Supplementary Information

Ultrasound Activated Bowl-like Nanomotors Release Nitric Oxide for Enhanced Thrombus Therapy

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Experimental

Materials

Tetraethyl orthosilicate (TEOS, ≥99%), resorcinol (≥99%), sodium citrate (≥99%), sodium borohydride ($NaBH_4$, ≥98%), and 3-aminopropyltriethoxysilane (APTES, ≥98%) were purchased from Sigma-Aldrich (Saint Louis, USA). Polyvinylpyrrolidone (PVP, Mr 55,000 Da), formaldehyde solution (37%), and calcium chloride ($CaCl_2$, ≥97%) were purchased from Aladdin. Thrombin (500 U/mL) was purchased from Yuanye. Ammonium hydroxide (28%), hydroxylamine hydrochloride ($NH_2OH·HCl$, ≥99%), and chloroauric acid hydrate ($HAuCl_4·3H_2O$) were purchased from Beyotime (China). Isopropanol and absolute ethanol (≥99.5%) were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). Hydrofluoric acid (HF, 40%) and ferric chloride ($FeCl_3$, ≥99%) were obtained from Macklin. Arginine (≥99%) and cRGD peptide (purity ≥95%) were obtained from Ruixi Biotechnology Co., Ltd. (Xi'an, China). Cell Counting Kit-8 (CCK8), Nitric Oxide Detection Kit (Griess), and Reactive Oxygen Species Assay Kit (DCFH-DA) were purchased from Beyotime. All chemicals were of analytical grade and were used directly without further purification.

Instruments and Equipment

Morphological and microstructural analysis was performed using a scanning transmission electron microscope (TEM, Hitachi HT7700, Hitachi, Japan). The optical properties of samples were evaluated by measuring ultraviolet-visible (UV-Vis) absorption spectra with a spectrophotometer (Model X-6, METASH, China). Fluorescence emission spectra were recorded on a fluorescence spectrophotometer (Model F-2700, Hitachi, Japan). The concentration of nitric oxide (NO) was determined using a full-wavelength microplate reader (Thermo Scientific™ Multiskan™, Thermo Fisher Scientific, USA). Cell viability was assessed via the CCK-8 assay, and the absorbance at 450 nm was measured with a microplate reader (Model ReadMax 300F, Shanghai Flash Spectrum, China). Fluorescence imaging was conducted using an inverted fluorescence microscope (Model Ts2R-FL, Nikon, Japan).

Preparation of SiO_2@RF Nanoparticles

Silica nanospheres were prepared using a modified Stöber method. Firstly, prepare a mixed solution containing anhydrous ethanol, deionized water, and concentrated ammonia. Then, add an ethanol solution containing TEOS (4.5 mL, ≥ 99%) to the above mixed solution. After stirring for 19 hours, the silica nanospheres were collected by centrifugation at 6000 rpm for 10 minutes and finally dispersed in 28 mL of deionized water. Disperse 14 mL of SiO_2 solution back into 44 mL of deionized water, add PVP solution (4 mL, 50mg/mL) and stir overnight. After centrifuging at 10000 rpm for 10 minutes, collect the product and store it in 56 mL of deionized water. Mix 16mL the SiO_2-PVP dispersion with resorcinol (0.02 g, ≥ 99%), formaldehyde solution (56 μ L, 37%), and ammonia water (200 μ L, 2.8%). The mixture was reacted at 60°C for 3 hours, centrifuged at 8000 rpm for 10 minutes, washed with ethanol and isopropanol, and finally redispersed in isopropanol (10 mL).

Preparation of SiO_2@RF@Au Shell Nanoparticles

Disperse 5 mL of SiO_2@RF in 95 mL isopropanol, add APTES (250 μL, ≥ 98%), reflux at 90°C for 3 hours, centrifuge at 8000 rpm for 10 minutes to obtain SiO_2@ RF-NH_2, wash with ethanol and water, and disperse in 25 mL of deionized water. A portion of the SiO_2@RF-NH_2 dispersion (5 mL) was mixed with $HAuCl_4$ (60 mL, 1 mM, ≥99%). Under ice bath conditions, sodium citrate (0.3 mL, 0.8 M, ≥99%) and $NaBH_4$ (1 mL, 0.12 M, ≥98%) were added, stirred for 6 minutes, centrifuged at 8000 rpm for 10 minutes, and redispersed in deionized water (5 mL). A portion of the Au seed dispersion (2.5 mL) was mixed with $HAuCl_4$ (25 mL, 1 mM, ≥99%), sodium citrate (1 mL, 0.8 M, ≥99%), and hydroxylamine hydrochloride (780 μL, 0.04 M, ≥99%) under ice bath, stirred for 10 minutes, centrifuged at 8000 rpm for 10 minutes, and redispersed in deionized water (10 mL). The entire volume of Growth Solution 1 was processed repeatedly using the same method and redispersed in deionized water (12 mL). Then, the product was mixed with HF (20 mL, 2%, 40%), stirred overnight, centrifuged at 8000 rpm for 10 minutes, and the precipitate was redispersed in deionized water (4 mL).

Preparation of AB Nanoparticles

Partial SiO_2@ RF@Au Shell dispersion was dried at 80 °C for 12 hours to obtain bowl shaped gold nanoparticles (AB). Calculate the mass of materials using the weighing method.

Preparation of AB-LC Nanoparticles

The dried material (10 mg) was dispersed in deionized water (10 mL). A portion of this dispersion (5 mL) was added to a 25 mL flask, followed by sh-L arginine (5 mg, ≥99%, dissolved in water (1 mL)), and stirred in the dark for 24 hours. Then, cRGD with thiol group (0.5 mg, purity ≥95%, dissolved in water (0.5 mL)) was added, and stirring was continued in the dark for 24 hours. The mixture was centrifuged at 10,000 rpm for 10 minutes, and the precipitate was resuspended in PBS (2.5 mL), sonicated for dispersion, and stored at 4°C in the dark (labeled as AB-LC).

ROS Generation Detection of AB-LC

The AB-LC (200 μL, 200 μg mL^{-1}) was mixed with the DCFH-DA (200 μL), treated with US (1.5 W cm^{-2}) for different minutes, and the fluorescence intensity was detected using a fluorescence spectrophotometer at Ex 488 nm/Em 525 nm. Similarly, AB-LC (200 μL) at different concentrations were mixed with the DCFH-DA solution (200 μL), treated with US (1.5 W cm^{-2}) for 3 minutes, and the fluorescence intensity was detected under the same conditions.

NO Release Detection of AB-LC

Groups were set as follows: deionized water, AB-LC (200 μg mL^{-1}), and AB-LC (200 μg mL^{-1}) + US (1.5 W cm^{-2}, 3 minutes). After centrifugation, the supernatant was collected, and the NO content was detected using a Griess kit.

Thrombolysis Therapy In Vitro

The fresh blood (200 μL) was mixed with calcium chloride (20 μL, 1 mol L^{-1}, ≥97%) and thrombin (10 μL, 500 U mL^{-1}), incubated at 37°C for 3 hours to form blood clots. The clots were washed with PBS until no blood was released, then weighed and photographed. Thrombi were divided into four groups: PBS, PBS + US, AB-LC, and AB-LC + US (1 W cm^{-2} for 10 minutes). They were weighed and photographed at 0, 3, and 5 hours, and the absorbance of the supernatants was measured at 450 nm (fibrin) and 540 nm (hemoglobin) using a microplate reader.

Hemolysis Experiment In Vitro

The red blood cell suspension (1 mL) was mixed with AB-LC (100 μL) at different concentrations, incubated at 37°C for 2.5 hours, centrifuged at 2000 rpm at 4°C for 10 minutes, and the absorbance at 545 nm was detected. Deionized water was used as a positive control, and PBS as a negative control to calculate the hemolysis rate.

Cell Cytotoxicity Test and Cell Live/Dead Staining

HUVECs were seeded in 12-well plates (2×10^5 cells/well) for 12 h. . After the cells were adherent, the medium was removed, the cells were washed twice with PBS, and then AB-LC solutions at concentrations of 0, 25, 50, 75, 100, and 200 μg mL^{-1} respectively were added. Subsequently, co-culture was maintained for 2 hours. Cells were then stained using a live/dead cell staining kit (Calcein AM/PI) and observed under a fluorescence microscope. Live cells and dead cells exhibited green and red fluorescence, respectively.

Cells were seeded in 12-well plates at 2×10^5 cells/well and cultured for 24 hours. The medium was replaced with fresh medium containing AB-LC (200 μg mL^{-1}), and cultured for 4 hours. Then, the cells were incubated with a Reactive Oxygen Species Assay Kit (DCFH-DA) for 30 minutes, irradiated with US (0.5 W cm^{-2}) for 10 minutes, and observed under a fluorescence microscope.

NO Release Capacity at the Cellular Level

Cells were seeded in 12-well plates at 2×10^5 cells/well and cultured for 24 hours. The medium was replaced with fresh medium containing AB-LC (200 μg mL^{-1}), and cultured for 4 hours. Then, the cells were incubated with a Nitric Oxide Detection Kit (Griess) for 30 minutes, irradiated with US (0.5 W cm^{-2}) for 5 minutes, and observed under a fluorescence microscope.

Thrombosis Model of Animals

All animal experiments complied with the Institutional Animal Care and Use Committee (IACUC) and the Administrative Committee of Laboratory Animals of Fujian Medical University (IACUC-FJMU 2024-Y-1519). BALB/C mice (male, 8-week-old, weight ≈20 g) were purchased from SLAC Laboratory Animal Co., Ltd. The mouse model of lower limb venous thrombosis was established as follows: after anesthesia, the mice were fixed in a supine position, the target artery of the left lower limb was exposed, and a filter paper (1 mm×1 mm) soaked with $FeCl_3$ (10%, ≥99%) was applied for 5 minutes, followed by rinsing the tissue with saline.

To evaluate the thrombolytic effect, mice with lower limb venous thrombosis were divided into four groups: tail vein injection of PBS (0.1 mL), AB-LC (0.1 mL), PBS (0.1 mL) + US, and AB-LC (0.1 mL) + US (1 W cm^{-2} for 5 minutes). Thrombus changes were observed within 12 hours. After euthanasia, blood vessels at the thrombus site were collected for H&E staining to observe thrombus morphology, thrombolysis rate and vascular recanalization were statistically analyzed. Heart, liver, spleen, lung, and kidney tissues were collected for H&E staining to evaluate organ damage.

Statistical Analysis

Data were obtained from at least three independent measurements (n=3). Statistical analysis was performed using Origin 2021 software. All experimental data are expressed as mean ± standard deviation (SD).

Ethical Statement

All animal experiments complied with the regulations of the Administrative Committee of Laboratory Animals of Fujian Medical University (IACUC FJMU 2024-Y-1519).

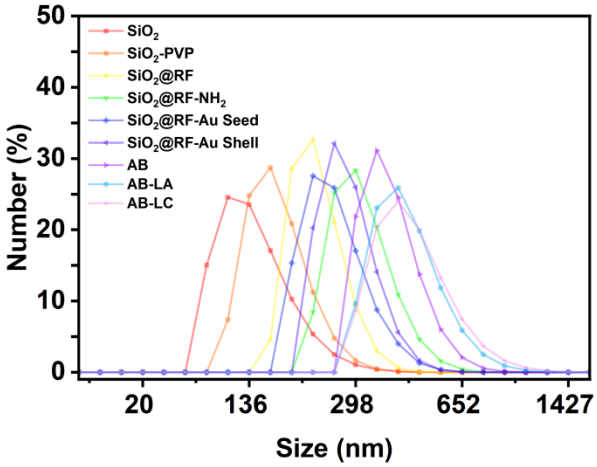

Fig.S1 The size distribution of SiO_2, SiO_2-PVP, SiO_2@RF, SiO_2@RF-NH_2, SiO_2@RF-Au Seed, SiO_2@RF-Au Shell, AB, AB-LA and AB-LC.

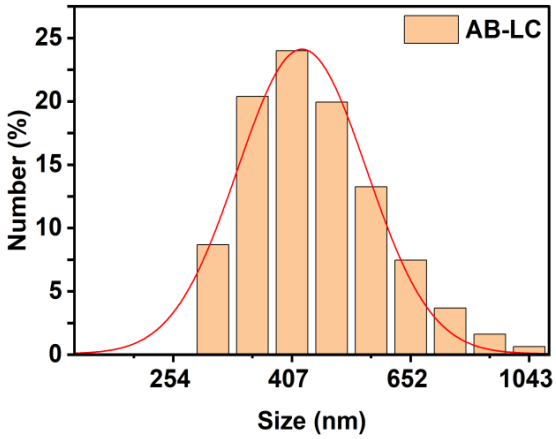

Fig.S2 The size distribution of AB-LC.

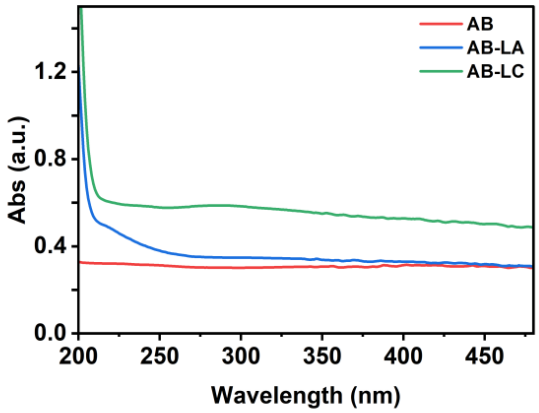

Fig.S3 UV–vis–NIR absorption spectra of AB, AB-LA and AB-LC.

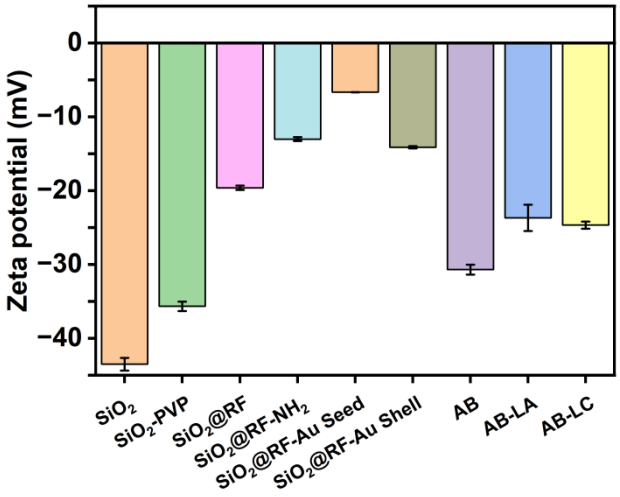

Fig.S4 The Zeta potential of SiO_2, SiO_2-PVP, SiO_2@RF, SiO_2@RF-NH_2, SiO_2@RF-Au Seed, SiO_2@RF-Au Shell, AB, AB-LA and AB-LC.

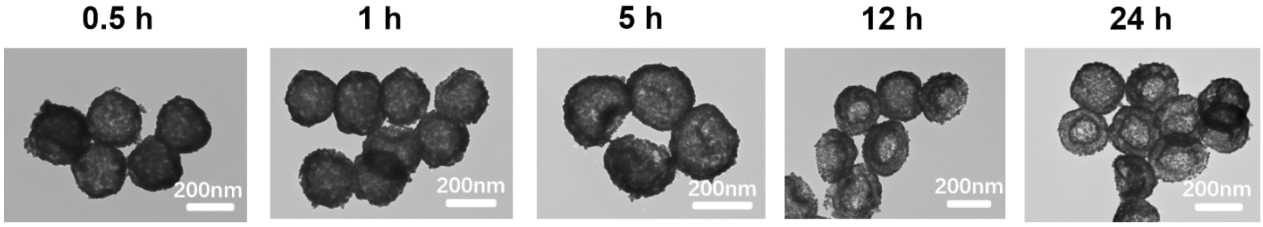

Fig.S5 The influence of different hydrofluoric acid etching conditions on the formation of bowl-shaped structures.

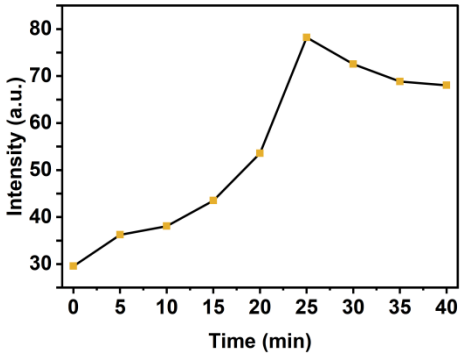

Fig.S6 The relationship between ROS generation in AB-LC and ultrasonic duration.

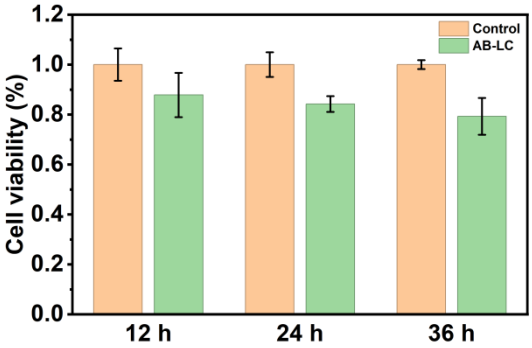

Fig.S7 Long-term cytotoxicity assessment of AB-LC on HUVECs.

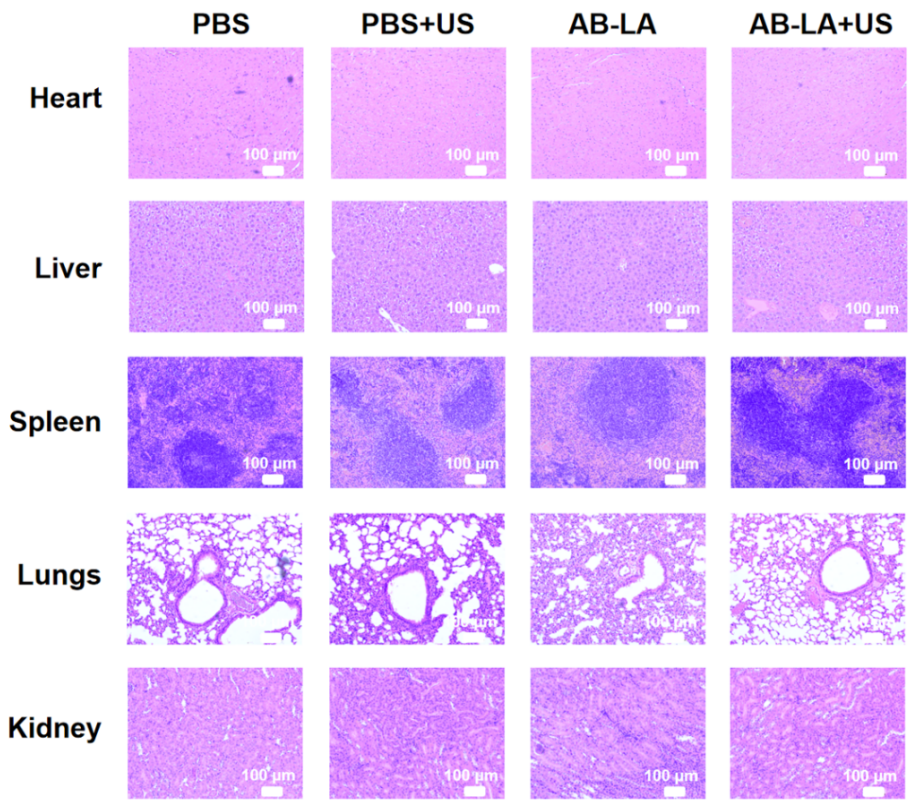

Fig.S8 The biological safety of the body was observed by staining different organs with hematoxylin and eosin.