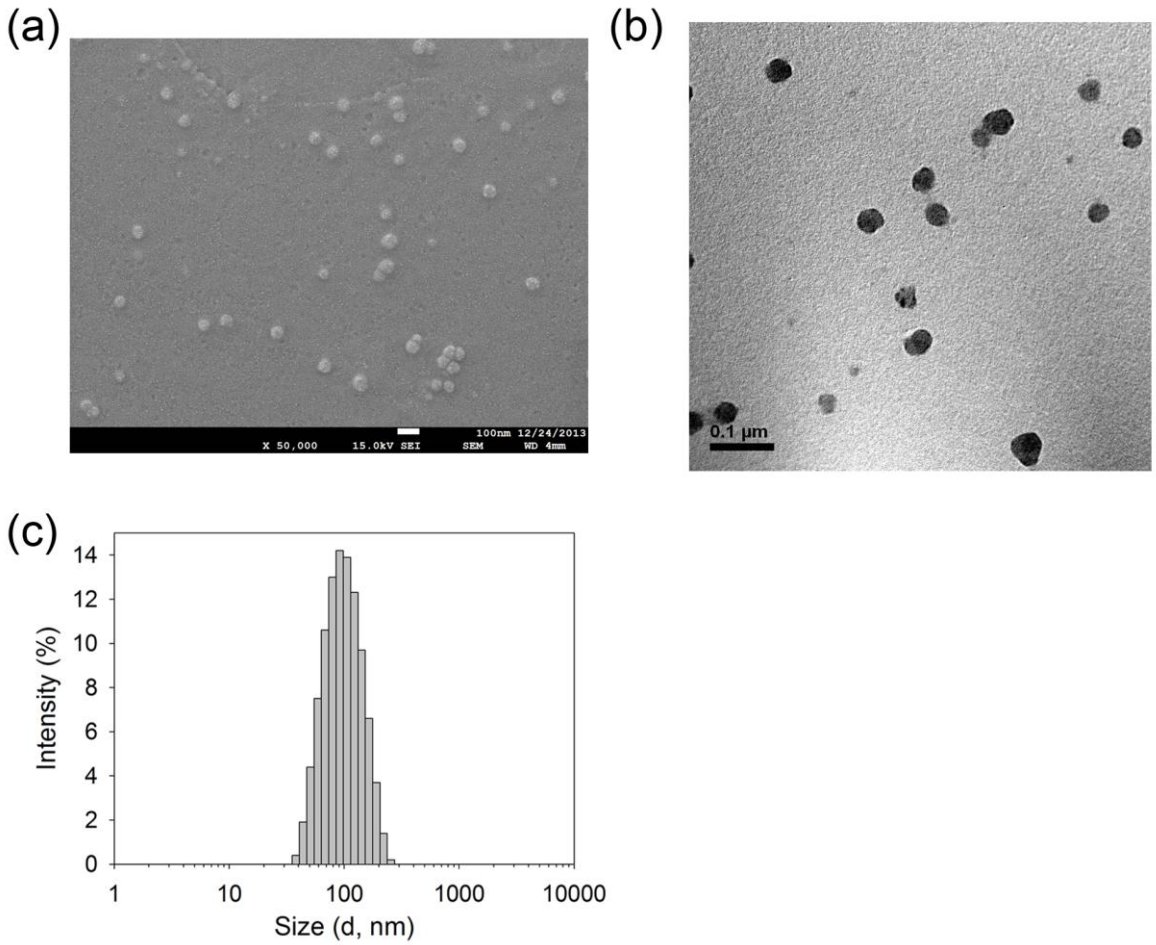

Fig. S1 (a) Scanning electron microscopy (SEM) and (b) transmission electron microscopy (TEM) images of HA-coated PPyNP (HA-PPyNP), and (c) the hydrodynamic size of HA-PPyNP.

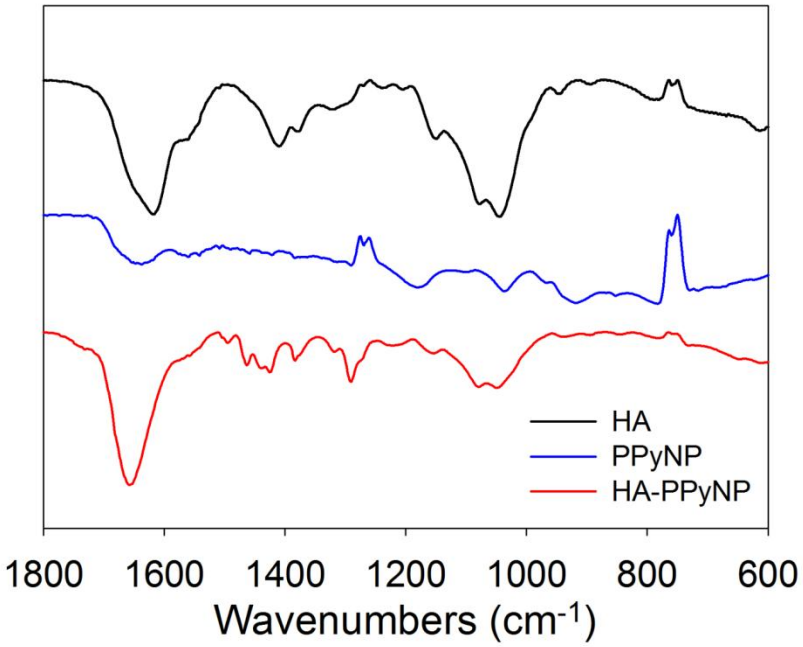

Fig. S2 FT-IR spectra of HA, PPyNP, and HA-PPyNP.

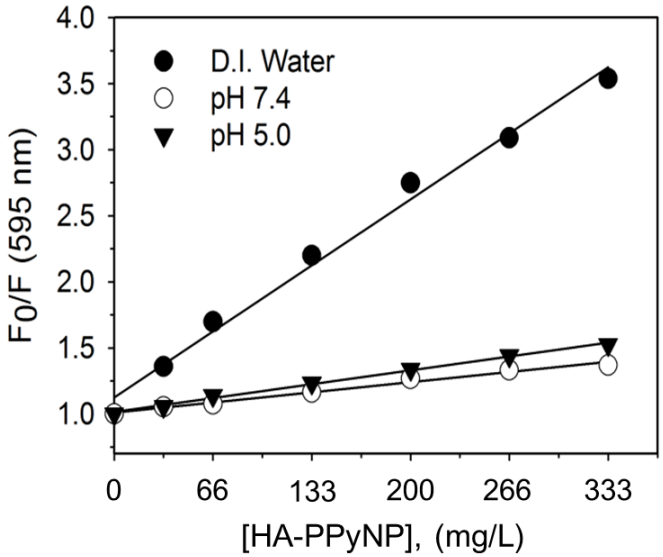

Fig. S3 Stern-Volmer plots showing the quenching efficiency of DOX fluorescence by HA-PPyNP in various solutions. Both DOX and HA-PPyNP were dissolved in deionized (DI) water, phosphate buffer (pH 7.4), and acetate buffer (pH 5.0) solutions, and then the quenching experiment was performed using various concentrations of HA-PPyNP. The quenching efficiency of HA-PPyNP was highest in the DI water condition, indicating that charge complex between DOX and HA-PPyNP formed best in DI water. The Stern-Volmer quenching constants (K_{sv}) calculated from the plots were 7.5×10^{-3} (DI water), 1.2×10^{-3} (pH 7.4), and 1.5×10^{-3} (pH 5.0).

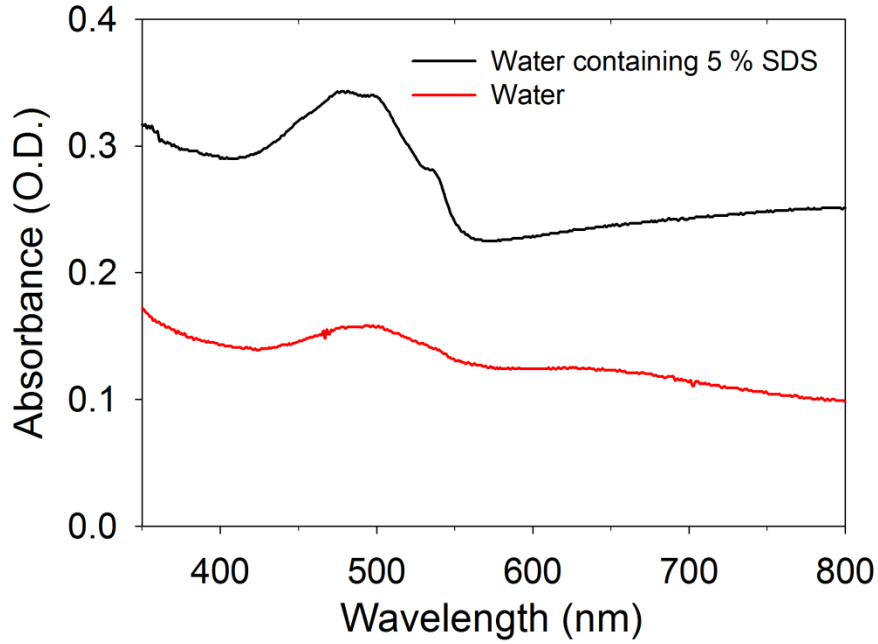

Fig. S4 UV/Vis absorption spectra of DOX@HA-PPyNP in water and water contained 5 (wt/v) % sodium dodecyl sulfate (SDS).

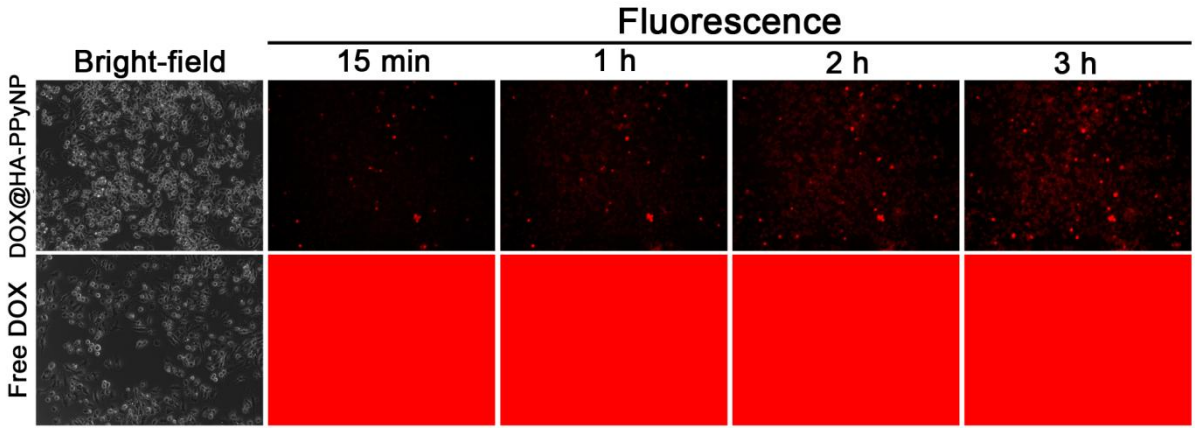

Fig. S5 Live cell images of Raw 264.7 cells incubated with either free DOX or DOX@HA-PPyNP (2 μM DOX equivalent). Fluorescence images were obtained every 15 min for 3 h without washing the cells. Red color indicates fluorescence signals from DOX.

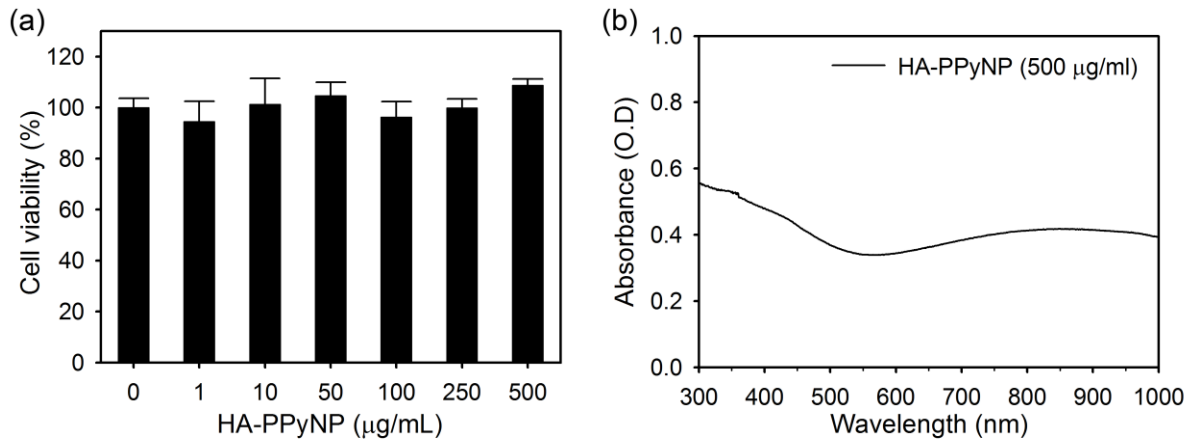

Fig. S6 (a) *In vitro* cytotoxicity test of HA-PPyNPs at various concentrations. Results represent mean±SD (n=4). For reference, the concentration of HA-PPyNP contained in DOX@HA-PPyNP at 5 μM DOX eq. is 41.7 μg/mL. (b) UV/Vis. absorption spectra of HA-PPyNP in water at a concentration of 500 μg/mL.

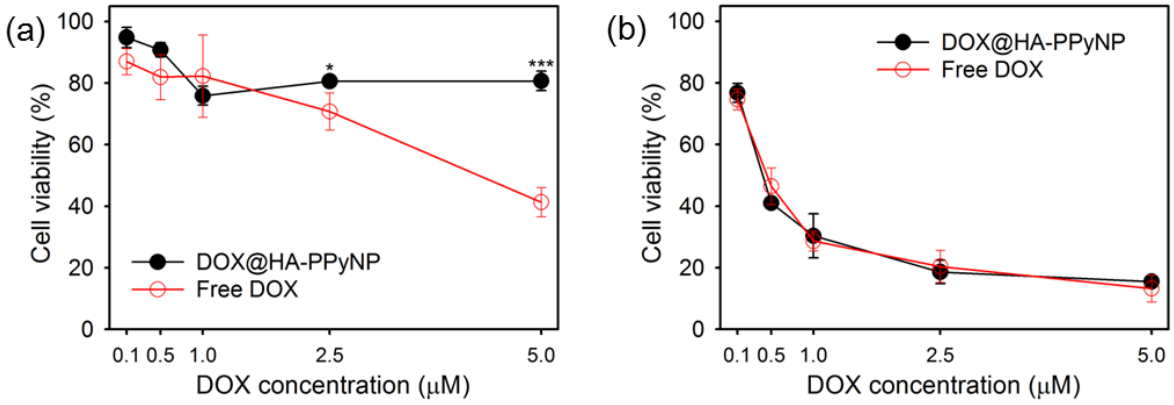

Fig. S7 *In vitro* cytotoxicity of DOX@HA-PPyNP and free DOX in (a) VSMCs and (b) macrophage cells (n = 4). Significant differences (*P < 0.05, ***P < 0.001) between free DOX- and DOX@HA-PPyNP-treated VSMC cells were observed at 2.5 and 5 μM DOX equivalent concentrations.

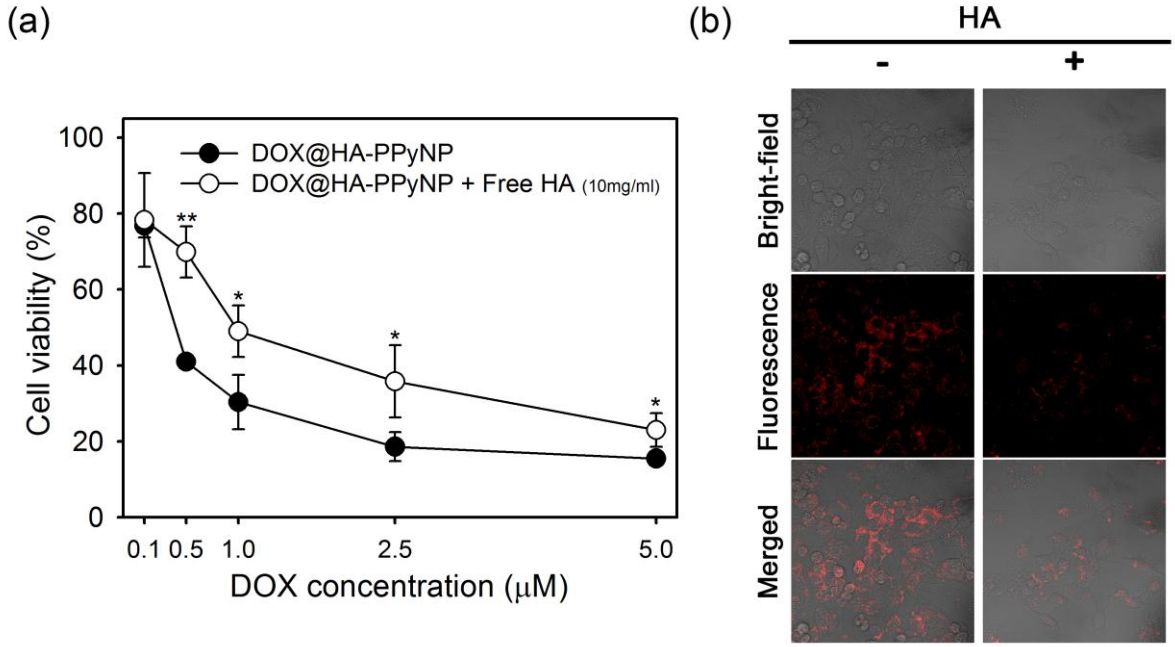

Fig. S8 (a) *In vitro* cytotoxicity test of DOX@HA-PPyNP. Macrophage cells were treated with DOX@HA-PPyNP at various concentrations in the absence and presence of free HA (10 mg/mL). $*P < 0.05$, $**P < 0.01$. (b) Confocal microscopy images showing co-treatment of free HA (10 mg/mL) reduces intracellular uptake of DOX@HA-PPyNP into macrophage cells.