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Construction of a novel "ball-and-rod" MSNs-pp-PEG: a promising antitumor drug delivery system with particle size switchable function

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Experimental section

Live Subject statement

All experiments were performed in compliance with the Guidelines of the Air Force Medical University Animal Use and Care Committee and international standards on animal welfare. Besides, Animal Research Committee of Air Force Medical University have approved the experiments.

Materials

Tetraethyl orthosilicate (TEOS), cetyltrimethyl ammonium bromide (CTAB), triton X-100, 3-Aminopropyl triethoxysilane (APTES) and Doxorubicin (DOX) were purchased from Sigma-Aldrich (Shanghai, China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl) and N-hydroxysuccinimide (NHS) were purchased from Energy Chemical (Shanghai, China). Matrix metalloproteinase-2 sensitive peptide (GPLGIAGQ) was purchased from Genscript. 3-arm polyethylene glycol (3-arm PEG-COOH) was purchased from Nanocs Inc. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS) and Cell Counting Kit-8 (CCK-8) were purchased from Hyclone Cell Culture and Bioprocessing (Thermo Scientific, USA). 4',6-diamidino-2-phenylindole (DAPI) was purchased from Gibco. Cy5.5-PEG_{2000}-NHS ester was purchased from Pure Chemistry Scientific Inc.

Cell lines and animals

The human lung epithelial tumor cell line A549, human leukemia monocytic cell line Thp-1, human umbilical vein endothelial cell line HUVEC and mouse melanoma cell line B16 were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A549 cells, HUVEC cells and B16 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Thp-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. In all experiments, cells were cultivated at 37 °C in a humidified atmosphere of 5% CO_2/95% air. All experiments were performed on cells in the logarithmic phase of growth.

The male C57 mice (6–8 weeks old) were purchased from the Experimental Animal Center of Air Force Medical University. Animals were housed in a temperature and light-controlled environment and were provided food and water ad libitum.

Synthesis of MSNs and MSNs-NH_2

Briefly, 0.1g of CTAB was dissolved in a solution composed of n-Hexyl alcohol (8 mL), cyclohexane (37 mL), deionized water (2 mL) and triton X-100 (12 mL). After stirring for 0.5 h, TEOS (0.5 mL) and ammonia water (0.5 mL) were added into the solution and stirred for another 24 h at 100 °C. After the mixture cooled down, the resultant complexes were separated by centrifugation at 12000 rpm for 10 min, then washed with ethanol and water for several times. To anchor -NH_2 onto the surface of silica nanoparticles, MSNs were functionalized with APTES. MSNs (10 mg) were dissolved in ethanol (30 mL) followed by the addition of APTES (400 μL) and reacted at 60 °C for 6h. The products were centrifuged at 12000 rpm for 10 min and washed with ethanol, then MSNs-NH_2 were obtained.

Synthesis of 3-arm PEG-matrix metalloproteinase-2 sensitive peptides conjugates （pp-PEG)

The 3-arm PEG-COOH and MMP-2 sensitive peptide (GPLGIAGQ, pp) (1:6, molar ratio), along with EDC•HCl and NHS, were reacted in dimethyl formamide (DMF) under nitrogen protection at room temperature for 24 h. After that, the mixture was dialyzed against deionized water by using a dialysis membrane (MWCO 2000 Da) for 48 h. After being frozen dried, the obtained pp-PEG conjugates were stored at −20°C for further use.

Synthesis of MSNs-pp-PEG and MSNs-PEG nanoparticles

The MSNs-pp-PEG was obtained by amidation between the carboxyl groups of pp-PEG and amino groups of MSNs-NH_2. The MSNs-NH_2 and pp-PEG (1:15, mass ratio), along with EDC and NHS•HCl, were reacted in the dimethyl sulfoxide (DMSO) under nitrogen protection for 24 h. The products were centrifuged at 12000 rpm for 10 min and washed with deionized water and ethanol several times, then MSNs-pp-PEG was obtained. MSNs-PEG was prepared similarly to MSNs-pp-PEG nanoparticles by replacing pp-PEG with 3-arm PEG-COOH.

Characterization of MSNs-pp-PEG

The structures of MSNs and MSNs-NH_2 were characterized by fourier transformed infrared spectroscopy (FTIR). The spectra were obtained using the IR spectrometer (Nicolet, Madison, USA) at a resolution of 2 cm^{-1} and a spectral range of 4000–400 cm^{-1}. The pp-PEG was verified by proton nuclear magnetic resonance (^{1}H-NMR). The ^{1}H-NMR spectra was obtained using Varian 400MHz (Bruker, Germany), with tetramethylsilane (TMS) as the internal standard. Chemical shifts were expressed as parts per million (ppm). The morphologies of MSNs and MSNs-pp-PEG with or without MMP-2 incubation were examined by transmission electron microscopy (TEM, JEM-1230, Japan). The Zeta potentials of MSNs, MSNs-NH_2 and MSNs-pp-PEG with or without MMP-2 were evaluated by dynamic light scattering (DLS) using the Delsa™ Nano C Particle analyzer (BECKMAN Coulter Instruments, USA).

Preparation of MSNs/Cy5.5, MSNs-PEG/Cy5.5 and MSNs-pp-PEG/Cy5.5 nanoparticles

Nanoparticles were labeled by Cy5.5. MSNs-pp-PEG (1.0 mg) and Cy5.5-PEG_{2000}-NHS ester (0.3 mg) were dissolved in DMF and stirred at room temperature for 4 h. The solution was centrifuged at 12000 rpm for 10 min and MSNs-pp-PEG/Cy5.5 nanoparticles were obtained. MSNs/Cy5.5 and MSNs-PEG/Cy5.5 nanoparticles were prepared similarly to MSNs-pp-PEG/Cy5.5 nanoparticles.

Doxorubicin loading and *in vitro* release

MSNs (10 mg) were added to 10 mL of DOX solutions (500 μg/mL) and stirred for 48 h at room temperature. Subsequently, the reaction mixture was centrifuged at 12000 rpm for 10 min and the sediment was washed with deionized water several times to remove redundant DOX. The supernatant was collected and unloaded DOX was estimated at 480 nm using UV–vis spectroscopy (Shimadzu, Japan). A similar method was followed for drug loading in MSNs-pp-PEG. Drug loading was calculated using the following equations:

Drug loading content (%) = (the total amount of DOX added − the amount of unloaded DOX) / the amount of carriers × 100.

For the *in vitro* DOX release experiment, 10 mg of DOX-loaded MSNs were suspended in 5 mL of PBS. These suspensions were then dialyzed against 50 mL PBS using a dialysis membrane (MWCO 2000 Da). The sample was shaken at 120 rpm in the dark. At predetermined time points, 2 mL of solution was taken

away and replenished with 2 mL of fresh PBS. The amount of DOX released was detected by UV–vis spectroscopy and the cumulative release of DOX was calculated. Each drug release test was performed thrice. A similar method was followed for drug release in MSNs-pp-PEG.

Cytotoxicity of MSNs-pp-PEG

The cytotoxicity of blank carriers was evaluated by a Cell Counting Kit-8 (CCK-8) assay on A549 cells, Thp-1 cells and HUVEC cells. A549 cells (5.0×10^3 cells per well) were seeded into 96-well plates and incubated for 24 h. Then the medium was replaced by MSNs, MSNs-PEG or MSNs-pp-PEG in cell culture medium with different concentrations and incubated for 24 h. After that, 10μL of CCK-8 solution was added to each well and incubated for another 2 h. The absorption was measured by scanning with a microplate reader (Thermo Scientific Varioskan Flash, USA) at 450 nm. By changing A549 cell to Thp-1 cells and HUVEC cells, the cytotoxicity of blank carriers to these cells was also evaluated.

Hemocompatibility of MSNs-pp-PEG

The whole mouse blood was collected into heparinized tubes to prevent coagulation and the obtained fresh red blood cells were diluted to 2% in saline. The MSNs, MSNs-PEG or MSNs-pp-PEG were added in 2% red blood cell suspensions at a final concentration of 1 mg/mL. Besides, red blood cells incubated with distilled water and saline were used as positive and negative controls, respectively. All samples were kept in static condition at 37 °C for 12 h. The red blood cells suspensions were centrifuged at fixed time points. The supernatants were collected and the absorbance values at 560 nm were determined by using a microplate reader. The hemolysis ratio was calculated using the following equation:

Hemolysis ratio (%) = (sample absorbance – negative control absorbance) × (positive control absorbance – negative control absorbance) × 100.

***In vitro* cytotoxicity of DOX-loaded nanocarriers**

A549 cells (5.0×10^3 cells per well) were seeded into 96-well plates and cultured for 24 h. Then the medium was replaced by free DOX, MSNs/DOX, MSNs-PEG/DOX or MSNs-pp-PEG/DOX in cell culture medium with different concentrations of DOX and incubated for 24 h. After that, CCK-8 assay was implemented by the method described in the " Cytotoxicity of MSNs-pp-PEG" section.

***In vitro* penetration**

The multicellular three-dimensional tumor spheroids were cultured using the liquid overlay method. Briefly, a 96-well plate was pre-coated by 1.5% hot sterilized agarose solution with 50 μL per well and cooled to room temperature. A549 cells were seeded at a density of 8.0×10^3 cells per well in 200 μL of DMEM medium and incubated. Three days later, uniform spheroids were obtained. Then, MSNs/Cy5.5, MSNs-PEG/Cy5.5, MSNs-pp-PEG/Cy5.5, MSNs-PEG/Cy5.5 and MSNs-pp-PEG/Cy5.5 incubated with MMP-2 (300 ng/mL) for 3 h (MSNs-PEG/Cy5.5 + MMP-2 3 h and MSNs-pp-PEG/Cy5.5 + MMP-2 3 h) were added to the medium 100 μL per well at a final concentration of 0.2 mg/mL. After 24 h of incubation, the spheroids were rinsed with cold PBS twice and fixed with 4% paraformaldehyde. Then the fluorescence intensity of the spheroids was observed via a confocal microscope (Nikon, Japan) using Z-axis scanning with 10 μm intervals from the top of the spheroids to the middle.

***In vivo* imaging and tumor penetration**

To develop the tumor model, a suspension of B16 cells (5×10^5 cells in 0.2 mL saline) was subcutaneously inoculated into the left flank of male C57 mice. When tumor reached designated volume (100 mm^3), mice were administrated with different Cy5.5-labeled carriers at an equivalent Cy5.5 of 30 μg per mouse through the tail vein. At 24 h after the injection, the mice were sacrificed, then the tumors were collected and imaged through an *in vivo* imaging system (IVIS Spectrum, PerkinElmer, Thermo Fisher, USA). To quantify the accumulation of nanoparticles, the fluorescence intensity of the tumor tissue was quantified. The camera was set a luminescent exposure time of 3 s.

To further determine the intratumoral distribution of nanoparticles, the tumors were sliced and stained with DAPI. Ultimately the tumor slices were observed by confocal microscopy.

***In vivo* tumor growth inhibition**

The B16 tumor-bearing mice model was established as described above. When the tumors reached a mean volume of approximately 100 mm^3, the treatment started. The mice were randomly divided into five groups (five mice each group) and were injected through the tail vein with saline, free DOX, MSNs/DOX, MSNs-PEG/DOX, or MSNs-pp-PEG/DOX respectively. The treatment was implemented with a DOX

dose of 3 mg/kg body weight every other days 7 times. The tumor volumes and body weights were measured every other day before every administration. One day after the last injection, the mice were sacrificed and the tumor tissues and major organs (heart, liver, spleen, lung and kidney) were collected and fixed with 4% paraformaldehyde for subsequent studies. Besides, tumors were weighed.

Survival data analysis

The survival time of the tumor-bearing mice after different treatments was recorded from the day of the tumor implantation to the 50th days. After that, survival curves of the five groups were plotted using the Kaplan–Meier method and were analyzed by the Mantel–Cox log-rank test. Besides, the medium survival times of five groups were compared.

Histology and immunohistochemistry

Major organs (heart, liver, spleen, lung and kidney) were subjected to hematoxylin and eosin (H&E) staining. The formalin-fixed tissues were embedded in paraffin blocks and then sliced. The slices were stained with H&E and imaged by optical microscope for histological examination.

The tumor tissues were also subjected to H&E staining. Besides, paraffin-embedded tumor sections were also subjected to the terminal transferase dUTP nick-end labeling (TUNEL) assay and Ki67 assay.

Statistical analysis

The results were presented as mean ± standard deviation of at least three repetitive experiments for all the treatment groups. Statistical analysis was conducted using the one-way ANOVA with Student's t-test using a SPSS 23.0 program. The differences between two groups were considered significant for $*P < 0.05$, and very significant for $**P < 0.01$, $***P < 0.001$.

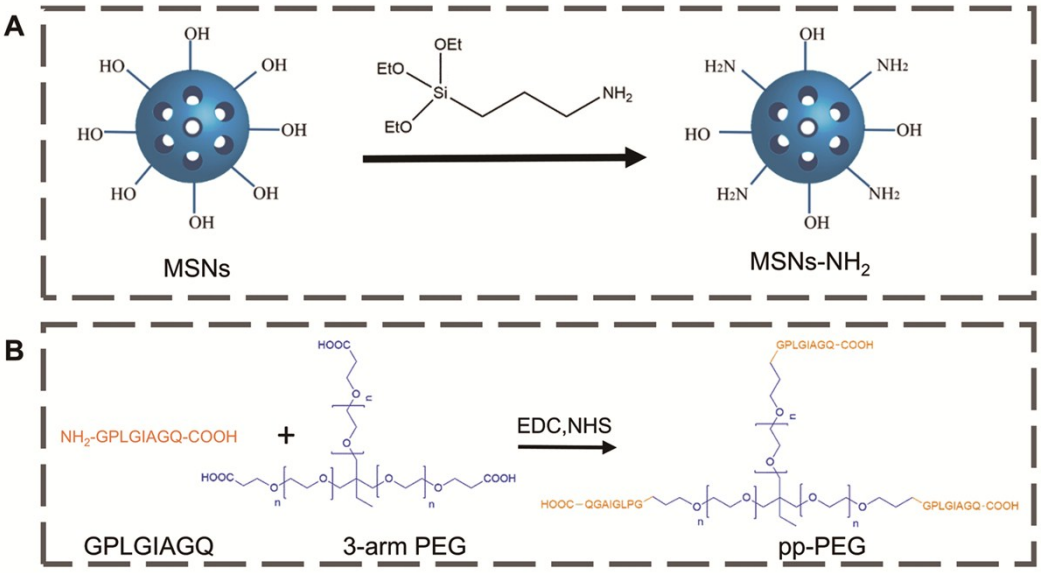

Figure S1 Synthetic scheme of (A) MSNs-NH_2 and (B) pp-PEG.

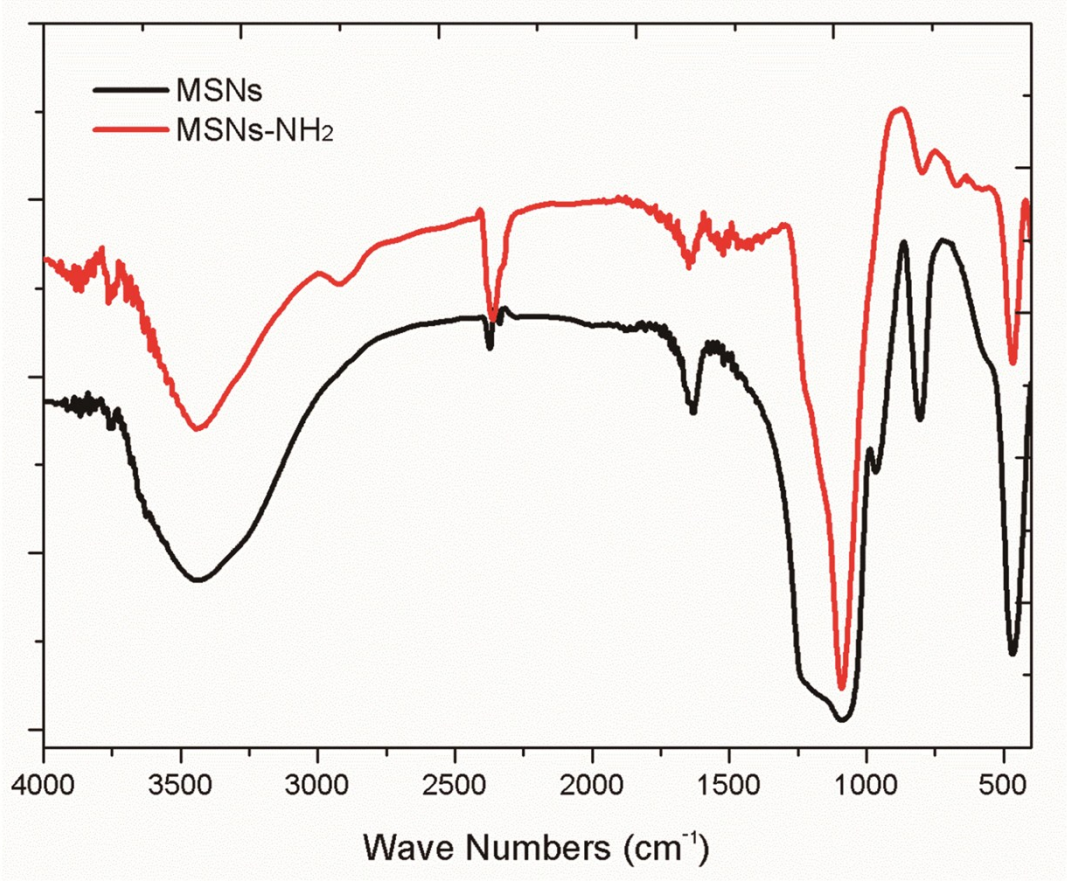

Figure S2 FTIR spectra of MSNs and MSNs-NH_2

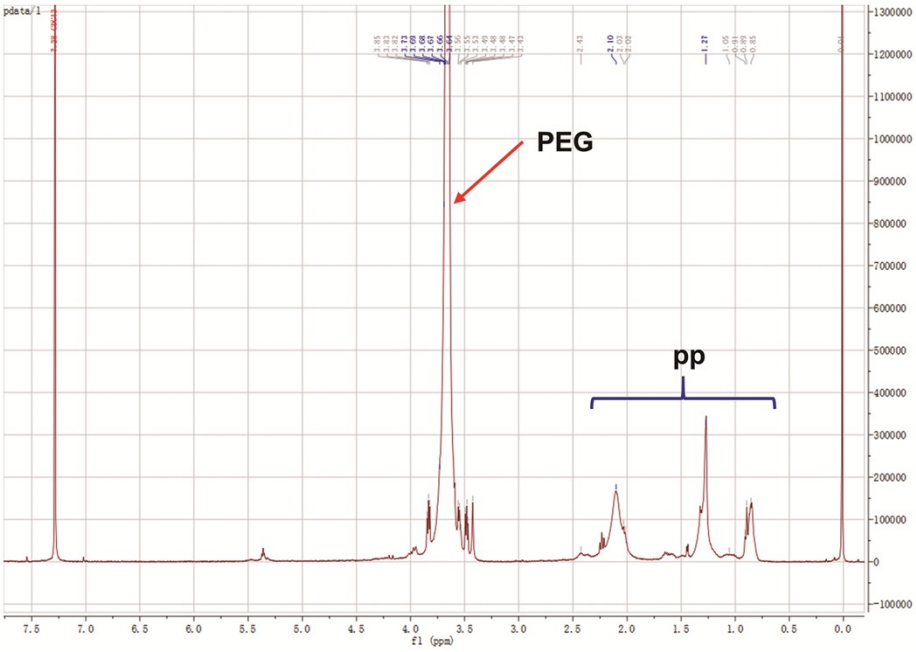

Figure S3 ^{1}H-NMR spectra of pp-PEG (solvent: $CDCl_3$, frequency of instrument: 400 MHz, concentration of compound: 5 mg/mL, temperature: 25°C).

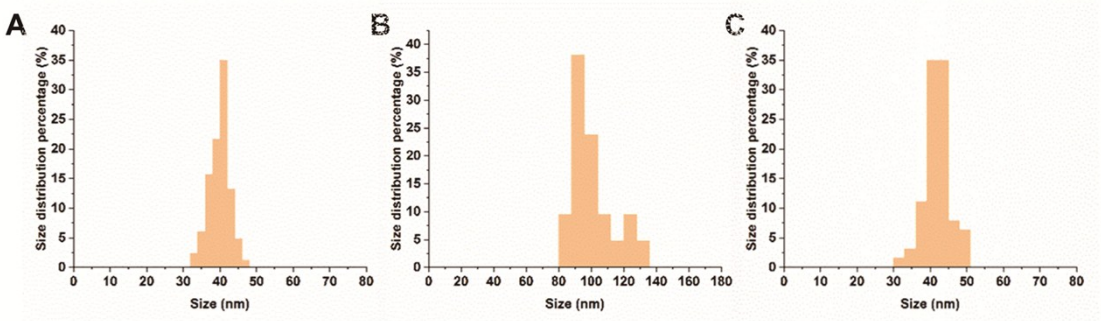

Figure S4 Size distribution of nanoparticles.

(A) MSNs. (B) MSNs-pp-PEG. (C) MSNs-pp-PEG (incubation with MMP-2 for 3 h).

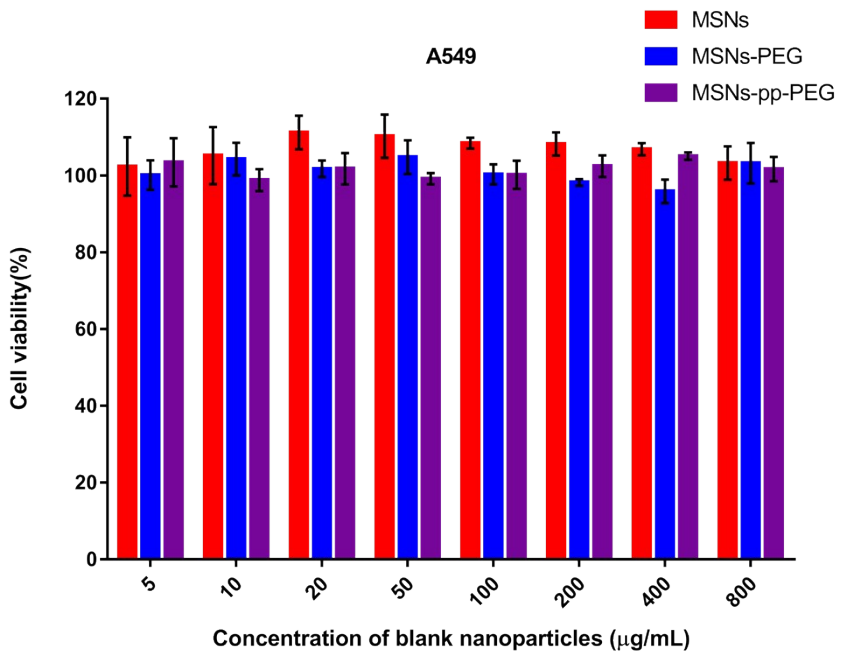

Figure S5 Cytotoxicity of blank nanocarriers against A549 cells at different concentrations.

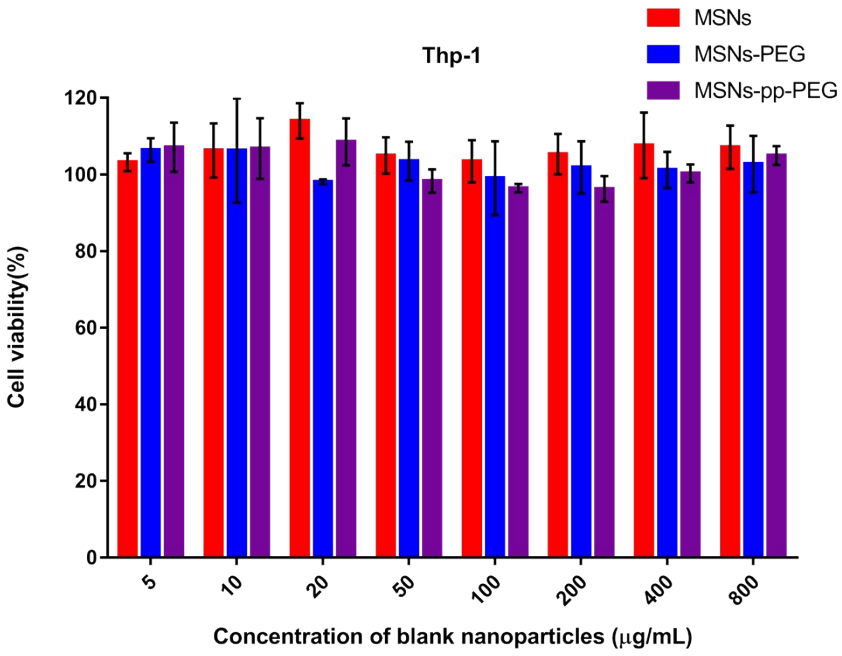

Figure S6 Cytotoxicity of blank nanocarriers against Thp-1 cells at different concentrations.

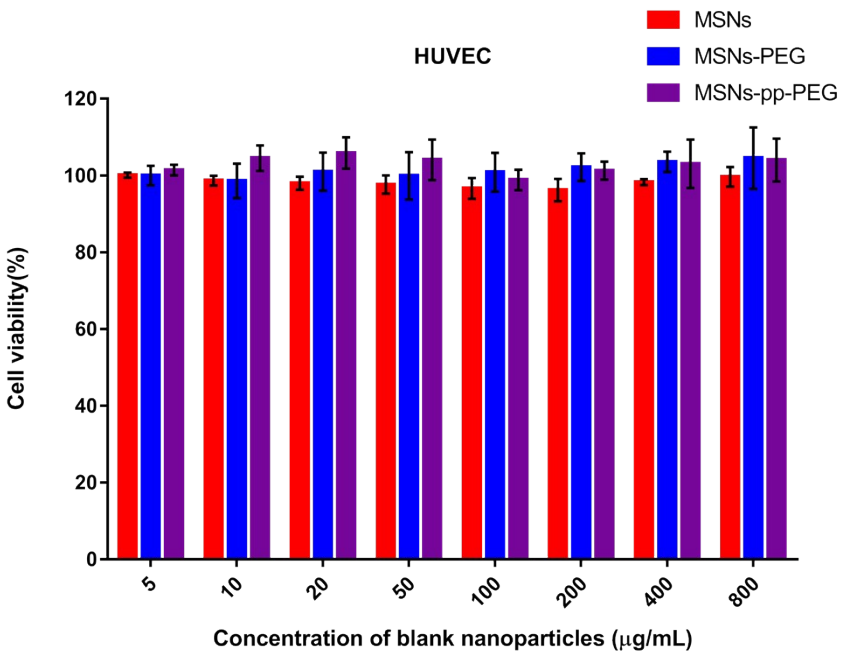

Figure S7 Cytotoxicity of blank nanocarriers against HUVEC cells at different concentrations.

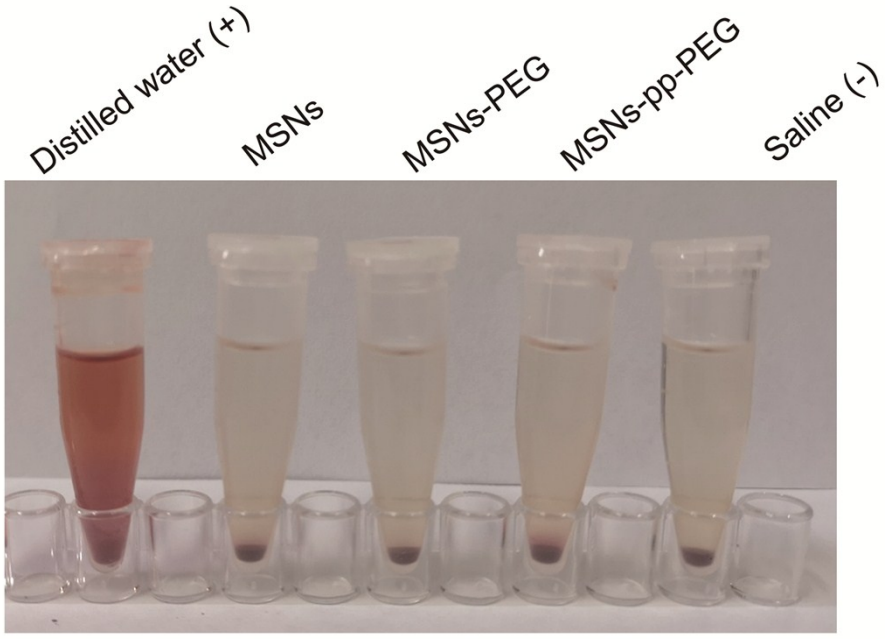

Figure S8 Image of red blood cells incubated with MSNs, MSNs-PEG and MSNs-pp-PEG for 12 h. Distilled water (+) and saline (-) were used as positive and negative control, respectively.

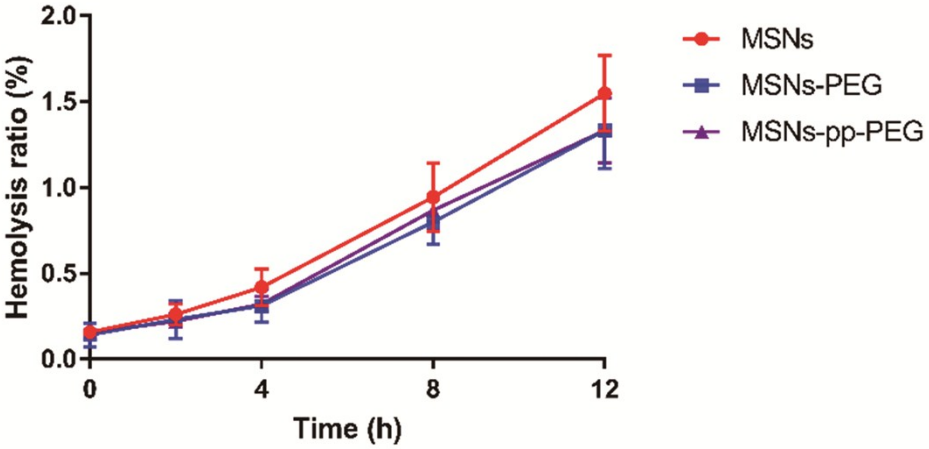

Figure S9 Hemolysis rates of blank nanocarriers for 12 h.

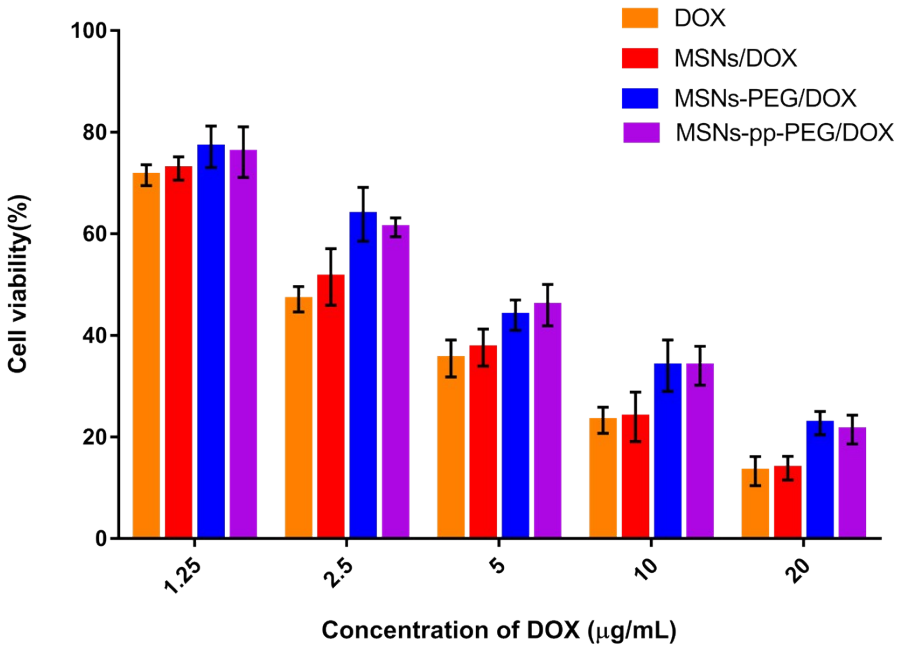

Figure S10 Cytotoxicity of DOX-loaded nanocarriers against A549 cells at different concentrations.

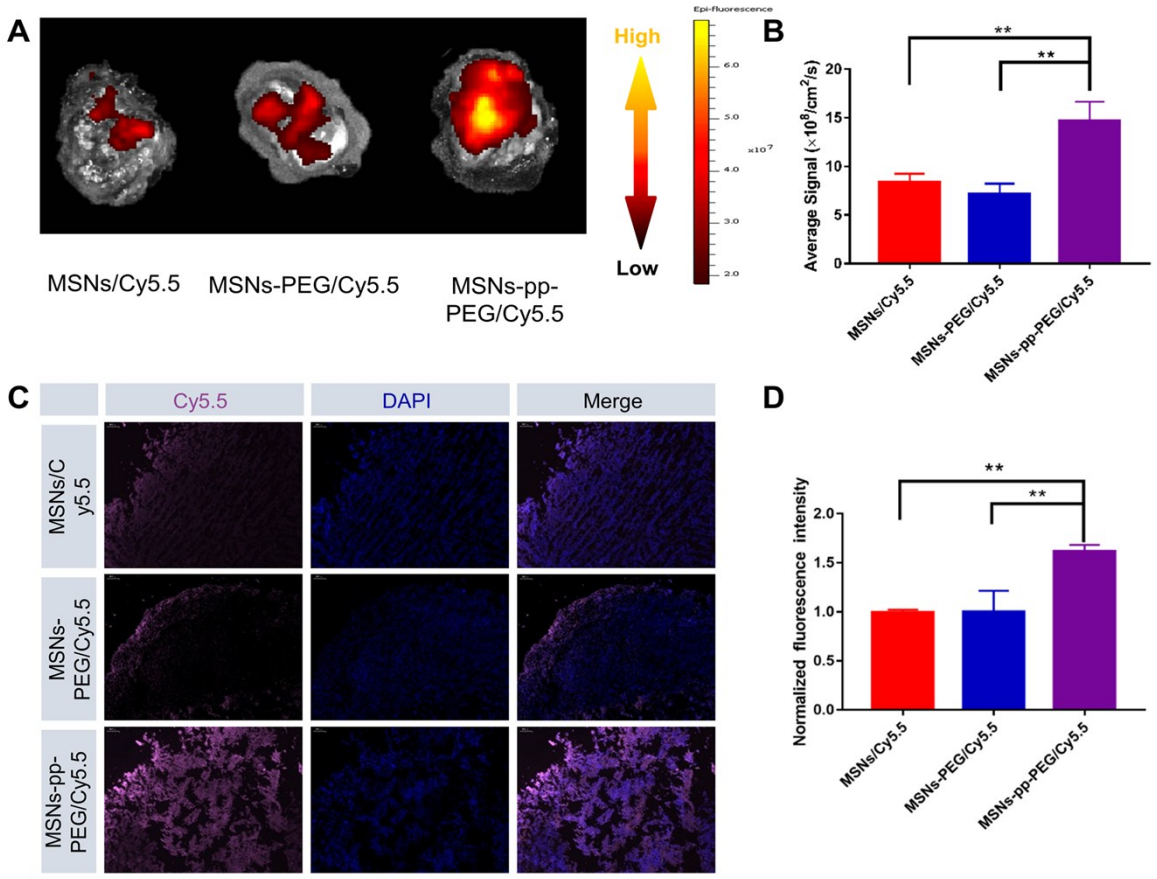

Figure S11 *In vivo* imaging and tissue penetration. (A) Fluorescence imaging of the tumor tissue at 24 h after the injection with MSNs/Cy5.5, MSNs-PEG/Cy5.5 or MSNs-pp-PEG/Cy5.5. (B) Semi-quantitative of fluorescence intensity in tumor tissue at 24 h after the injection. (C) Fluorescence distribution in edge region of tumor slices. (D) Semi-quantitative data of signals in tumor slices. Tumor tissues were stained With DAPI.

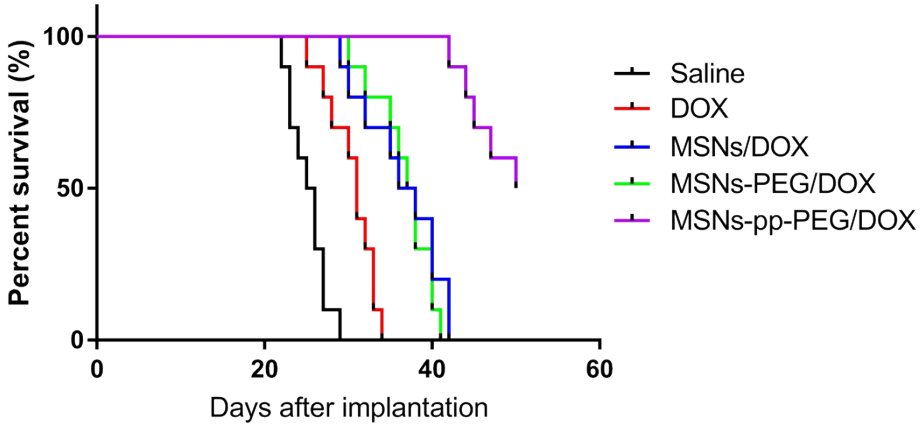

Figure S12 The Kaplan-Meier survival curves of tumor-bearing mice in each treatment group.

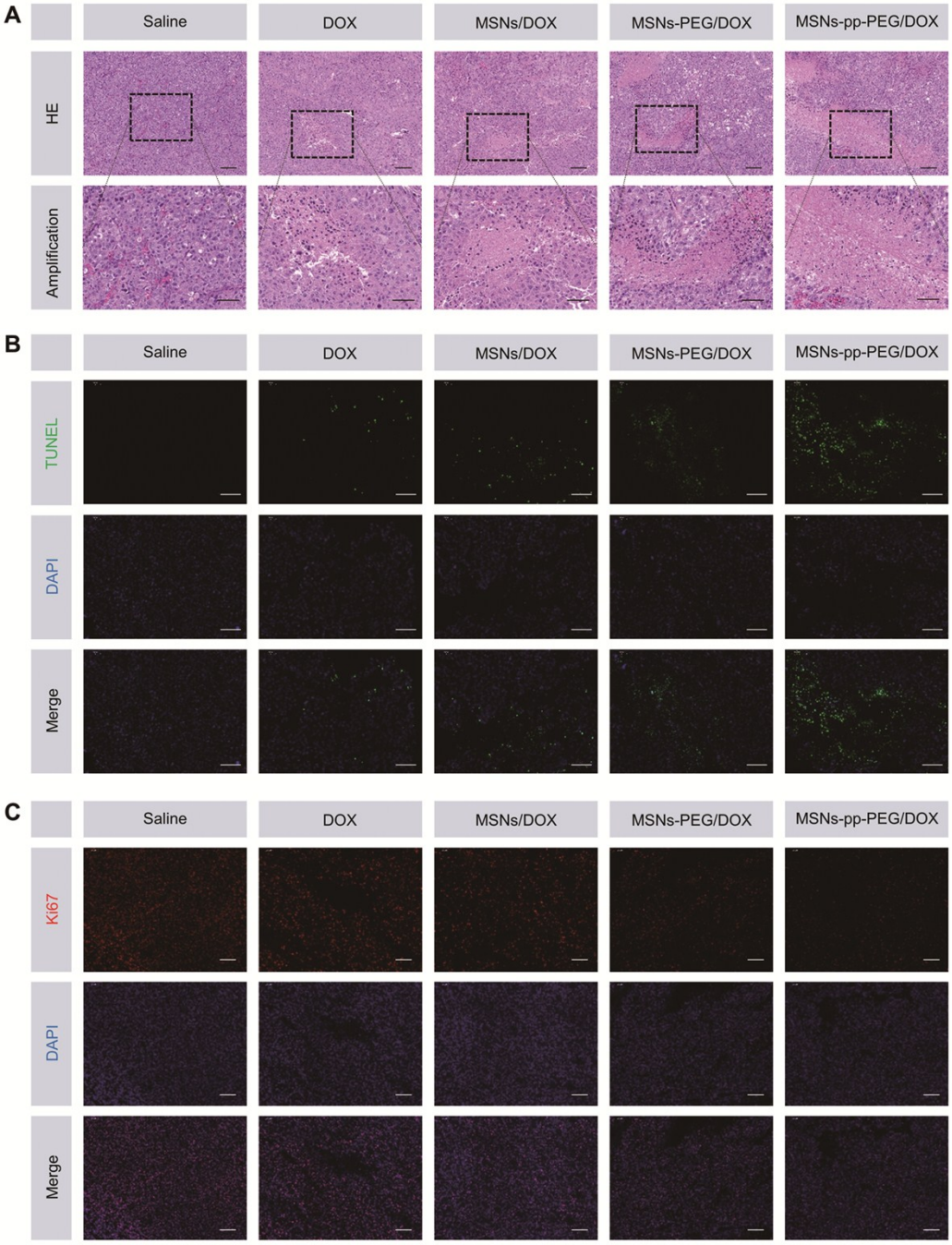

Figure S13 Histopathological analysis. Images of H&E staining (A), TUNEL staining (B) and Ki67 staining (C) of the tumors slices from mice after treatments with Saline, Free DOX, MSNs/DOX, MSNs-PEG/DOX and MSNs-pp-PEG/DOX. The scale bar represents 100 μm.

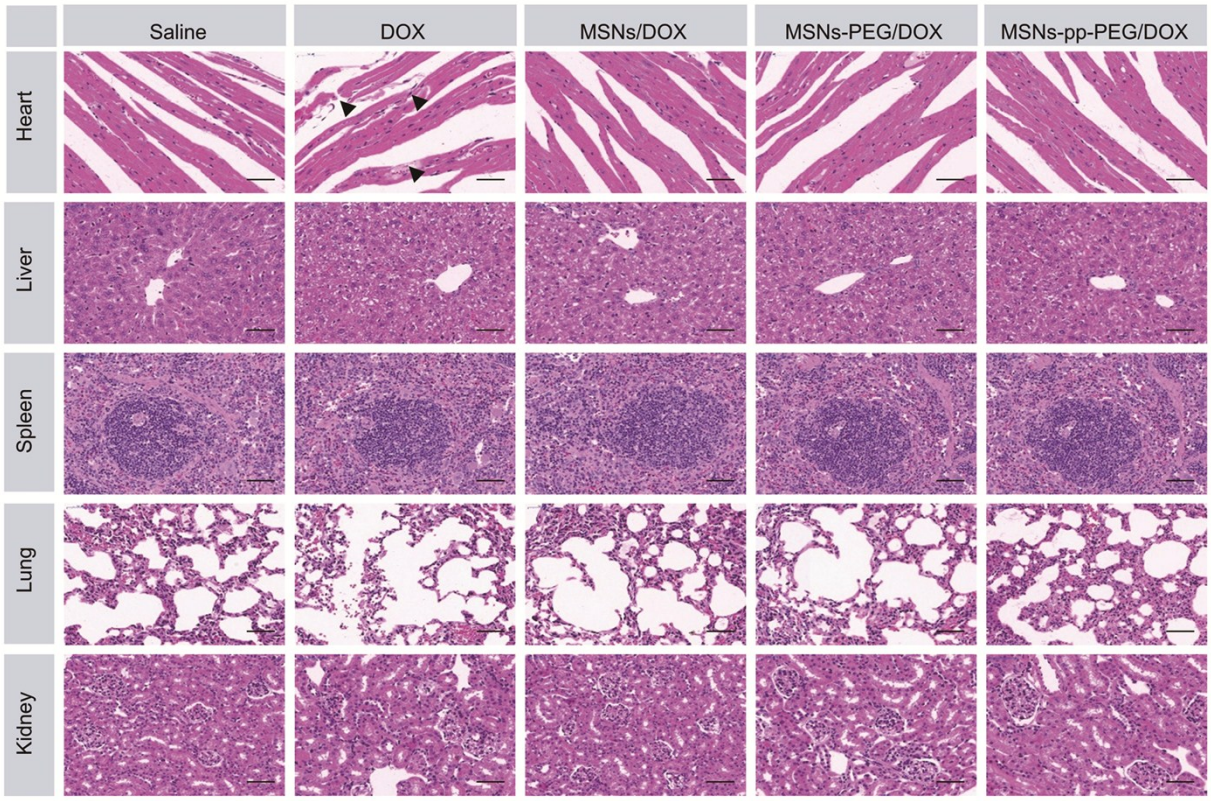

Figure S14 Images of tissue sections stained with H&E after treatments with Saline, Free DOX, MSNs/DOX, MSNs-PEG/DOX and MSNs-pp-PEG/DOX. The scale bar represents 100 μm.