Supporting Information

Deep Penetration of Targeted Nanobubbles Enhanced Cavitation Effect on Thrombolytic Capacity

Ling Ma [1, †], *Yinjie Wang* [2, 4, †], *Shengmin Zhang* [1, 3, †], *Xuechen Qian* [1, 3], *Nianyu Xue* [1], *Zhenqi Jiang* [2, 4], *Ozioma Udochukwu Akakuru* [2, 4], *Juan Li* [2, *], *Youfeng Xu* [1, 3, *], *Aiguo Wu* [2, *]

1. Department of Ultrasound, Ningbo First Hospital, Ningbo 315010, China

2. Cixi Institute of Biomedical Engineering, CAS Key Laboratory of Magnetic Materials and Devices, Key Laboratory of Additive Manufacturing Materials of Zhejiang Province, Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences, Ningbo 315201, P.R. China

3. Medical School of Ningbo University, Ningbo 315211, China

4. University of Chinese Academy of Sciences, Beijing 100049, China

† These authors contributed equally to this work.

*Corresponding author.

E-mail: aiguo@nimte.ac.cn, 2371334413@qq.com, lij@nimte.ac.cn

EXPERIMENTAL METHODS

PLGA50/50-COOH was purchased from Shanghai Yare Biotech., Inc. (Shanghai, China). Cyclic Arg-Gly-Asp-D-Tyr-Glu (RGD) peptides were purchased from Nanjing Peptide Biotech. Co. Ltd (Jiangsu, China). Agar powders, sodium cholate hydrate, polyvinyl alcohol (PVA), and sorbitan monooleate (Span 80) were purchased from Aladdin Industrial Inc. (Shanghai, China). Dichloromethane (DCM), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), and N-hydroxysuccinimide (NHS) were purchased from Sino Pharm Chemical Reagent Co., Ltd (Shanghai, China). Fetal bovine serum (FBS) was purchased from Invitrogen™ (Carlsbad, USA), while urokinase (UK) was purchased from Nanjing Nanda Pharmaceutical Co. Ltd (Jiangsu, China).

Preparation of micro-scaled or nano-scaled bubbles

The PLGA micro-scaled bubbles (Mbs) were made by solvent emulsion/evaporation method[1]. Typically, 2 mL of 50 mg/mL PLGA and 50 mg/mL Span80, both in DCM solutions were prepared, respectively. These two solutions were mixed in 0.5 mL of water. Then, the mixture was emulsified; a pre-emulsion solution was formed under 20 000 rpm after a 1 min spin by using a superfine homogenizer (F6/10, FLUKO, China). Afterwards, the solution was added to a 20 mL of 3 % PVA solution to increase its viscosity, and then they were mixed again under the superfine homogenizer (10 000 rpm, 3 min) to obtain a multiple emulsion which was continuously stirred for 8 h at 25 ℃ to form Mbs. The Mbs were washed 3 times with pure water before use and separated by centrifugation (3500 rpm, 15 min, 4℃).

Pure Mbs was re-suspended in 5mL of water and lyophilized. After 24 h of lyophilization, powder-like PLGA Mbs were obtained.

The PLGA nano-scaled bubbles (Nbs) were also made by solvent emulsion/evaporation method[1]. In a typical experiment, 4 mL of 25 mg/mL PLGA in DCM was prepared. The above solution was added to 20 mL of 1. 5% sodium cholate solution, and then emulsified by a superfine homogenizer (10 000 rpm, 2 min) to form a pre-emulsion. This pre-emulsion was sonicated in an ultrasonic cell pulverizer at 648 W for 1 min for further emulsification. The dichloromethane solvent was then evaporated by magnetic stirring for 4 h at room temperature. Afterwards, 0.3 g of PVA was added to the emulsion with continued stirring for 1 h to promote dissolution. The mixture was stored for 5 days at 4℃ which can enable the PVA to replace sodium cholate. In the end, Nbs were washed with pure water before use and separated by centrifugation (10 000 rpm, 1 h, 4℃). Pure Nbs were re-suspended in 5mL water and lyophilized. After 24 h of lyophilization, powder-like PLGA Nbs were obtained.

Preparation of cRGD-targeted Mbs or Nbs

To prepare the cRGD-targeted Mbs or Nbs, the carboxylic groups on the surface of Mbs or Nbs were activated through an EDAC/NHS activation method[2]. The functionalized carboxylic groups were then conjugated with the amine group of cRGD. Briefly, 2 mL EDAC (1.25 mg/mL in PBS) and 1 mL NHS (2.5 mg/mL in PBS) were used to active 50 mL Mbs or Nbs solution (1 mg/mL in PBS). After 20 min of stirring in ice water bath, 5 mL cRGD solution (1 mg/mL in PBS) was added to the above mixture, and continuously stirred for 16 h at room temperature. The obtained

targeted Mbs or Nbs were washed with pure water before use and separated by centrifugation (12 500 rpm, 45 min, 4℃).The storage conditions were the same as for the Mbs. In addition, the supernatants were retained, freeze-dried and re-dissolved in 10mL pure water. The cRGD concentration was determined by high performance liquid chromatography (AJL1260, Agilent, USA). The content of unreacted cRGD in the supernatant was calculated from the standard curve.

Characterization

A metallographic microscope and dry-wet laser particle size analyzer (S3500, Microtrac, America) were used to preliminarily observe the particle sizes and distributions of Mbs and Mbs-cRGD at room temperature. Meanwhile, SonoVue was also observed in order to be better compared. Zeta potentials and particle sizes of Nbs and Nbs-cRGD were determined by Dynamic Light Scattering (DLS) using a particle size-zeta potential analyzer (Nano-ZS, Malvern, England).

Then, the morphology of Mbs and Nbs was further characterized by a scanning electron microscope (S4800, Hitachi, Tokyo, Japan and Verios G4 UC, Thermo scientific, US). A transmission electron microscope (TF20, FEI, USA) was used to observe the structure of Nbs and Nbs-cRGD. Briefly, 5 μL of Nbs solution was dropped on a copper sheet coated with ultra-thin carbon film, and a drop of 75 % ethanol solution was dripped onto the top of the sample droplet to dry it.

Cytocompatibility test

In vitro erythrocyte compatibility experiment was performed. 1mL of 5% erythrocyte suspension was added in each tube. 2 mL pure water was added into the

Tube 1, and 2mL PBS solution was added into the Tube 2. 2 mL PBS solution of 0.5 mg/mL Mbs, Mbs-cRGD, Nbs or Nbs-cRGD was added into the Tube 3 to 6, respectively. All tubes were incubated at 37℃for 24 h.

Rabbit clot preparation

A total of 25 mL of whole blood from rabbits was sampled in five different 5 mL syringes without any anticoagulant and kept in a water bath at 37 ℃. After 3-5 h in the water bath, the blood clots were stored at 4 ℃ for 2 days, ensuring maximal clots retraction, lytic resistance, and stability. After the serums were discarded, all of the coagulated blood clots were cut into 30 equal pieces by length and kept in 0.9% sodium chloride at 4 ℃ prior to experiments[3].

Sonothrombolysis model development

In order to simulate the thrombus in human blood flow, an in vitro loop-closed flow system was built in our study (Fig. 1). The circulating device consisted of a micro peristaltic pump (BT 100-2J, Baoding Dirui Electronics Technology Co., Ltd, Hebei, China), circulation pipes (peristaltic pump hose inner diameter 3.2 mm, medical rubber pipe inner diameter 5×7 mm) and an agarose model. Medical rubber tube was fixed with 1.5 % agarose clot at 37 ℃ in a water bath to simulate human soft tissues and blood vessels, ensuring good penetration of ultrasonic irradiation. The thrombus was placed in the rubber pipe, and the agarose model was connected with a peristaltic pump through three-way cocks. In each group, PBS containing 10 % bovine serum as circulating fluid was slowly injected into the lumen through the

three-way cock A, and excess air in the tube was discharged with three-way cock B at the same time. The pump created a pulsatile flow rate of 8 mL/min. The probe was dipped into the water and adjusted to a distance of 40 mm to the clot.

A commercial medical diagnostic ultrasound device (M9CV, Mindray, China) with a mechanical index (MI) of 1.1 and equipped with a 1.3 MHz probe was used for thrombolytic treatment. Moreover, ultrasound imaging of thrombus also used a diagnostic ultrasound device (DP-50, Mindray, China) which was transmitted (5 MHz, mechanical index = 0.8).

Sonothrombolytic experiments

Six groups of clots (five for each group) were set up and treated with 1.3 MHz and MI 1.1 ultrasound for 30 min. To observe the changes in the clots, 5 MHz and MI 0.8 ultrasound was used at 0, 15, and 30 min for imaging in thrombus major and minor axis. In ultrasound minor axis imaging, ultrasound device was performed to measure the cross-sectional area of lumen (A_0) and the cross-sectional area of thrombus (A_1). The cross-sectional area thrombolysis rates (%) were calculated as following: $(A_0\text{-}A_1)/A_0 \times 100$. The control group was only exposed to ultrasound for 30 min (US group). In another group, 5000 I.U. of UK was injected into the model prior to insonation (US+UK group). The US+Mbs+UK, US+Nbs+UK, US+Mbs-cRGD+UK, and US+Nbs-cRGD+UK were treated with 0.5 mg/mL of Mbs, Nbs, Mbs-cRGD or Nbs-cRGD with 5000 I.U. of UK at once, respectively. Other conditions were the same as the control group.

The mass loss of clot

The thrombi were weighed before treatment (W_0) and immediately after 30 min insonation (W_1) with an analytical balance (AL104, METTLER TOLEDO, Switzerland). All clots were rinsed with physiological saline once, and the water was removed before measuring. The relative reduction of clot weight (W_{loss} (%)) was used to show the efficiency of thrombolysis ability and calculated with the following equation:

$$W_{loss}(\%) = (W_0 - W_1)/W_0 \times 100$$

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed for the blood clots of six groups after treatment, in order to compare the changes in internal structure. The clots from each group were immediately fixed with 2.5 % glutaraldehyde solution at 4 °C for 24 h after therapy. They were subsequently dehydrated with ethanol, dried at room temperature, and plated to observe the morphology of the thrombus with a SEM (S4800, Hitachi, Tokyo, Japan and Verios G4 UC, Thermo scientific, US) operating at 4.0 kV.

Statistical analysis

Data were presented as mean ± SD. One-way ANOVA followed by post hoc test of Dunnett were performed for the sonothrombolytic experiments using SPSS 18.0 software, and values of $p<0.05$ were considered statistically significant.

References

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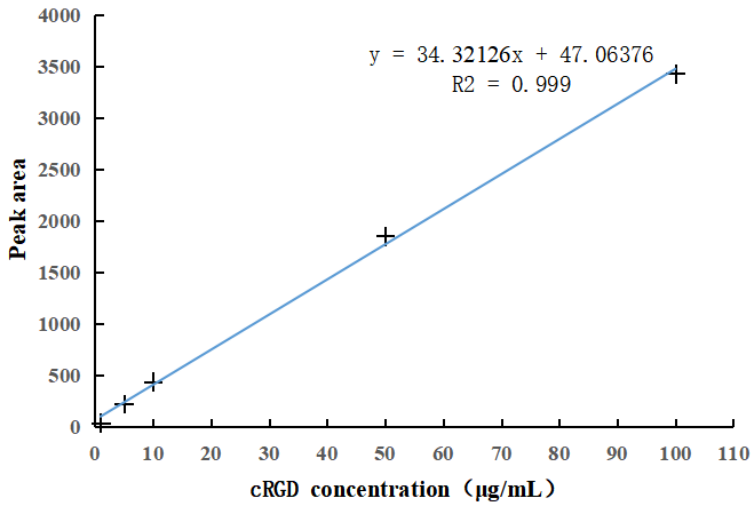

Fig. S1 The standard curve of cRGD by HPLC. The cRGD concentration is varied from 1.0 to 100.0 μg/mL.

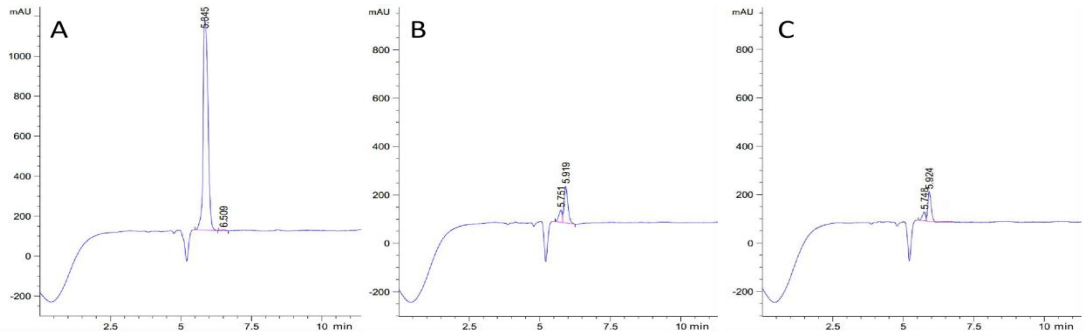

Fig. S2 HPLC images of cRGD. (A) cRGD before conjugation; (B) cRGD after conjugation to Mbs; (C) cRGD after conjugation to Nbs.

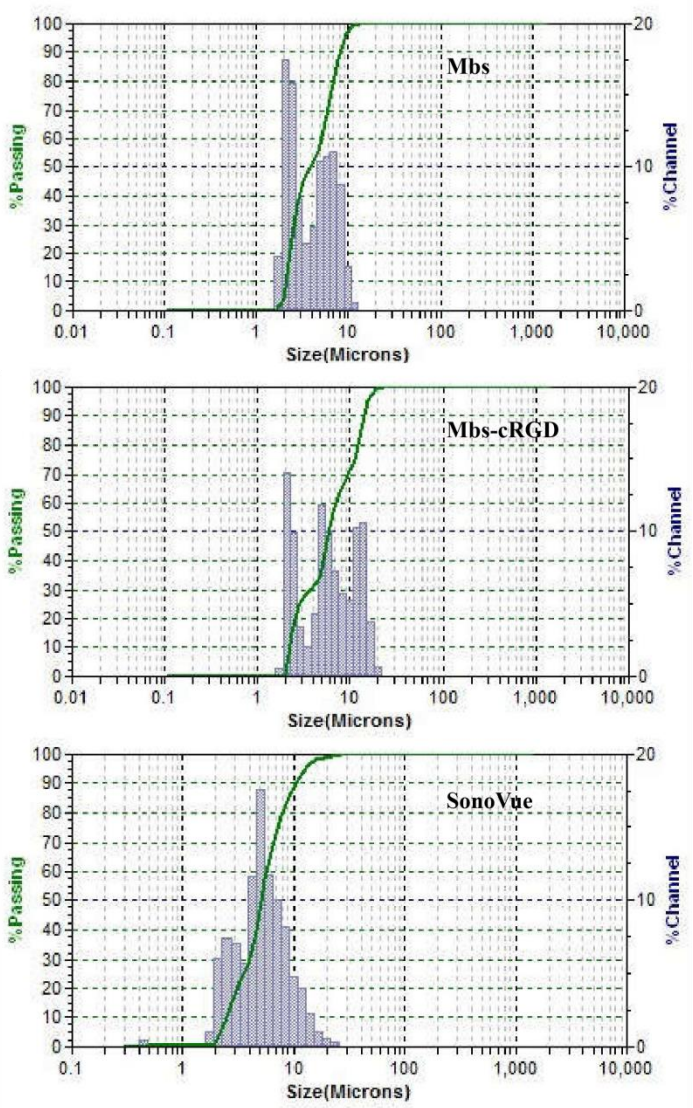

Fig. S3 Size distributions of Mbs, Mbs-cRGD and SonoVue by dry-wet laser particle size analyzer.

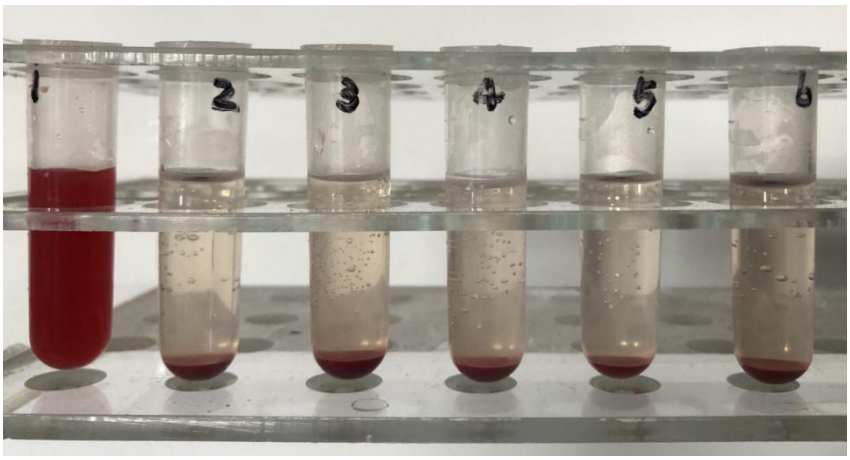

Fig. S4 Erythrocyte compatibility test of different bubbles. Tube 1: positive control group; Tube 2: negative control group; Tube 3: Mbs group; Tube 4: Mbs-cRGD group; Tube 5: Nbs group; Tube 6: Nbs-cRGD group.

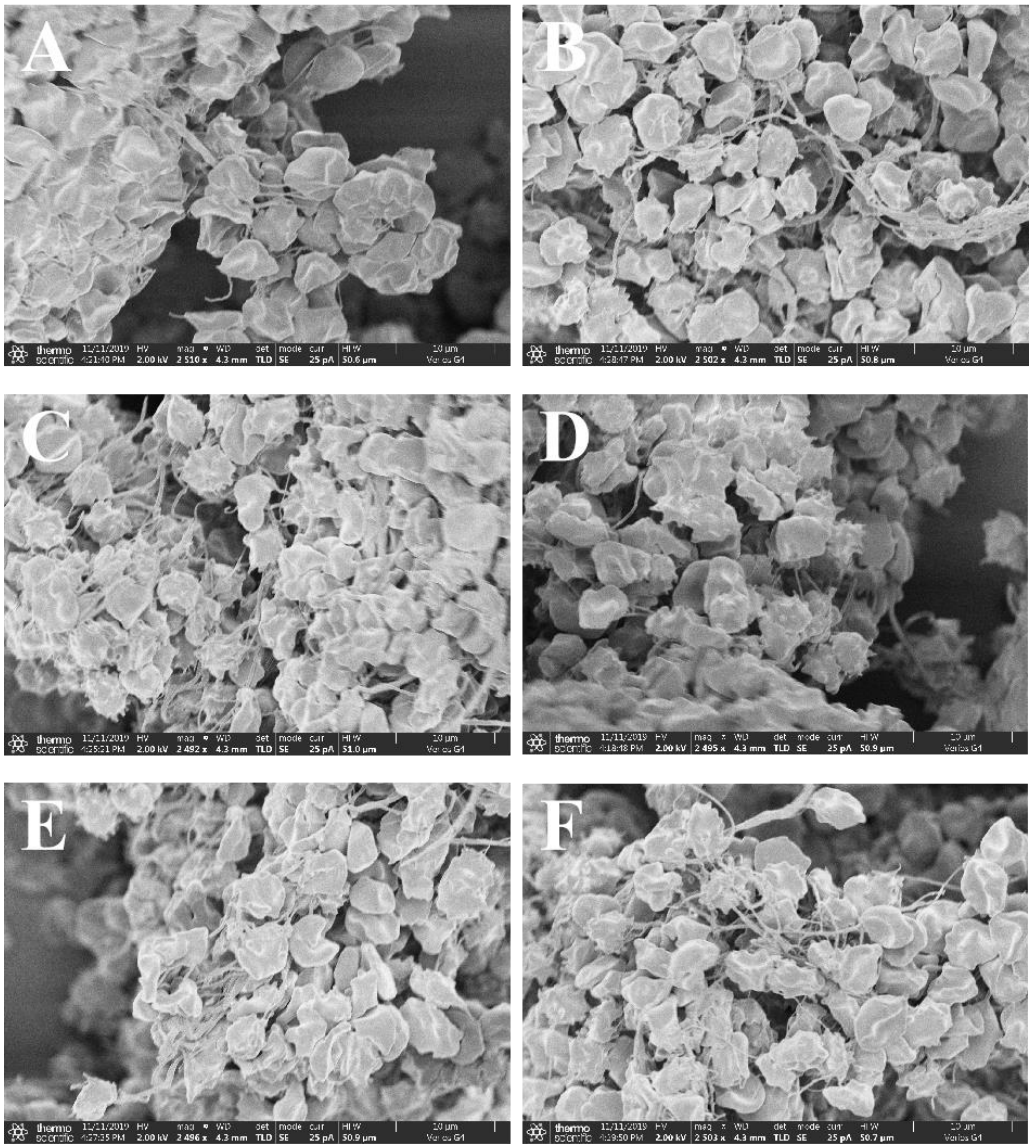

Fig. S5 Scanning electron microscopic images of thrombi in each group before therapy (2500×). (A) US；(B) US+UK；(c) US+Mbs+UK; (D) US+Mbs-cRGD+UK; (E) US+Nbs+UK; (F) US+Nbs-cRGD+UK.