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Supplementary Information

Mercaptopyrimidine-templated gold nanoclusters for antithrombotic therapy

Jianghong Dai[1,3,a], Jiaojiao Lei[1,2,a], Tianyan Zhang[1,2], Jingcan You[1], Dalian Qin[2], Ya Wu[3], Yong Liu[3,*], Youkun Zheng[1,2,*]

[1] *Basic Medicine Research Innovation Center for Cardiometabolic Diseases, Ministry of Education, Southwest Medical University, Luzhou 646000, China*

[2] *Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou 646000, China*

[3] Department of Vascular Surgery, The Affiliated Hospital, Southwest Medical University, Luzhou 646000, China

[a] Jianghong Dai and Jiaojiao Lei contributed equally to this work.

Experimental section

Materials

Chloroauric acid ($HAuCl_4$), 4,6-diamino-2-mercaptopyrimidine (DAMP), collagen, and other common reagents were purchased from Aladdin. Coomassie brilliant blue, Cell counting kit-8 (CCK-8) and BCA protein assay kit were obtained from Beyotime Biotechnology Co. Ltd. Fibrinogen (human plasma), thrombin, carrageenan, heparin sodium, and normal saline were purchased from Shanghai Yuanye Biotechnology Co., Ltd. Mouse cyclic adenosine monophosphate (cAMP) ELISA kit, 5-Hydroxytryptamine (5-HT) ELISA kit, and P-selectin ELISA kit were obtained from Beijing Qisong Biotechnology Co., Ltd. Ultrapure water (18.2 MΩ) was used throughout the experiment.

Instruments

UV-vis and fluorescence spectra were obtained using a UV 3600 plus spectrophotometer (Shimadzu, Japan) and a FS5 fluorescent spectrometer (Edinburgh, UK), respectively. The X-ray photoelectron spectroscopy (XPS) and zeta potential were measured on a Thermo Fisher K-Alpha (USA) and a Malvern Nano ZS Zetasizer 90 (UK), respectively. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out on a JEM-2100 Plus TEM (JEOL, Japan) and a Zeiss Sigma 300 (Germany), respectively. Elemental analysis was performed using an inductively coupled plasma mass spectrometer (ICP-MS, Germany). The CCK-8 cytotoxicity assays were executed using a MK3 microplate reader (Thermo Fisher, USA). The thromboelastography was drawn on a CFMSLEPU-8800 analyzer (Lepu, China)

Animals

Male ICR mice of 6–8 weeks, male Sprague-Dawley (SD) rats of 6–8 weeks, and male rabbits of 2.0-3.0 kg were used in these studies. These animals were specific pathogen-free and were purchased from HFK Bioscience Co., Ltd. (Beijing). All animal experiments were performed according to the guidelines for Animal Experimentation with the approval of the Animal Care Committee of Southwest Medical University.

Synthesis of DAMP-AuNCs

The synthesis of DAMP-AuNCs was carried out according to our previous approach [1]. For a typical synthesis procedure, a 200 mL mixed solution containing 1 mM of $HAuCl_4$ and 1.5 mM of DAMP is reacted for 10 h under continuous stirring (300 rpm) at 70 °C. After the completion of the reaction, a Millipore ultrafiltration tube with a molecular weight cut-off of 3000 Da was used to purify the prepared product. The purified DAMP-AuNCs solution was stored in a refrigerator for further use and characterization.

Cytotoxicity of DAMP-AuNCs

Human umbilical vein endothelial cells (HUVECs) were routinely cultured in Dulbecco's Modified Eagle Medium

(DMEM, Gibco) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C and 5% CO_2. Cell viability after treatment with DAMP-AuNCs at gradient concentrations (0–500 μg/mL) was evaluated by the CCK-8 assay. 100 μL HUVECs suspensions were inoculated in a sterile 96-well plate for 8 h and subsequently treated with different concentrations of DAMP-AuNCs in the medium for 24 h at 37 °C and 5% CO_2. Then, 10 μL CCK-8 was separately added to each well and further incubated for 1 h. After the incubation, the absorbance at 450 nm (A_{450}) of the solutions was recognized by a ThermoFisher MK-3 reader. The viability (%) was calculated by linear interpolation of the absorbance data from the cells treated with DAMP-AuNCs versus the control sample (100% viability).

Hemolysis Assay

Hemolysis assays were performed according to our previously reported method [2]. The red blood cells were obtained from human blood by centrifugation at 2000 rpm for 15 min, gently washed for 3 times with normal saline, and the subsequent re-suspended by using normal saline to prepare erythocyte suspension with a hematocrit of 2%. Then, different concentrations of DAMP-AuNCs were incubated with isovolumetric 2% erythrocyte suspension at 37 °C for 2 h. Ultrapure water and normal saline were employed as controls, respectively. After incubation, the supernatant was obtained by centrifugation at 3000 rpm for 5 min, and transferred to a 96-well plate. The absorbance of supernatant was measured at 450 nm (A_{450}). The hemolysis percentage was calculated as follows:

$$\text{Hemolysis (\%)} = (A_{\text{test}} - A_{\text{negative control}}) / (A_{\textit{positive}\ \text{control}} - A_{\text{negative control}}) \times 100\%,$$

where A represents the mean of absorbance value at 450 nm.

Blood smears

To examine the effect of DAMP-AuNCs on the morphology and number of blood cells, blood smears were performed. After intravenous injection of different concentrations of DAMP-AuNCs for 12 h, blood from the inferior vena cava of rats was collected, and blood smears were prepared and observed using a Blood Analysis Pipeline (Mindray CAL 7000).

Thromboelastography

Blood samples were obtained from a healthy volunteer. Different concentrations of DAMP-AuNCs were mixed with blood and incubated for 1 h. Thromboelastography was performed according to the instrument's operating manual, and coagulation-related parameters were recorded.

Coagulation parameter assay

Prothrombin time (PT), activated partial thromboplastin time (APPT), thrombin time (TT), fibrinogen (Fib), fibrinogen degradation products (FDP), D-dimer, and antithrombin III (AT-III), which were analyzed with automatic

coagulation analyzer (Mindray CX-9000). Blood samples were mixed with different concentrations of DAMP-AuNCs for testing, and each sample was tested within 30 min. The specific operation was carried out according to the instrument's operating manual.

SDS-PAGE assay

The sedimentation obtained by centrifugation was reconstituted in SDS-PAGE sample loading buffer and heated at 95 °C for 5 min. An equal volume of fibrinogen solution without DAMP-AuNCs was used as a control. Subsequently, all samples were separated by electrophoresis. Finally, the gels were dyed with Coomassie brilliant blue solution for 60 min and decolorized until the protein bands were clear.

Protein adsorption quantification

The concentration of protein adsorbed on DAMP-AuNCs was determined with a commercial BCA detection kit (Beyotime, China) according to the manufacturer's instructions.

Platelet function test

Platelet aggregation measurements is often used as a key indicator to evaluate the anti-platelet aggregation activity of drugs. Obtaining rat platelet-rich plasma (PRP) by centrifugation at room temperature for 5 min at 1100 rpm. Obtaining rat platelet-poor plasma (PPP) by centrifugation at room temperature for 15 min at 3000 rpm. After PRP was adjusted (1.0×10^8 platelets per mL) by PPP, added different concentrations of DAMP-AuNCs and collagen, immediately use the platelet aggregometer analyzer (Techlink LBY-NJ4, China) to obtain the aggregation process curve. Platelet aggregation was observed in vitro by naked eye and microscope after addition collagen to PRP. ELISA detection of platelet activation, aggregation, and cascade reaction-related factors (P-selectin, 5-HT, and cAMP) in mice serum.

Tail thrombosis assay in mice

The inhibition of tail thrombosis assay in mice was performed by the reported method [3]. After 1 h tail vein injection of DAMP-AuNCs (25 mg/kg), heparin (100 U/kg) or 0.9% normal saline (equal volume), 0.2% carrageenan (40 mg/kg) was injected intraperitoneally to induce tail thrombosis. The length of tail thrombus length and blood flow intensity were recorded by Laser Doppler perfusion imaging system after 24 h of injecting carrageenan. Mice lung tissue samples were collected and fixed with 4% paraformaldehyde for 48 h and tail collected and fixed with Decalcifying solution (JYBL-Ⅰ Decalcifying Solution, Solarbio) for 48 h. After embedding in paraffin, the samples were cut into slices of 4 μm thickness. Next, all slices were stained with either hematoxylin and eosin (H&E, Beyotime). Finally, all slices image acquisition through automatic slide scanner.

Histopathology

The in vivo toxicity of the DAMP-AuNCs in mice was also investigated through histopathological analysis. After 24 h tail vein injection of the DAMP-AuNCs, major organs (heart, liver, spleen, lung, and kidney) were fixed in 4% paraformaldehyde solution, processed routinely into paraffin, and stained with H&E. The pathologies were examined using an optical microscope.

Biodistribution

The organs (heart, liver, spleen, lung, and kidney) and blood samples of the DAMP-AuNCs treated mice were digested by using aqua regia. The Au content was measured with an ICP-MS (Agilent 7500 CE, Germany).

Bleeding risk evaluation

A mice-tail amputation model was employed to evaluate the bleeding risk. First, different drugs after intravenous injection for 1 h, trim the mouse tail 1.5 cm from the distal end. Then, the mice were placed in air for 10 s to ensure normal bleeding. Finally, absorb bleeding with filter paper on the mouse tail wound. After 10 min, the weight of samples and filter papers were measured. All tests were repeated six times for each group.

Fibrin polymerization assays

Fibrinogen was mixed with increasing concentration of DAMP-AuNCs (0–300 μg/mL) in 200 mL of 50 mM sodium phosphate buffer (PBS, pH = 7.2) in siliconized glass tubes. Fibrin polymerization was induced by addition of thrombin at 1 U/mL and measured as time taken to form an insoluble mass of clot in the tube. The status of each tube at different times was photographed with a mobile phone. The fluorescence spectra of the mixed system were also record using a fluorescent spectrometer. After washing several times, each sample was dehydrated in graded ethanol solutions and then observed with a transmission electron microscope (JEM-2100 Plus TEM, Japan).

Molecular docking

To calculate the interaction between 4,6-diamino-2-mercaptopyrimidine and crystal structure of human fibrinogen, molecular docking was performed. First, all protein and molecular files were converted into pdbqt format with all water molecules excluded and polar hydrogen atoms were added. The grid box was centered to cover the domain of 3GHG and to accommodate free molecular movement. Then the protein geometries were downloaded from a public database and geometry optimization was performed. Then molecular docking was performed to obtain the docking structure and binding energy. Molecular docking studies were performed by Autodock Vina 1.2.2.

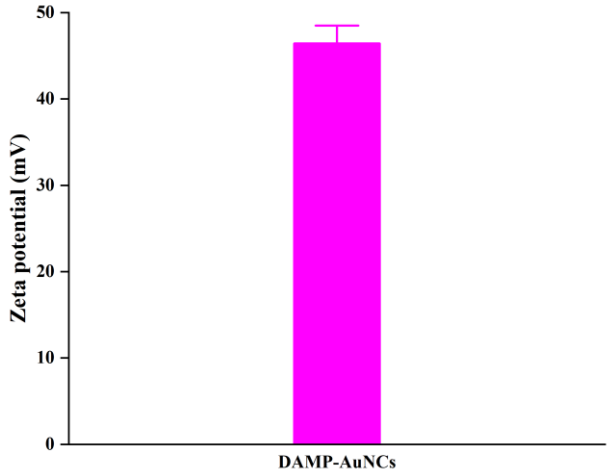


Fig. S1. The zeta potential (46.4 ± 2.2 mV) of DAMP-AuNCs.

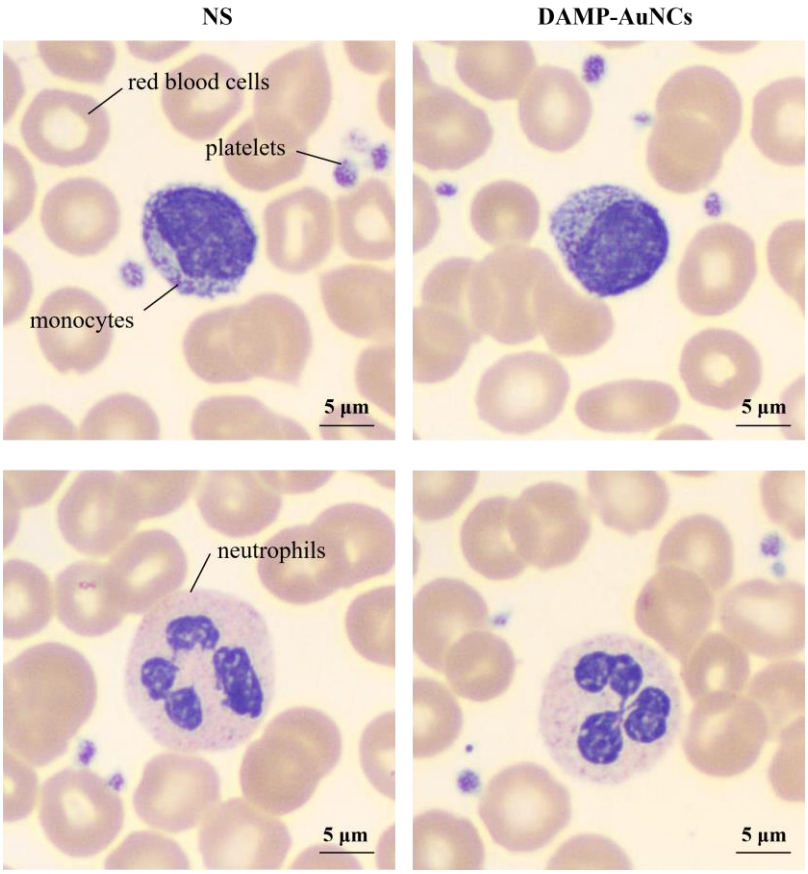


Fig. S2. Optical microscopic images (blood smear) of blood cells treated with normal saline (NS) and DAMP-AuNCs (500 µg/mL).

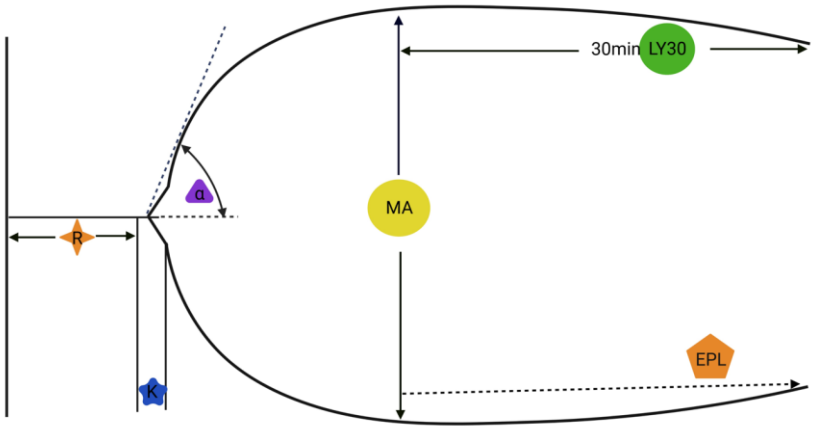


Fig. S3. Schematic diagram of the basic parameters of thromboelastography.

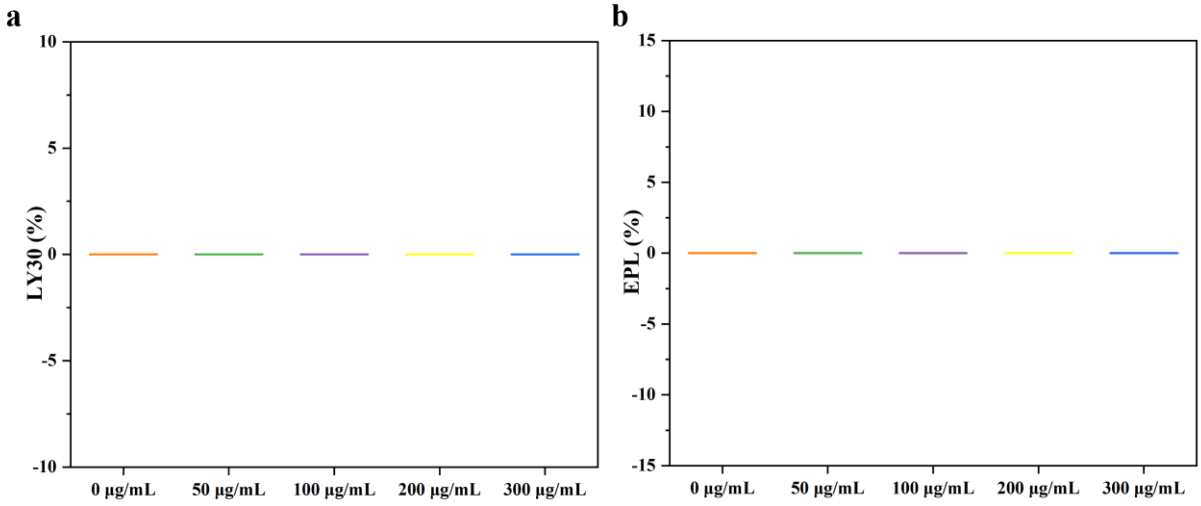


Fig. S4. Fibrinolytic indicators LY30 (a) and EPL (b) of thromboelastography.

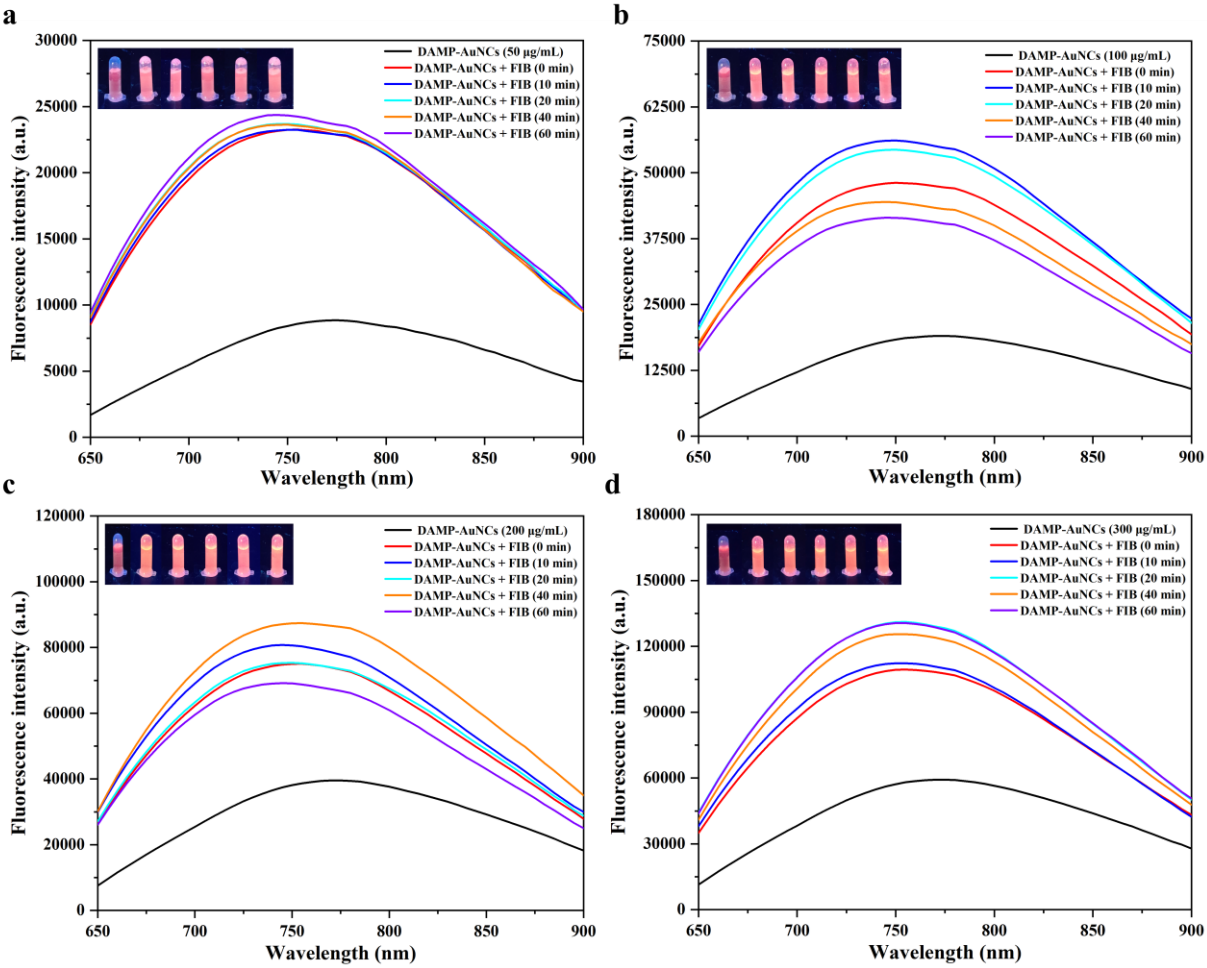


Fig. S5. Time-dependent fluorescence spectra of DAMP-AuNCs interacting with fibrinogen at different concentrations ((a) 50 μg/mL, (b) 50 μg/mL, (c) 50 μg/mL, and (d) 50 μg/mL). Insets: Photographs of fibrinogen-treated DAMP-AuNCs under UV illumination.

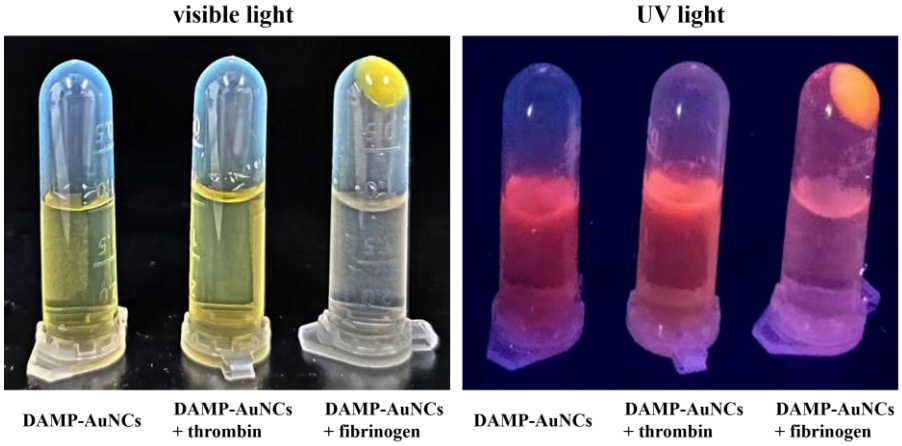


Fig. S6. Photographs of DAMP-AuNCs, DAMP-AuNCs + thrombin, and DAMP-AuNCs + fibrinogen under visible light (left panel) and 365 nm UV light (right panel).

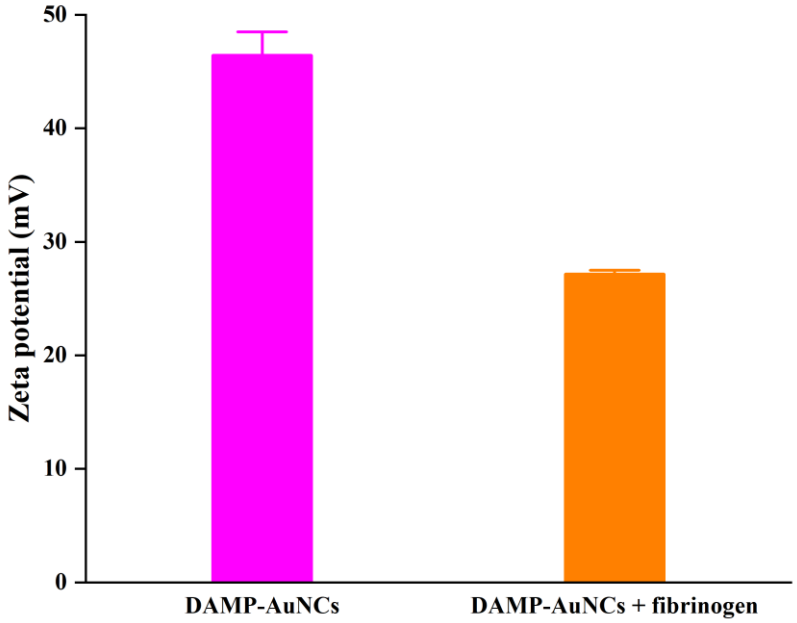


Fig. S7. The zeta potentials of DAMP-AuNCs without and with fibrinogen.

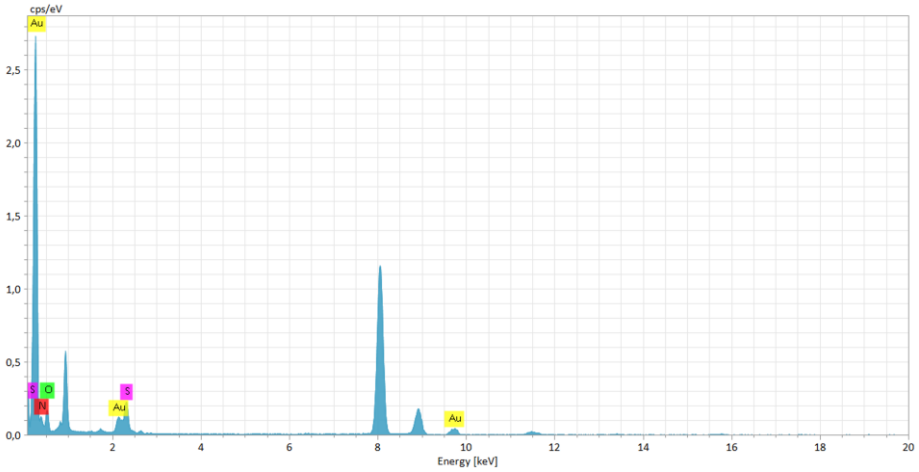
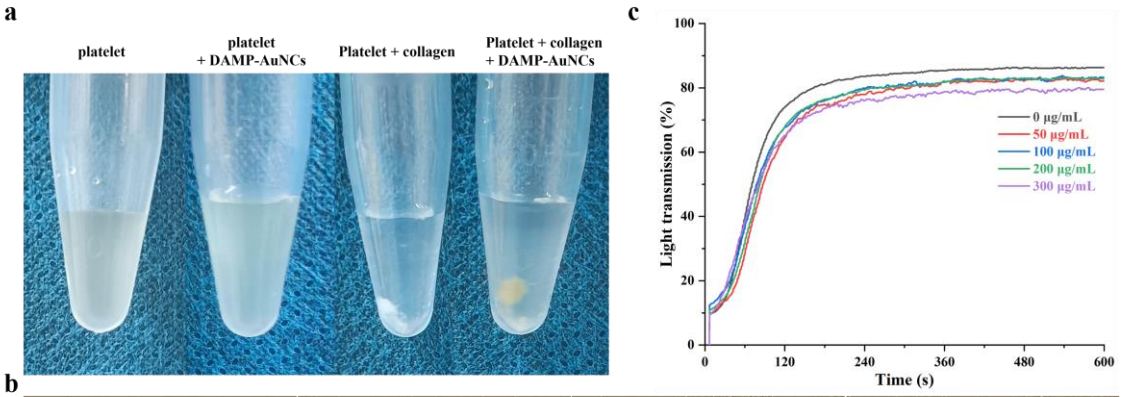


Fig. S8. Elemental analysis of fibrinogen treated with DAMP-AuNCs (50 μg/mL).



d

	P-selectin (ng/mL)	5-HT (ng/mL)	cAMP (nmol/mL)
NS	24.03 ± 0.38	38.44 ± 1.43	1.45 ± 0.07
DAMP-AuNCs	23.85 ± 2.56	37.46 ± 3.19	1.56 ± 0.15
p value	0.87	0.48	0.14

Fig. S9. Effect of DAMP-AuNCs on platelet aggregation. (a) Representative photographs (a) and micrographs (b) of platelet aggregation. (c) 0, 50, 100, 200, and 300 μg/mL DAMP-AuNCs were added before collagen induction and the platelet aggregation was measured by turbidimetry. The representative curve of one out of three experiments. (d) Effects of DAMP-AuNCs on platelet activation-related factors P-selectin, 5-HT, and cAMP. Data are means ± SD, *n* = 6, Student's *t* test.

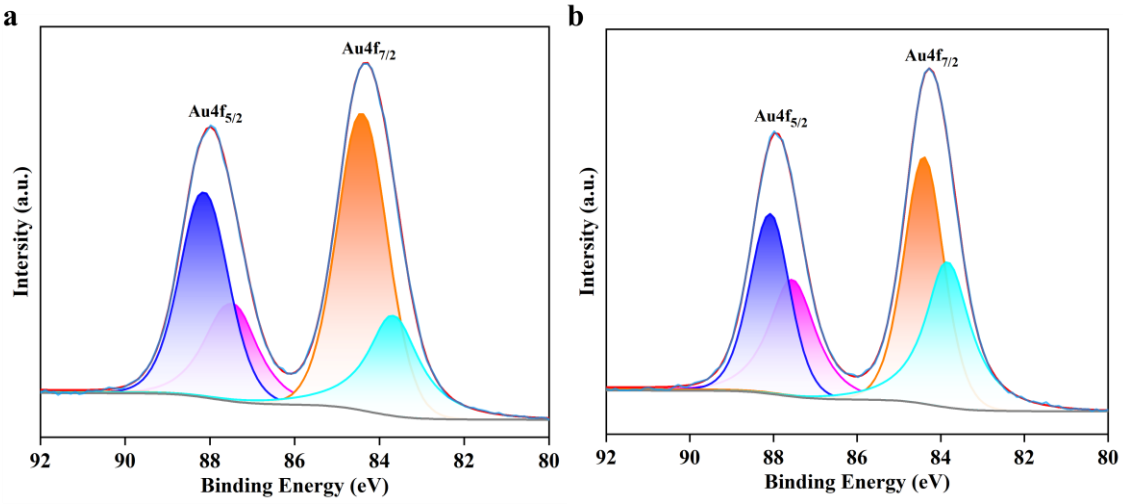


Fig. S10. XPS spectra (Au4f) of DAMP-AuNCs without (a) and with (b) carrageenan.

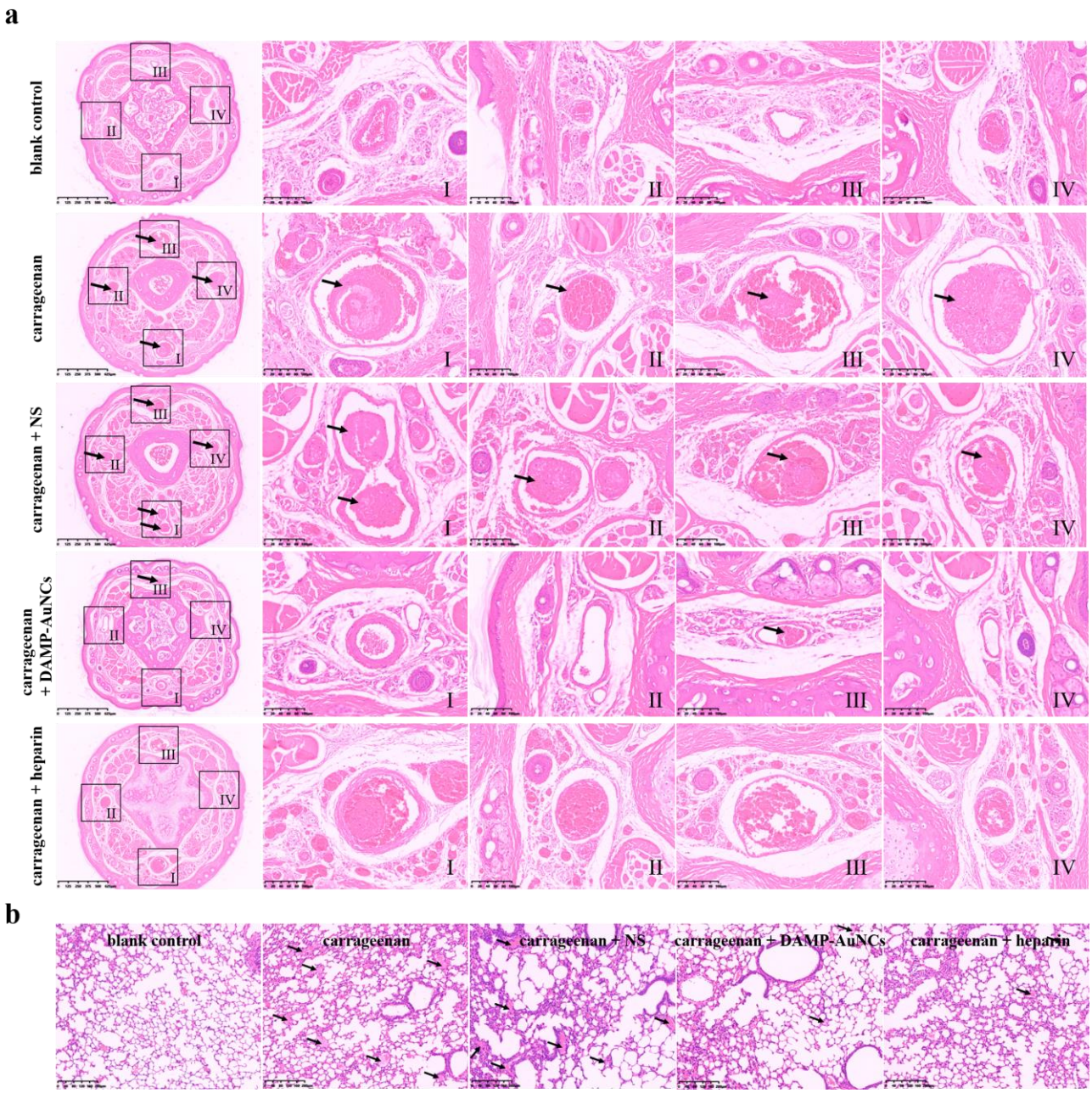


Fig. S11. Partial enlarged images of tail tissue sections (a) and lung arterioles (b) of mice in different treatment groups. The arrow points to the blood clot.

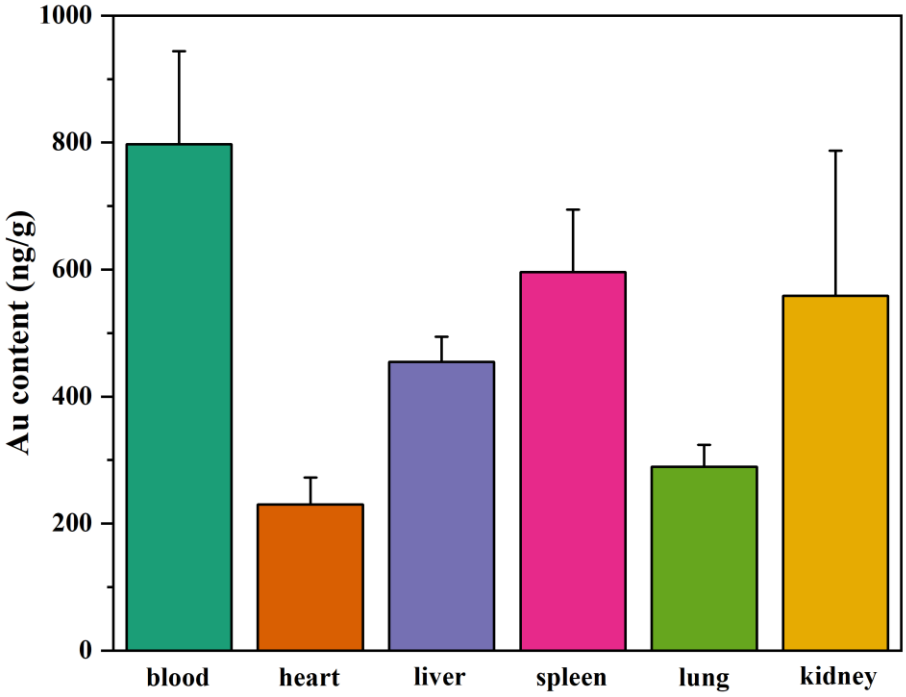


Fig. S12. Biodistribution of the DAMP-AuNCs treated mice at 24 h.

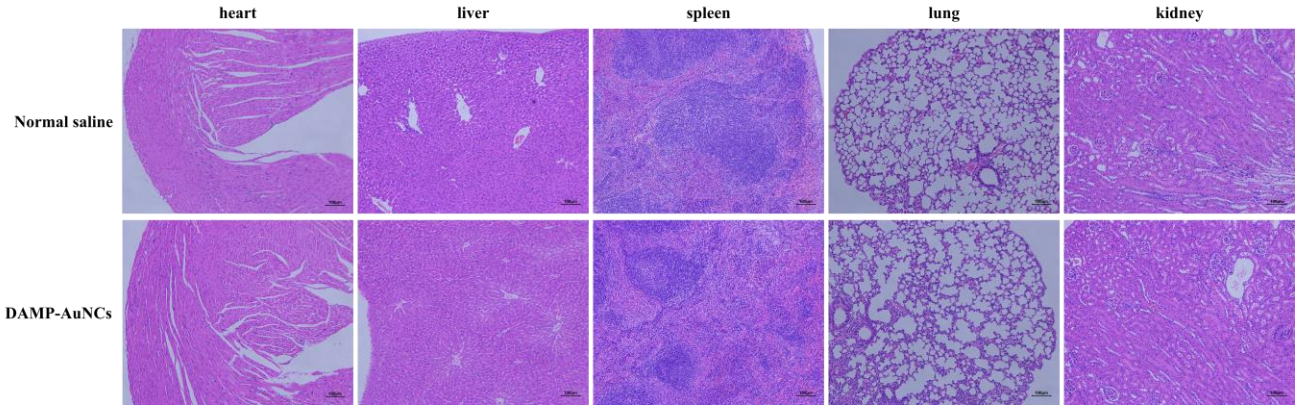


Fig. S13. H&E staining images of the main organs (heart, liver, spleen, lung, and kidney) after 24 h of treatments. Scale bar, 100 μm.

References

[1] Y. Zheng, W. Liu, Z. Qin, Y. Chen, H. Jiang, X. Wang, *Bioconjugate Chem.* 2018, **29**, 3094-3103.

[2] Y. Zheng, Y. Zhu, J. Dai, J. Lei, J. You, N. Chen, L. Wang, M. Luo, J. Wu, *Int. J. Biol. Macromol.* 2023, **230,** 123452.

[3] Y. Tian, Y. Zhao, W. Zheng, W. Zhang, X. Jiang, *Nanoscale* 2014, **6**, 8543-8550.