Supplementary Information

Platelet membrane-coated nanoparticles enable vascular disrupting agent combining antiangiogenic drug for improved tumor vessel impairment

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1. Materials and methods

1.1 Materials

Tetraethylorthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), Prostaglandin E1 (PGE1) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Shanghai, China). Combretastatin A4 and apatinib were obtained from Selleck Chemicals (Shanghai, China). Dulbecco's modified Eagle medium (DMEM), F-12K medium and phosphate buffer saline (PBS) were purchased from Wisent Corporation (Wisent, Canada). The 10 cm cell culture dishes and 3.5 cm confocal dishes were obtained from Corning (Shanghai, China). Human umbilical vein endothelial cells (HUVECs) and MHCC-97H liver tumor cells were purchased from American type culture collection (ATCC, USA). Hoechst 33342, DiO, cy5.5 and recombinant VEGF were bought from Beyotime Institute of Biotechnology (Shanghai, China). Anti-mouse CD62p antibody (ab6631) and anti-mouse integrin α6 (ab181551) antibody were purchased from Abcam (Shanghai, China). The AngioSense680 Ex (NEV10054EX) agent was purchased from PerkinElmer (Massachusetts, USA).

1.2 Instruments

A Zetasizer Nano-ZS Particle Sizer and Zeta Potential Analyzer (Malvern Instruments, UK) and a T20 transmission electron microscope (Jeol Ltd., Tokyo, Japan) were used for characterization of nanoparticles. The LSM 710 confocal laser scanning microscope (Carl Zeiss, USA) was used to acquired confocal fluorescence images. The Accuri C6 flow cytometer (BD Biosciences, USA) was applied to collect flow cytometry information. The Maestro imaging system (CRi Maestro, USA) was used for *in vitro* and *in vivo* animal imaging.

1.3 Synthesis of MSN nanoparticles

For the synthesis of Mesoporous silica Nanoparticles (MSN), 0.7 mL sodium hydroxide solution (2 M) and 200 mg CTAB were added in 100 mL deionized water and heated to 75 °C. Then, 1 ml TEOS was added into the reaction solution containing CTAB. To remove the free CTAB surfactant from the pores, the solution was heated in acidic ethanol for 36 hours.

1.4 Acquisition and purification of platelet membrane

The whole blood was collected from BALB/c nude mice by the retro-orbital venous plexus and then transferred into the anti-coagulant tube containing 10 % citric acid glucose buffer (ACD buffer, 75 mM sodium citrate, 39 mM citric acid and 135 mM dextrose, pH = 7.4). After that, the solution was centrifuged at 120 g for 20 min to obtain the platelet-rich plasma (PRP). The PRP was then transferred into the new tube that rinsed with ACD buffer,

then centrifuged at 800 g for another 20 min to precipitate platelets. The platelet precipitation was resuspended in Tyrode's buffer (12 mM $NaHCO_3$, 10 mM HEPES, 134 mM NaCl, 1 mM $MgCl_2$, 0.34 mM Na_2HPO_4, 2.9 mM KCl, pH = 7.4) and quantified by protein content. To prevent activation or membrane protein degradation of the platelets, 1 μM prostaglandin E1 (PGE1, Aladdin, China) and 1 mM phenylmethylsulfonyl fluoride (Solarbio, China) were added to the solution. To extract and purify platelet membrane, the platelet suspension was frozen at -80 °C and then placed at 25 °C for five cycles. The platelet suspension was centrifuged at 12,000 g for 30 min at 4°C to collect the platelet membrane. The platelet membrane is stored at -80 °C and used for preparation of different formulation nanoparticles.

1.5 Drug loading and platelet membrane coating

The platelet membrane was suspended in deionized water and sonicated for 10 min at 100 W and 42 kHz (the equipment name and details) before using. Drug loaded MSN were prepared by stirring continuously. The required amount of CA4 and apatinib were dissolved in the absolute ethanol and then the MSN nanoparticles were added. The mixture solution was stirred at 3000 rpm for six hours. In order to explore the optimal ratio of platelet membrane to MSN, 10 mg MSN nanoparticles were dispersed in 1 mL absolute ethanol solution that containing different amount of platelet membrane. After sonication and stirring for 15 min, the PM-coated nanoparticles were washed two times with PBS.

1.6 Characterization of platelet membrane proteins

The protein expression profiles of platelets, platelet membrane and MSN@PM were

analyzed by Western blot and SDS-PAGE experiments. Firstly, the different suspended samples were centrifuged at 15,000 g for 20 min and the precipitates were dissolved in pre-cold RIPA buffer on ice for 30 min. The protein solution was collected by centrifuged at 15,000 g for 30 min and the supernatant was boiled in protein loading buffer for 5 min. The 10% polyacrylamide 10-well gel was used to separate the proteins by the Novex Xcell Surelock Electrophoresis System (Bio-Rad, USA). After that, the protein gel was placed in the Coomassie brilliant blue protein staining buffer for 6 h and destained with destaining buffer for another 6 h. The gel was imaged *via* the Bio-Rad ChemiDoc Touch Imaging System. To the western blot experiment, protein gel was transferred into a poly (vinylidene difluoride) membrane and then was blocked with 5% BSA for 1 h. The membrane was then incubated with the antibodies of platelet membrane protein markers integrin α6 and CD62p for 2 h at room temperature. The membrane was washed three times with TBST solution (Tris-buffered saline with 0.02% Tween 20) and incubated with the IgG-HRP secondary antibody (goat anti-rabbit) for another 1 h. The blot images were visualized by the Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad, USA).

1.7 Detection of drug loading and release efficiency

To quantify the contents of CA4 and apatinib in the nanoparticles, the suspensions including nanoparticles were centrifuged at 13000 g for 20 min. The amounts of CA4 and apatinib in the supernatant were measured by high-performance liquid chromatography (HPLC). To analyse the drug release, nanoparticles were placed in the solutions with different pHs. The drug release profiles were determined by similar measurements at different time intervals.

1.8 Cell culture

MHCC-97H cells were cultured in DMEM cell medium with 10% fetal bovine serum (FBS). The F-12K cell medium with FBS (10%), heparin (0.1 g/ml) and endothelial cell growth supplement factor (5%) were used to culture HUVEC cells. All cells were cultured with 1% penicillin/streptomycin at 37 °C in 5% humidified CO_2. VEGF (10 nmol) was added to HUEVC cells (V-HUVEC) and cultured for 7 days for a cell model of tumor vascular endothelium.

1.9 Cytotoxicity assay

V-HUVECs were seeded in the 96-well cell culture plates at a cell density of 5×10^3 per well for 24 h and deal with different formulations or PBS as a control. After incubation for different time intervals, the cell counting kit-8 (CCK-8) (Dojindo, Japan) was used to analyze cell viability.

1.10 Cellular targeting and internalization

To visualize the cell targeting and internalization ability of the nanoparticles to V-HUVECs, different formulations were loaded with cy5.5 dye. V-HUVECs were seeded in the confocal dishes or 10 cm cell culture dishes and cultured for 24 h. For cell targeting analysis, the V-HUVECs were treated with cy5.5-loaded nanoparticles on ice for 30 min or 60 min. For cell internalization analysis, the V-HUVECs were treated with cy5.5-loaded nanoparticles on 37 ℃ for 2 or 4 h. The V-HUVECs in the 10 cm dishes were then collected and analyzed by a flow cytometry. For laser confocal imaging, the cells were washed two times with PBS and stained with Hoechst 33342, DiO or cy5.5 dyes then imaged by

confocal microscopy.

1.11 Tubule formation assay

Each well of the 24-well plates was pre-coated with 200 μl matrigel. V-HUVECs treated by different formulations were seeded into the plates at a cell density of 5×10^4/well and then cultured for 12 h, in the medium containing VEGF. The images of tubule formation were obtained by light microscopy. Three random fields in each image were acquired. The length of tubule in each filed was analyzed by AngioSys 2.0 Image Analysis Software.

1.12 *In vivo* plasma pharmacokinetics and biodistribution

MHCC-97H liver tumor bearing nude mice were intravenously administered with MSN-C-A, MSN-liposome-C-A or MSN-PM-C-A nanoparticles labeled with cy5.5 fluorescent dye (n = 3). Blood samples were repeatedly acquired by tail snip at different time points and cy5.5 fluorescent signal was analyzed by Maestro *in vivo* imaging system (Cri Maestro, USA). To determine the biodistribution of the MSN-PM-C-A, MHCC-97H tumor-bearing mice were divided into different groups randomly (n = 3) and intravenously administered saline, free cy5.5, MSN-C-A-cy5.5, MSN-liposome-C-A-cy5.5 or MSN-PM-C-A-cy5.5. At the indicated time points, the mice were sacrificed to collect major organs (heart, liver, spleen, lung, kidney) and the tumors for *ex vivo* imaging.

1.13 Tumor blood perfusion

MHCC-97H tumor-bearing mice were injected 2 nmol AngioSense680 Ex agent (PerkinElmer, Germany). After 24 h, the tumor fluorescence intensity was measured by a

Maestro CRi imaging system (680 nm excitation/720 nm emission) and recorded the fluorescence intensity. The tumor-bearing mice were then given different drug loading nanoparticles, and tumor fluorescence intensity was measured after a further ten hours.

1.14 *In vivo* anti-tumor experiment

2×10^6 MHCC-97H liver tumor cells were subcutaneous injected in BALB/c nude mice to establish liver tumor model. The tumor volume of mice was measured by a vernier caliper, and the mice were randomly divided into seven groups when the average volume reached about 200 mm^3 (n = 6). The mice were injected with saline, MSN-PM, CA4 plus apatinib, MSN-CA4-PM, MSN-CA4-apatinib, MAN-CA4-apatinib-liposome or MAN-CA4-apatinib-PM (the dosage of CA4 was equivalent of 24 mg kg^{-1}). The body weight and tumor volume were measured every three days during the experiment. After the treatment, the animals were sacrificed and the tumors were removed for further study. The tumor volume was calculated by the formula: Tumor volume = (length × $width^2$)/2. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of National Center for Nanoscience and Technology.

1.15 Blood coagulation assay

The standard coagulation indicators of healthy BALB/c mice were estimated by measurement of prothrombin time, thrombin time, activated partial thrombin time and fibrinogen with their responsive kits, with the semi-automatic coagulation analyzer (LG-PABER-I, Taizhou, China).

1.16 Histological analysis

Heart, liver, spleen, lung, kidney and the tumors were collected and sectioned for hematoxylin-eosin (H&E) staining. Tumor tissues also were stained for endothelial cell marker CD31 with Anti-CD31 antibody (ab182981). All the histological analysis was performed by Servicebio company (Wuhan, China).

1.17 Statistical analysis

SPSS 17.0 statistical analysis software was used to analyze data. Student's t-test or one-way ANOVA was used for comparison between two groups or among multiple groups, respectively: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ are considered significant. All error bars are presented as the mean ± s.d.

Supplementary Tables and Figures

Table S1. Drug encapsulation efficiency and drug loading efficiency with different mass ratios of MSN, CA4 and Apa (mass ratios of CA4: Apa = 5:1).

MSN/Drug	CA4 EE (%)	CA4 DLR (%)	Apa EE (%)	Apa DLR (%)
5:4	18.98	11.28	16.19	2.06
5:2	34.20	10.14	25.07	1.72
5:1	48.76	7.65	35.27	1.05
10:1	64.98	5.12	50.54	0.85

EE: encapsulation efficacy; DLR, drug loading rate; CA4: combretastatin A4; Apa: apatinib

Table S2. The size, polydispersity index (PDI) and zeta potentials of the nanoparticles with different mass ratios of platelet membrane and MSN nanoparticles.

MSN/PM	Size (nm)	PDI	Zeta potential (mv)
5:0	132.5±0.4	0.21±0.02	-26.9±0.9
5:1	141.8±2.8	0.23±0.02	-21.4±1.0
5:2	140.4±3.9	0.16±0.02	-40.6±2.5
5:3	147.0±0.4	0.26±0.01	-41.9±0.4
5:4	216.9±20.9	0.40±0.05	-41.6±1.2
0:5	476.8±94.7	0.51±0.12	-40.5±0.4

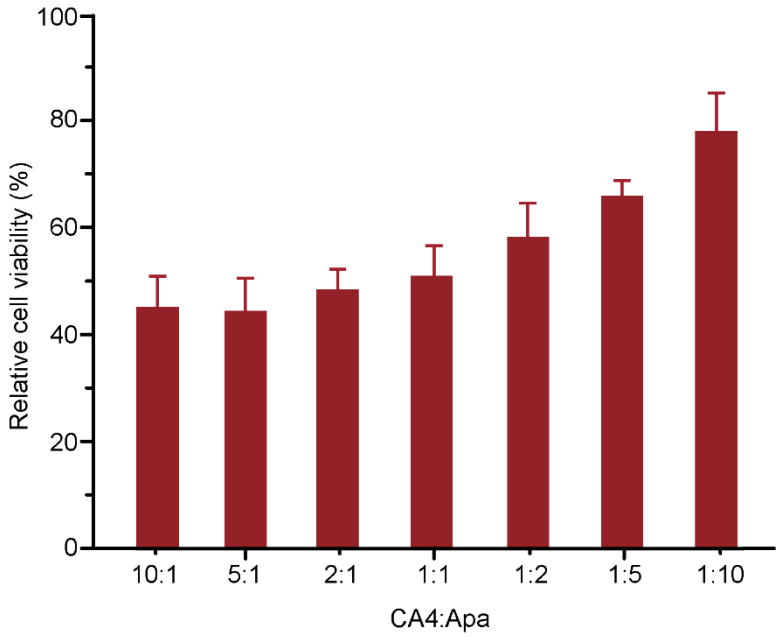

Figure S1. Screening for the optimal dose ratio of CA4 and apatinib. The cytotoxicity experiment was used to explore the best ratio of CA4 and apatinib to achieve maximum cell killing effect.

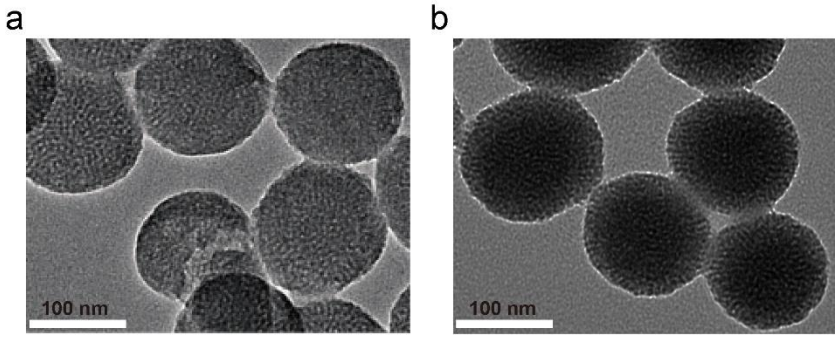

Figure S2. TEM images of MSN@PM-C-A before (a) and after incubation for 48 h (b) in PBS with 10% FBS were acquired to explore the stability of MSN@PM-C-A.

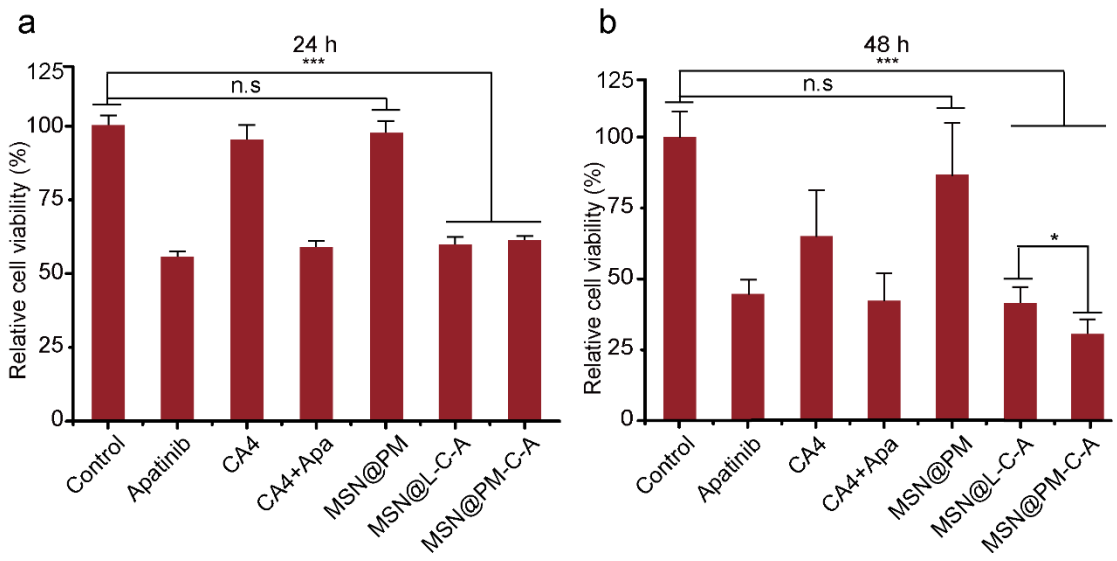

Figure S3. Cytotoxicity of MSN@PM-C-A against tumor vascular endothelial cells. Cell viability of V-HUVECs treated with different formulations for 24 h (a) or 48 h (b). Error bars represent the mean ± s.d. Student's t-test or one-way ANOVA was used for comparison between two groups or among multiple groups, $*p < 0.05$, $***p < 0.001$. The cell viability was measured by CCK-8 assay.

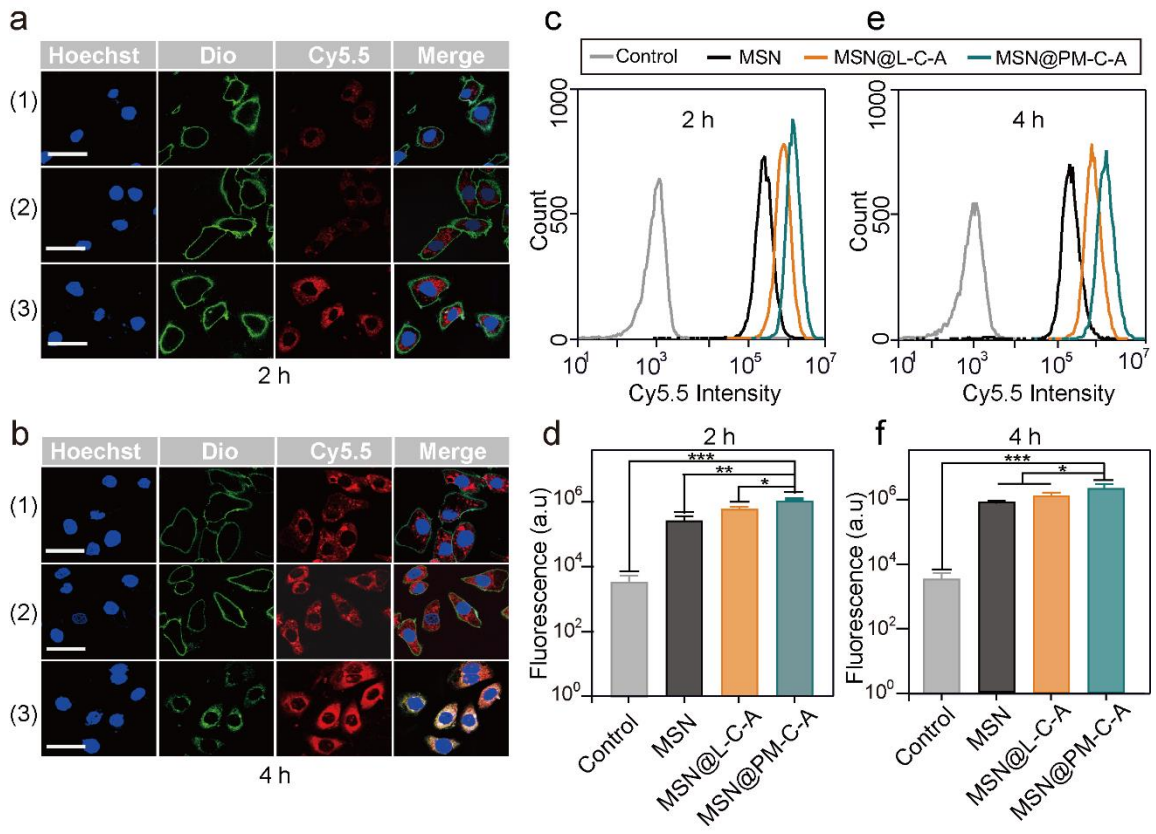

Figure S4. Confocal laser scanning microscopy images and flow cytometry analysis of V-HUVECs incubated with the indicated formulations. (a-b) Confocal images of V-HUVECs after incubation with different formulation nanodrugs, MSN-C-A (1), MSN@L-C-A (2) or MSN@PM-C-A (3) for 2 h or 4 h. Nanoparticles were labeled by the red florescence of cy5.5. Cell nucleus were stained with Hoechst 33342 and cell membranes were stained with DiO. (c-f) Flow cytometry analysis of the uptake of different nanoparticles after incubation with V-HUVECs for 2 h or 4 h. Error bars represent the mean ± s.d. One-way ANOVA was used for multiple comparisons, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

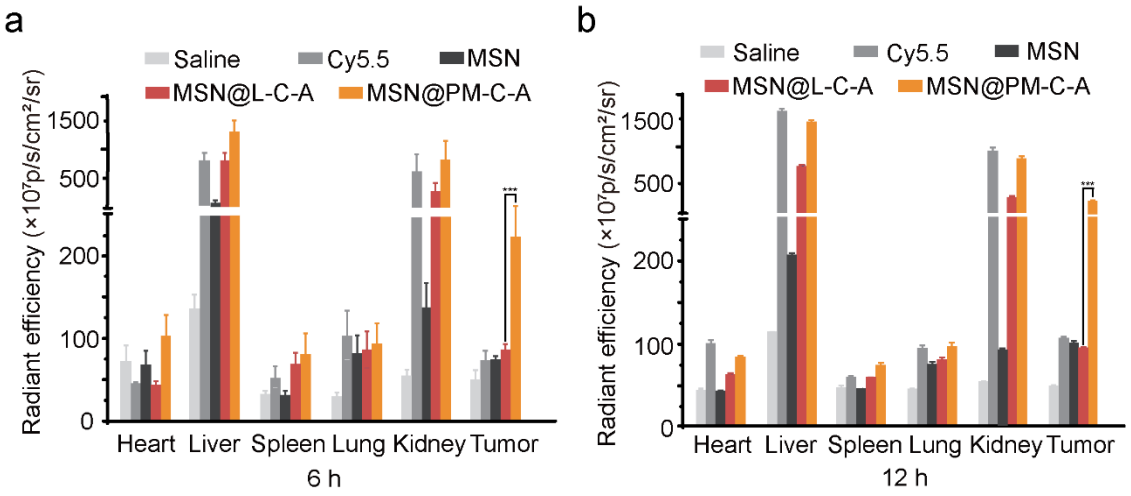

Figure S5. The quantified fluorescence results of tumors and major organs from mice after treatment with the indicated formulations for 6 h (a) or 12 h (b). Student's t-test was used for comparison between two groups, ***$p < 0.001$ (n = 3).

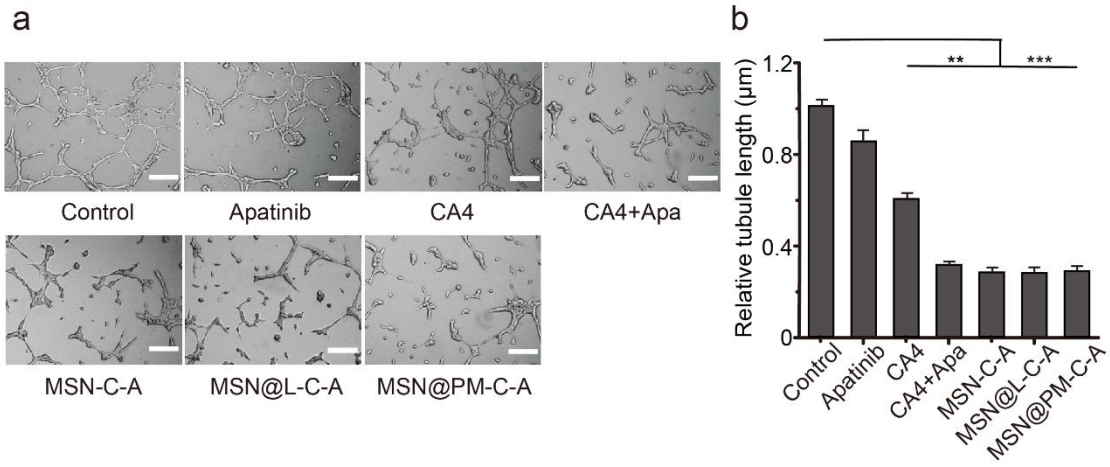

Figure S6. Effects of MSN@PM-C-A treatment on V-HUVEC activity. (a) The tubule formation of V-HUVECs in matrigel was analyzed after treatment with different nanoparticles for 24 h. MSN@PM-C-A was able to obviously suppress the endothelial tubule formation. (b) Quantitative statistics of the tubule length. Scale bars, 50 μm. One-way ANOVA was used for multiple comparisons, **$p < 0.05$, ***$p < 0.01$.

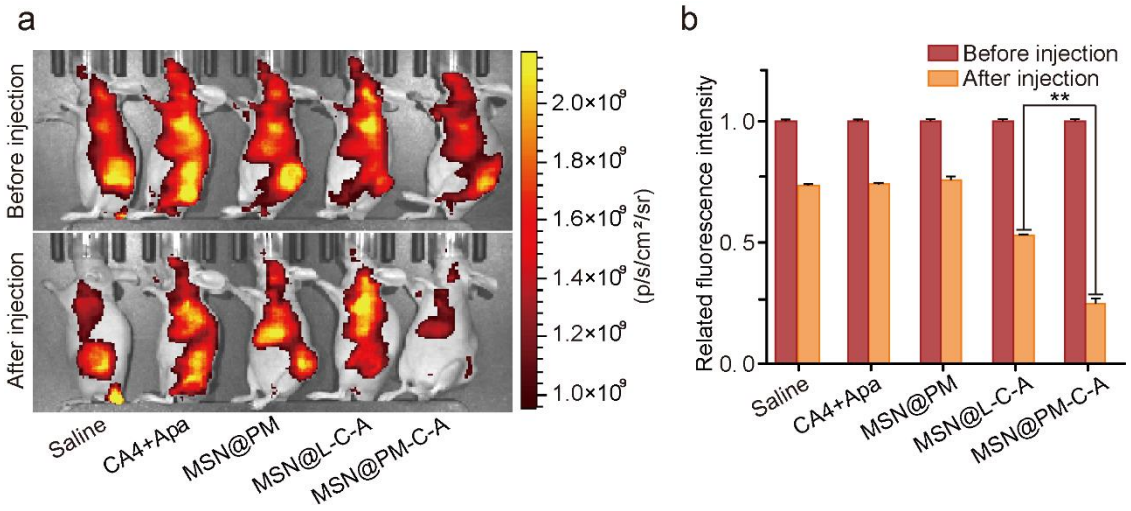

Figure S7. MSN@PM-C-A significantly destroyed tumor blood vessels and reduces blood perfusion. (a) Representative fluorescence images of MHCC-97H liver tumor-bearing nude mice that were given vascular imaging agent (Angio-Sense 680EX) before and after treatment with the indicated formulations. (b) Quantitative analysis of the tumor fluorescence intensities of the AngioSense 680EX (n = 3). Error bars represent the mean ± s.d. Student's t-test was used for comparison between two groups. $^{**}p < 0.01$.

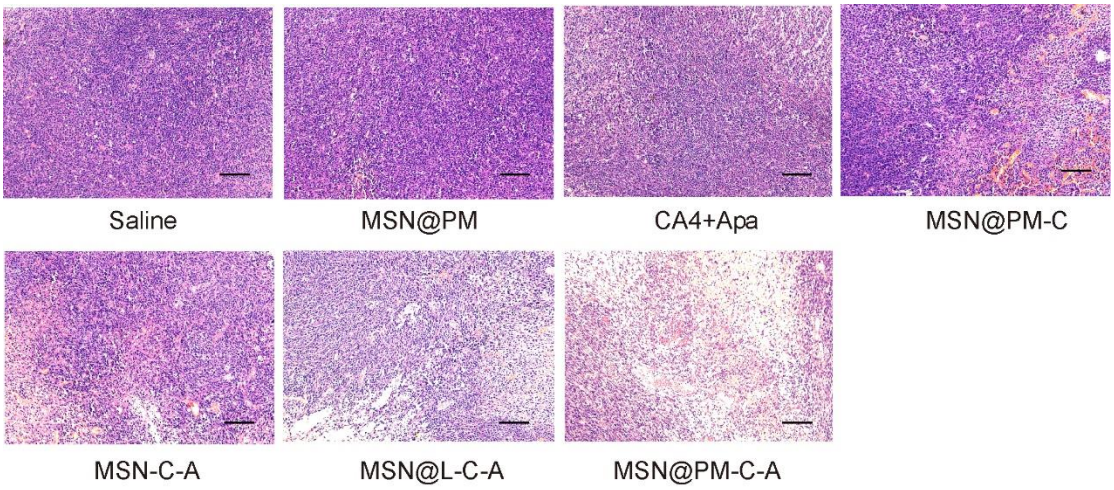

Figure S8. Tumor tissues were harvested at the end of treatment and were subjected to H&E staining for detecting tissue necrosis. Scale bars, 100 μm.

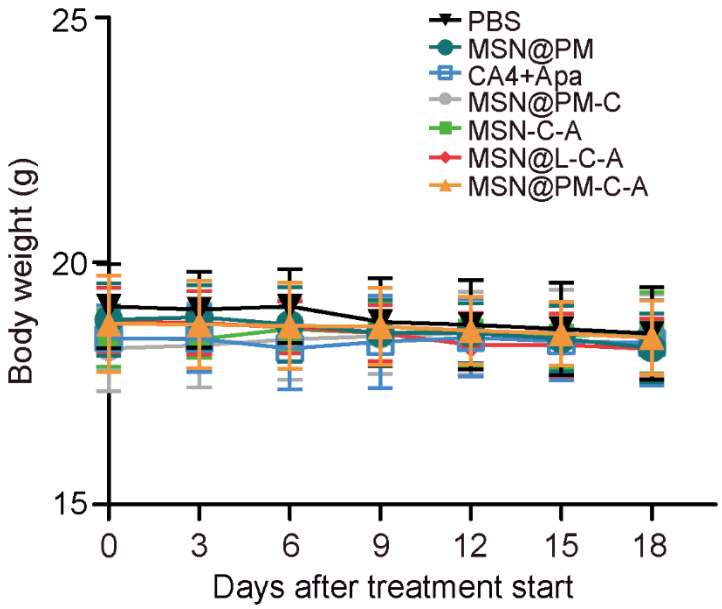

Figure S9. Body weight change of MHCC-97H liver tumor-bearing mice during the treatment process. There was no significant change of the body weight of various groups.

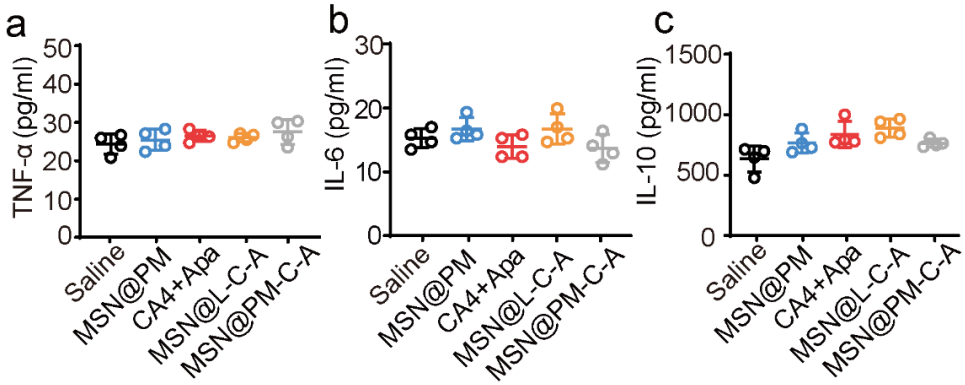

Figure S10. Healthy BALB/c mice were intravenously injected with saline or different formulations every three days for five total treatments (n = 4). The immunostimulation indicators TNFα (a), IL-6 (b) and IL-10 (c) were measured after the last treatment. No apparent change was observed in any measured immunostimulation indicator in MSN@PM-C-A treated group compared with saline group.

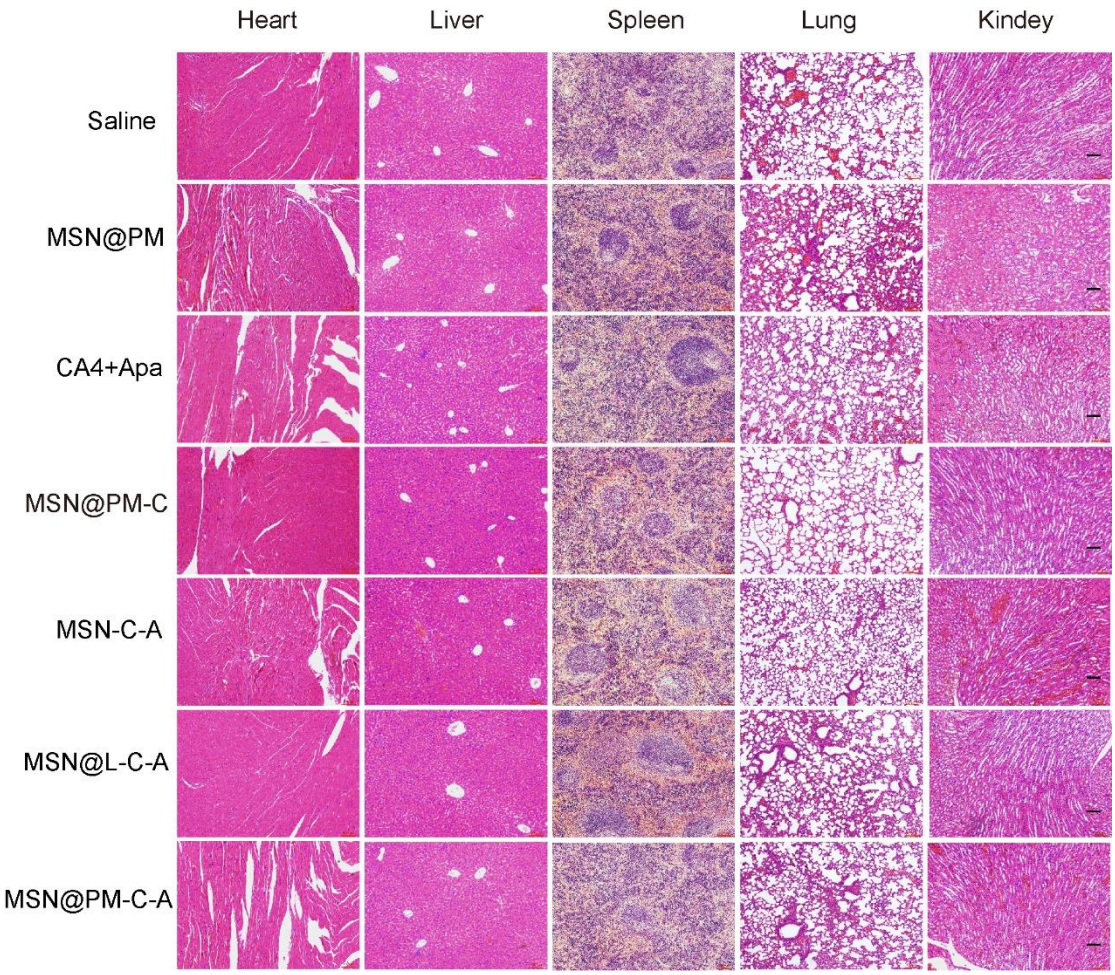

Figure S11. Healthy BALB/c mice were intravenously injected with saline or different formulations every three days for total of five treatments. The major organs, including the heart, liver, spleen, lung and kidney were collected for histological examination after the last treatment. No apparent organ damage was observed in any group. Scale bars, 50 μm.

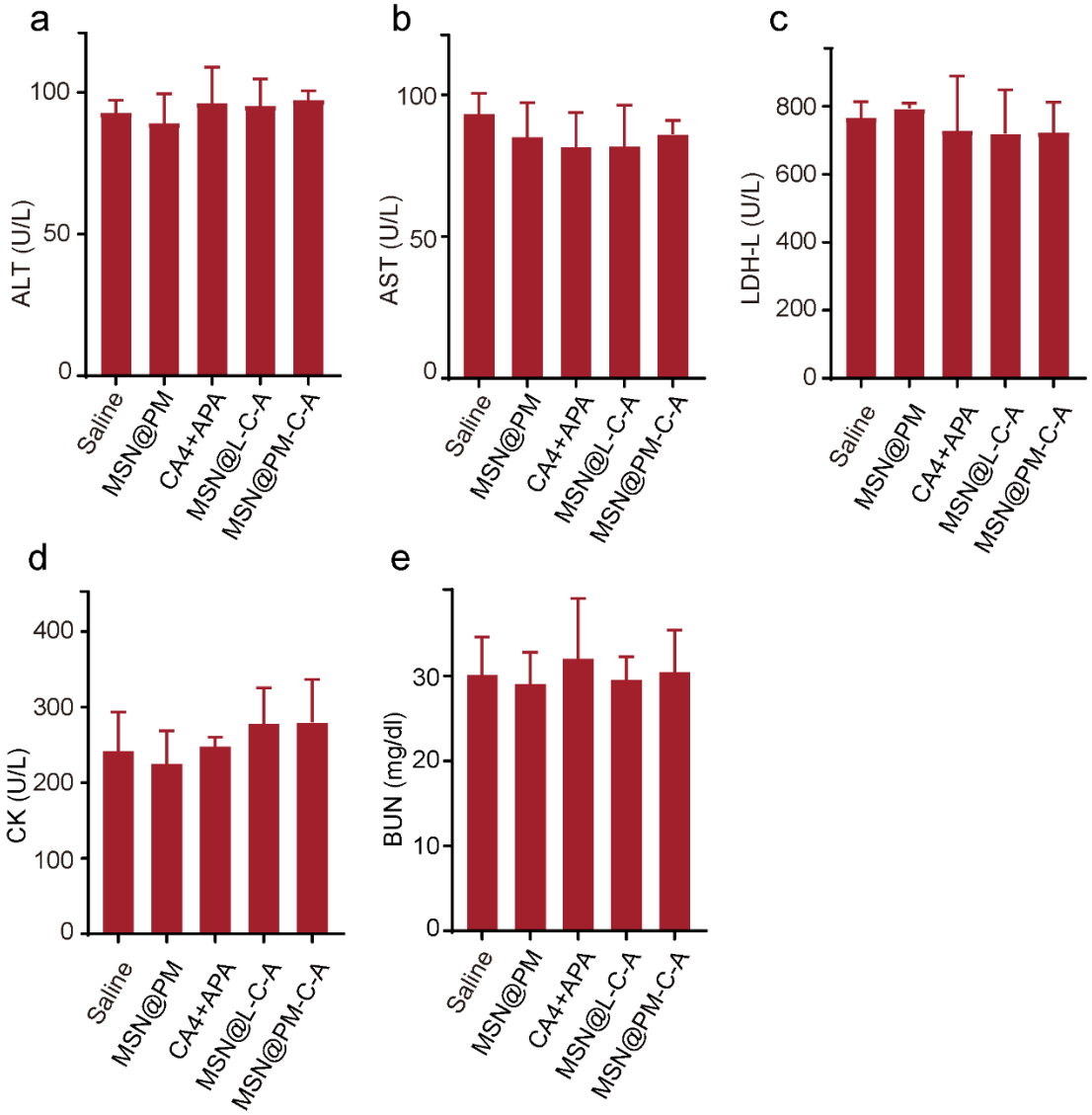

Figure S12. Serum biochemistry indicators of healthy mice treated with the indicated formulations. ALT (a), AST (b), LDH-L (c), CK (d) and BUN (e) in healthy mice serum were showed after treatment (n = 4). The results show that MSN@PM-C-A posses favorable biological safety profile.

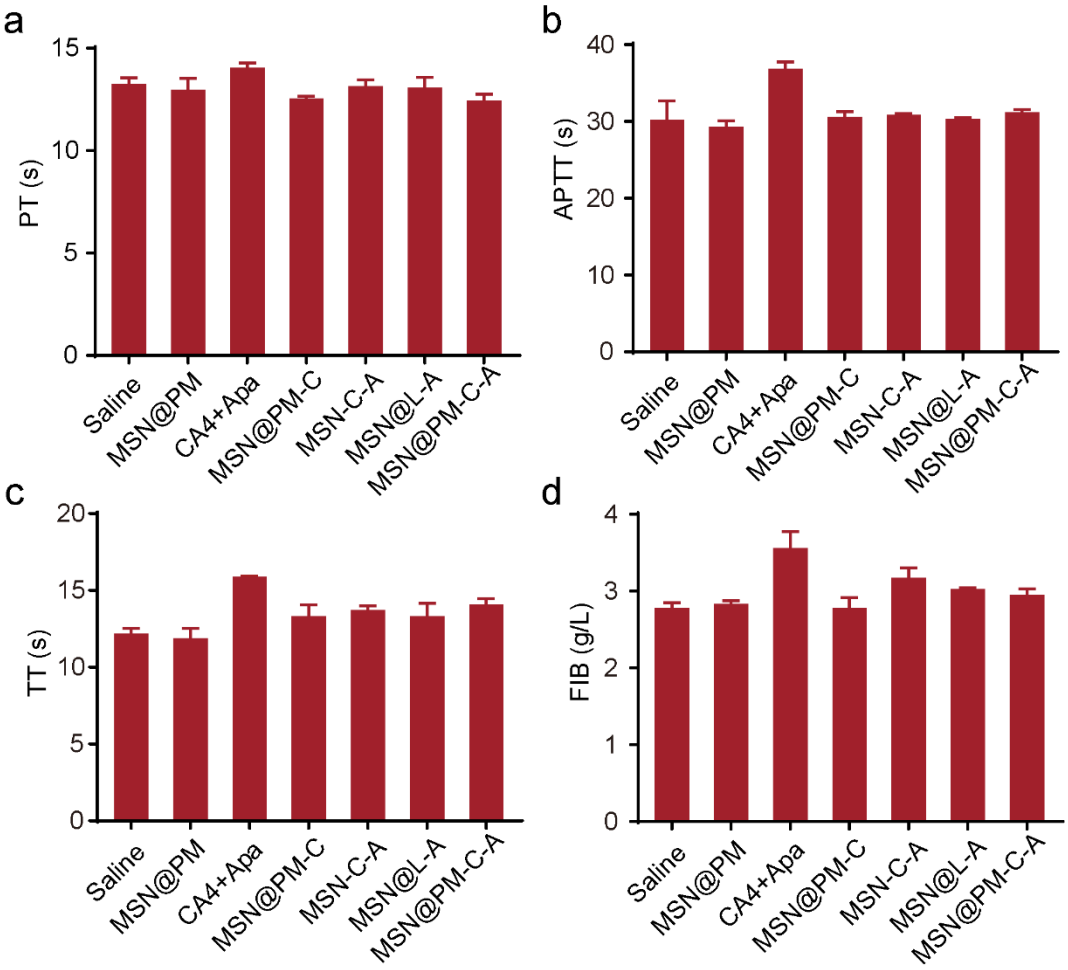

Figure S13. Indicators of blood coagulation, including PT (a), APTT (b), TT (c), and FIB (d) in healthy mice plasma after treatment with the indicated formulations (n = 4).