

Peer Review File

Ultrasound-responsive theranostic platform for the timely monitoring and efficient thrombolysis in thrombi of tPA resistance



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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This experimental study evaluates the efficacy of nanoparticle targeted to thrombi, with ultrasound mediated thrombolysis. This is done ex vivo, using human samples, rat and pig arterial thrombus.

1. Please consider, given the role that NETs play in DVT, to evaluate your agents on DVT in mice or rat model -- perhaps a flow restricted IVC model

2. Can you elaborate on the age of the human post stroke thrombi? How were they treated in the humans; eg, heparin, lysis, antiplatelet therapies prior to harvest?

3. In the pig model, can you determine the function of the vessels post targeted nanoparticles as compared with control? For example, do they have intact endothelium, and produce nitric oxide, prostaglandins, etc? This would be a bit more directed than the bulk rna sequencing.

4. For the experiments evaluating the breakdown on NETs, you should compare with a control such as DNase.

5. It would be of interest to define your agent in the setting of heparin use and antiplatelet use, as this is directly translatable to humans. In other words, within these models, what do the standard agents procedure in terms of patency?

6. From a practical standpoint, what is the depth limit of the ultrasound energy to penetrate to the vessel and activate the nanoparticle agent?

7. Conceptually, it seems the thrombus is mainly fibrin with platelets, rbc, and less so with NETs -- thus, do you have an agent that mainly 'attacks' the fibrin crosslinking, and not NET digestion?

Reviewer #2 (Remarks to the Author):

Specific Comments: Title Is awkward. Simplify and avoid jargon

Line number: 19-36 Several terms in the abstract are not in general use. Thus, the text is hard to follow.

What is meant by “programmed thrombolysis”? Why were TEVGs employed? What is meant by “multifunctional nanoplatform”? Are there more than two functions (diagnosis and therapy)? If not “dual-function submicron particles” may be a more accurate description. Are particles that have a diameter of 200 nanometers considered “nanoparticles”? In my opinion, these are submicron particles. I recommend reserving the term “nanoparticles” for particles on the order of 1 to 10s of nanometers, not 100s of nanometers.

52 What is mean by “valid time”? The authors should cite a broader clinical literature regarding efficacy of thrombolysis approaches, such as sonothrombolysis. (See for example,

<https://doi.org/10.1161/STROKEAHA.120.030960> Stroke. 2021;52:3786– 3795, and others)

569 Reference 11 is incomplete

67 Sonodynamic therapy to enhance rt-PA thrombolysis is termed “sonothrombolysis” in the literature.

Please cite more relevant work, such as <http://dx.doi.org/10.1016/j.ultrasmedbio.2014.08.018> .

72 The term “Drug-resistant thrombi” needs to be clearly defined in the introduction.

72ff The seminal work by Liebeskind et al. needs to be cited

(<https://doi.org/10.1161/STROKEAHA.110.605576> Stroke. 2011;42:1237–1243)

45 “scouring of blood flow” is not an appropriate description in English

90 “gas-blasting” no clear. Are you referring to acoustic activation of nanodroplets into microbubbles? Or acoustic cavitation? Clarify.

94 Thrombolysis with ultrasound exposure alone has been shown to be ineffective, unless the peak rarefactional pressure amplitude is quite high (> 17 MPa) such as pulses used in histotripsy (See for example, Kenneth B Bader et al 2016 Phys. Med. Biol. 61 5253 DOI 10.1088/0031-9155/61/14/5253, and Henley et al, IEEE TRANSACTIONS ON ULTRASONICS, FERROELECTRICS, AND FREQUENCY CONTROL, VOL. 68, NO. 9, SEPTEMBER 2021). It is unclear what is meant by thrombi “soften under the mechanical action of US.” No such evidence of a decrease in Young's modulus of blood clots exposed to US has been demonstrated in the literature.

94ff Define all acronyms on first usage. What is “RB”? Retinoblastoma protein? Why did you choose this particular therapeutic approach? Overall, the description of your approach is disorganized and the text is hard to follow.

94 “Sensibilizer” is not a word in English. Please spell check your manuscript carefully using MS Word.

96 Define PFH. Are these perfluorocarbon nanodroplets? Provide composition details. In general, not enough detail in the synthesis of the submicron particles was provided.

97 The use of perfluorocarbon nanodroplets to enhance rt-PA thrombolysis is not novel. Previous literature should be cited.

103 What is meant by “transplanted blood vessels”? Clarify.

Fig. 1b: In my opinion, particles that are 200 nm in diameter should be called “submicron” particles, not “nanoparticles”.

117-22 There are many undefined acronyms, making the components impossible to discern.

148 Too many undefined acronyms. What is DPBF? What is SOSG?

155 The term “echo contrast agent” is used in English, not “imaging enhancer agent”.

177-8 The nanoparticles alone were not tested as a control. This is an important limitation of the study.

194 Why was the degree of clot retraction not assessed?

230 What is meant by “the density and thickness of fibrin skeleton was significantly improved”? How were fibrin density and fibrin thickness quantified?

257 MSB, not H&E is the stain of choice to image fibrin. It is difficult to differentiate fibrin from hemoglobin on H&E (both appear red).

276ff The motivation for flushing the carotid arteries post treatments is not well justified.

305ff The acoustic insonation parameters were not provided in detail, other than the 1 MHz center frequency. See Padilla and ter Haar, Ultrasound in Med. & Biol., Vol. 48, No. 7, 3 pp. 1299–1308, 2022, <https://doi.org/10.1016/j.ultrasmedbio.2022.03.001> for guidance on acoustic parameter reporting.

339 TEVGs undefined (Tissue-engineered vascular graft?). No details were provided for this tissue construct.

357-63 GO, KEGG, CSEA acronyms undefined

376 Citation needed for “CCK8 method”.

Reviewer #3 (Remarks to the Author):

L. Lin et al. present in this article an interesting non-invasive treatment activated by ultrasounds for drug-resistant thrombi. According to the authors, NETS (formed by neutrophils) and isopeptide bonds are the key elements that make thrombi drug resistant. Thus, they designed nanoparticles (hmSi-CREKA-RB-PFH) which are supposed to produce ROS and gases under ultrasound stimulation for a combined chemical

and mechanical action on both NETs and isopeptide bonds in the thrombus. This innovative therapy has interesting characteristics such as the targeting of the thrombus, the combination of two different modes of action on the thrombus, the absence of fibrinolytic agent, the thrombolytic action controlled by ultrasound that have a deep tissue penetration and the focus on drug-resistant thrombi.

First, the authors presented the different thrombus therapies, their limits, as well as the composition of a blood clot and the various technics to diagnose it. Then, the synthesis and characterization of the nanoparticles are presented. Secondly, they studied the degradation of NETs and isopeptide bonds by their treatment ex vivo (on clinical thrombi), in vitro and in vivo. Then, they tested their combined therapy on a fibrin scaffold ex vivo (on clinical thrombi) and in vitro. The authors assessed the therapeutic efficacy of their treatment on thrombi induced in vivo in rats and pigs, but also on thrombi induced in tissue-engineered vascular grafts implanted in rats. They finally evaluated the biosafety of their nanoparticles in vitro (hemocompatibility test) and in vivo (biodistribution and RNA sequencing of the recanalized vessels).

Despite some very interesting results like the therapeutic effect of the combined treatment in a rat occlusion model, the authors made too many hasty conclusions which are not supported by their data, while some results are impossible to analyze due to poor data quality or no explanation on how they were obtained. Furthermore, some experiments lack necessary controls, the description of the methods is uncomplete, the number of experiment repetitions is sometimes not mentioned or too low and the analysis of the results is too poor.

Major points:

- Introduction:

- o the authors present the mechanism of action of their treatment, but the experiments presented don't allow to justify it (at least, they should say that this is a hypothetical mechanism). For example, in the different figures, there is almost never any significant difference between the treatment without and with PFH, and this is not interpreted.

- o Moreover, the treatment with PFH and without RB has never been tested, which would have been interesting to distinguish the ROS effect and the mechanical action

- o Furthermore, since barely any exploration of mechanism of action exist, claims about how these objects work can only be hypothetical; this is especially true for lines 93-98.

- Lack of experiment or experiment persistence:

- o TEM images are not enough to assess size, and the quantification method of size (total and shell) by TEM is not given. DLS size measurements are only given in the supplementary but without mention of solvent, whether these are intensity, volume or number distributions. Solvent and pH for Zeta potential are not mentioned, nor the explanation of variations. A more thorough size, shape and physico-chemical analysis is necessary.

- o It lacks an experiment that proves the link between NETs / isopeptide bonds degradation and fibrin degradation, which is claimed by the authors. Indeed, they set up experiments to show the efficacy of their combined therapy on NETs, isopeptide bonds and fibrin separately but they didn't prove that NETs and isopeptide bonds degradation increases the lysis of the fibrin network and thus the thrombus.

- o In addition, the liquid-to-gas transformation of PFH under US has not been tested in the article.

- o Part 2.7 (Biosafety assessment), there is no study of the biosafety of the nanoparticles under US

stimulation

- ♣ Notably, it would be expected that safety and cytotoxicity experiments be performed and presented BEFORE switching to in vivo experiments, including big mammals.

- o Line 371-373 / Figure S11: the maximum dose tested is 400 µg/mL for hemolysis study, but in vivo experiments were done at 5 mg/mL. Furthermore, the authors make conclusions on this figure, but no statistical test was performed

- o In the in vivo study on pigs, n = 1

- Uncomplete methods.

- o Example: Figure 6 / Paragraph 2.6: The purpose of the study on thrombi induced in TEVGs is not clear. There is no explanation about the procedure of vascular grafting and about its treatment after thrombus induction, neither in the Materials & Methods nor in the article

- o The explanation of immunofluorescence markers used here is not given, nor the interpretation of their presence/absence in staining. In particular, SytoxGreen is used to stain nucleic acid: what are the authors trying to see when co-staining with Hoechst) (supposedly, in Fig. 2C)?

- o Quantitative analysis of images is not explained: how do the authors quantify the NET area (fig. 2B)

- o How are dipeptides stained in Fig. 3A?

- Too many hasty conclusions. Indeed, they hardly analyzed the results of their experiments and go straight to the conclusion. Sometimes, even false conclusions are made or conclusions on results without statistical tests. Examples:

- o Line 179 / figure 2B, they conclude that the combined treatment significantly destroyed NETs but there is no significant difference between US only and US+hmSi-CREKA-RB-PFH, and they don't mention that it can be the ultrasounds that degrade NETs and not the combined treatment. This happens several times in the article, and would suggest that US only are responsible for the in vitro results.

- o Fig. S4: Nothing can be deduced from the images. There is a clear overexposition and unspecific staining with the MPO channel (which also questions other results in the manuscript). The magnification is not enough to see anything, let alone a NET (where are the cells, where is the NET?) Side-by-side monochannel images are necessary as good practice. The lack of control (non-induction of NETs) is critical here.

- o Line 235-236 / figure 3F: there is no significant difference between US+hmSi-CREKA-RB and US+hmSi-CREKA-RB-PFH. Thus, it is false that "The proportion of isopeptide bonds had dropped from 12.22% to 0.02% when PFH blasting was added (Figure 3F)". The drop corresponds to the control compared to the combined therapy

- o Line 301-303: the specific targeting ability of CREKA has not been demonstrated because there is not the condition hmSi-RB-PFH (without CREKA) as control of passive targeting

- o Line 420-423: in figure 8D, there is no error bar, no statistical tests, ... The conclusion is hasty, they can't conclude that the thrombus was fully dissolved because there is no control of complete thrombolysis

- o Figure 5: Since no imaging of the PFH signal enhancement has been done in vivo without thrombus, it can't be said that the change in ultrasound signal is due to PFH bubbles or only to blood reperfusion due to the combined treatment

- Some results are clearly indicating that the experiment did not work or that the results do not follow the authors' interpretation.

- o Fig. 2C: no cells are apparent in SEM images for US with NPs: are they all dead (thus cytotoxicity issues) or are no cell present (then obviously no NET can be formed)?

o Figure 2E: same issues: no more apparent nuclei in the combined treatment conditions. What are the cell types that are present in the control group and not anymore in the treatment group (no more nuclei)? Are they neutrophils? If yes, why are they all dead?

Minor points:

- Introduction:

o the authors justified the use of sonodynamic therapy by the fact that PTT induces fragments larger than 10 μm but the reference of this statement claims this without any reference. In addition, they did not present how singlet oxygen produced by their therapy are supposed to destroy the NETS and isopeptide bond (at least, they should present the literature on this topic). Also, the authors designed CREKA-functionalized nanoparticles, but they didn't explain how this peptide targets the thrombus. The different components of the nanoparticles are not presented either.

o Certain words are used seemingly to increase complexity but are not justified or relevant:

“spatiotemporal thrombolytic method” for instance.

o The claim that NET and isopeptide bonds are contributor to drug resistance is a conjecture: it might have to do with mechanical strength, secondary networks, etc. but not drug resistance.

- Regarding the organization of the results of the article, the authors made the choice not to separate in vitro and in vivo experiments, which is surprising but not irrelevant. However, the order of the different parts is sometimes not very persistent like the parts on tissue-engineered vascular grafts and biosafety between the therapeutic efficacy tests on rats and those on pigs. Furthermore, the experiments of RNA sequencing have been added in the biosafety part whereas these tests are focused on inflammation and don't seem to be very connected to this part. Finally, some figures should be in the article instead of the supplementary data, for example the figure S8 that compares the authors' treatment with tPA or the figure S11 on blood compatibility that is the main element of the part on biosafety.

- Also, the organization of the Materials & Methods does not follow always the one of the articles (example: Characterization before Synthesis whereas it's the opposite in the article).

- Some acronyms like RB, DPBF or PFH are not explained in the article

- Line 140: Partial analysis. Explain more in detail why DLS and ELS measurements confirm the “stepwise construction”. Nanoparticles are less positive when CREKA is added, is it because CREKA is negatively charged? Why are there Zeta changes when RB and PFH are added considering that, in Scheme 1, they seem to be incorporated inside the particles and not on their surface?

- Line 148-151: The relationship between IO_2 generation and DPBF & SOSG evolutions are not explained, neither in Materials & Methods nor in the article.

Reviewer #4 (Remarks to the Author):

This article integrates combination therapy of SDT and gas-blasting for real-time feedback of ultrasound imaging to achieve deep tissue penetration depth of thrombosis treatment and validates the therapeutic effect of combined therapy in vascular recanalization.

Major comments

1. Using CREKA to target thrombus and ultrasound to induce PFH thrombolysis have been reported (ACS

Nano. 2019 Mar 26;13(3):3387-3403). Here in this manuscript, RB and PFH are combined, and CREKA-RB is used as a control. Why not use CREKA-PFH as a control?

2. RB, as a photosensitizer, is also a commonly used method in vascular injury thrombus model. Why should it be used for thrombolysis, will it induce apoptosis and local inflammatory reaction, and long-term detection of blood vessels after thrombolysis? Although the author has sequenced the common transcriptome of vascular tissue in the US+hmSi-CREKA-RB-PFH group 1 and 7 days after thrombolysis, it cannot be explained whether the use of RB has a significant impact on the safety of blood vessels.

3. RB is not only a photosensitizer, but also a fluorescent dye. If RB reaction is incomplete, or RB in the blood continues to reach the thrombolytic site after ultrasonic treatment, will it affect the late staining and cause false positive results?

4. How to control the concentration of injected nanoparticles and the amount of nanoparticles that can reach the thrombus site and play an effective role? What is the retention and clearance time of nanoparticles in other organs?

5. In the in vitro cytotoxicity assay, it is impossible to predict the toxicity of nanoparticles to local cells only with co-cultured nano-particles and HUVEC without the application of ultrasound.

6. In Figure 4E, why are the particles produced after thrombolysis in US+hmSi-CREKA-RB-PFH group larger than those in the other three groups, and the size is mostly greater than 1000 nm? However, Figure 4G and 4H show that the particle size after thrombolysis is less than 1000 nm.

7. In general, if the animal has no other special conditions (such as continuous inflammatory reaction), the thrombus induced by FeCl₃ will be gradually absorbed. Therefore, the thrombus still completely occluded after 30 days shown by the author in Figure S9 is puzzling. The thrombus that is not absorbed after 30 days will become more compact. Does the author detect the amount of nanoparticles entering the thrombus at this time?

8. Figure 8, n=1 ?

9. It is suggested that the author should try to unify the results of animal experiments, such as blood vessel recanalization rate, serum D-dimer, FDP level, and vascular pathological sections.

Minor

1. Dipeptide antibody information is missing.

2. In Figure 2E, high magnification figures do not match the low magnification figures.

3. The same problem in Figure S5A, day 3, and S5B, day 4.

Response to reviewer #1:

Dear Reviewers,

We appreciate your clear and detailed feedback and hope that the explanation can fully address all of your concerns. In the remainder of this letter, we discuss each of your comments individually along with our corresponding responses.

To facilitate this discussion, we first retype your comments and then present our responses and the corresponding revision to the comments. The relevant literature in the Revision has been annotated in the revised manuscript.

Comment 1: Please consider, given the role that NETs play in DVT, to evaluate your agents on DVT in mice or rat model -- perhaps a flow restricted IVC model

Response: Thank you for the above suggestion. We think your suggestion is reasonable and the shear stress needs to be taken into account. Therefore, we further confirm the therapeutic efficacy on the rat IVC model. And we have added this result as Figure S17 and depicted it in the revised manuscript.

Revision: It is considered that shear stress may favor the thrombolysis in arterial occlusion model, we further verified the therapeutic efficacy in the inferior vena cava (IVC) model. The immunofluorescence staining revealed that NETs also existed in FeCl₃-induced IVC thrombus (Figure S17A). After three times non-invasive treatment, Doppler ultrasound imaging showed the reperfusion of blood flow (Figure S17B). Histological staining showed that the patency rate increased from 40.86% to 74.89% (Figure S17C and D), indicating that the combined therapy can achieve satisfactory thrombolysis efficiency despite the exclusion of shear stress.

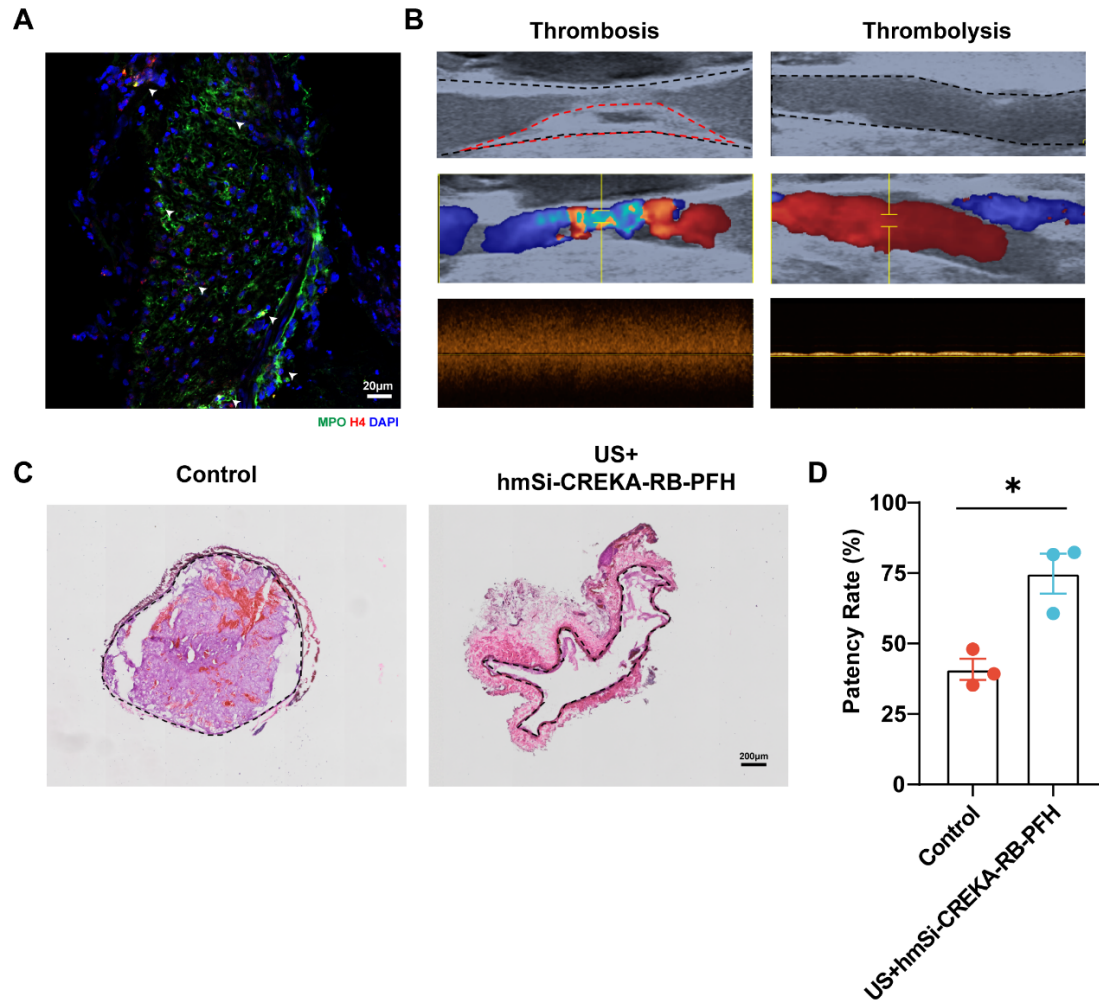


Figure S17. Therapeutic effect on IVC thrombus. A) Immunofluorescence of NETs in IVC thrombus. B) Doppler ultrasonography of IVC vessels before and after treatment. C) H&E staining and D) the patency rate of IVC vessels. mean $\pm$ SEM, n=3, Independent samples t test, *P<0.05, **P<0.01, ***P<0.001.

Comment 2: Can you elaborate on the age of the human post stroke thrombi? How were they treated in the humans; eg, heparin, lysis, antiplatelet therapies prior to harvest?

Response: Thank you for your question. It's difficult to elaborate the age of human thrombi because we usually don't know the time of thrombus onset. Instead, the clinician indirectly defines the age of thrombi through the reperfusion time of thrombectomy and histological staining. We have previously reported that the composition of thrombi changes in a time-dependent manner¹. Stroke patients

received intravenous thrombolysis (such as recombinant tissue plasminogen activator) within 4.5 hours from symptom onset and endovascular thrombectomy within 24 hours from symptom onset will be considered if the thrombi are tPA-resistant or out of therapeutic time window. Heparin (anticoagulant) and antiplatelet therapies are used for stroke prevention. We have depicted the preoperative treatment in the revised manuscript to better define the tPA-resistant thrombi.

Revision: Thrombi were obtained from AIS patients who have failed intravenous thrombolysis within the therapeutic time window (4.5h) and underwent endovascular thrombectomy within 24 hours from symptom onset.

Comment 3: In the pig model, can you determine the function of the vessels post targeted nanoparticles as compared with control? For example, do they have intact endothelium, and produce nitric oxide, prostaglandins, etc? This would be a bit more directed than the bulk rna sequencing.

Response: Thanks for your suggestion. Intact endothelium, production of nitric oxide and prostaglandins are important indicators of vascular function. In rat occlusion model, blood vessels at 0, 7 and 28 days after thrombolysis were harvested for the immunofluorescence staining of endothelial cells, endothelial nitric oxide synthase and detection of prostaglandins, we have supplemented the results in Figure 7 in the revised manuscript.

Revision:

Experimental part: The treated vessels and the contralateral normal blood vessel were harvested at Day 0, Day 7 and Day 28 after recanalization. A portion of the blood vessels were fixed with 4% paraformaldehyde and cut into frozen sections for immunofluorescence staining of CD31(1:100, abcam, ab281583) and eNOS (1:50, abcam, ab300071) to testify the morphology of endothelial cells and its ability to generate nitric oxide. The recanalized-vessels and normal-vessels of same length were soaked in DMEM culture medium in CO₂ incubator for 24h. The supernatant was obtained through centrifugation (1000g, 20min, 4°C), which can be used for the detection of prostaglandins using an ELISA kit (BBI, D751014-0048).

Result analysis: Given the importance of endothelial cells (ECs) in maintaining the long-term patency of the vessels, the morphology of ECs and its biological functions were tested. Almost no ECs were observed at the completion of treatment due to the damage brought by thrombosis. ECs with poor continuity and large gaps were observed after 7 days of reperfusion. After 28 days, there was a distinct layer of ECs with tight connections (Figure 7F). The number of endothelial cells per unit area increased with time (Figure 7H). As an important enzyme in cardiovascular system, endothelial nitric oxide synthase (eNOS) can catalyze the generation of nitric oxide (NO) to regulate vasomotion and the display of eNOS showed a consistent reconstruction trend with the ECs (Figure 7G and 7I). In addition to endothelial cells, other types of cells, especially invasive inflammatory cells, can produce prostaglandins E₂ (PGE₂), which is conducive to the regeneration of various organ systems after injury. At the beginning, the concentration of PGE₂ was about 1.8 times higher compared with normal blood vessels, which may be attributed to the upregulation of COX-2 expression during inflammation stage. The relative concentration of PGE₂ decreased over time, which was consistent with the subside of inflammation indicated by Bulk RNA sequencing. After 28 days, the PGE₂ production was close to normal blood vessels (Figure 7J).

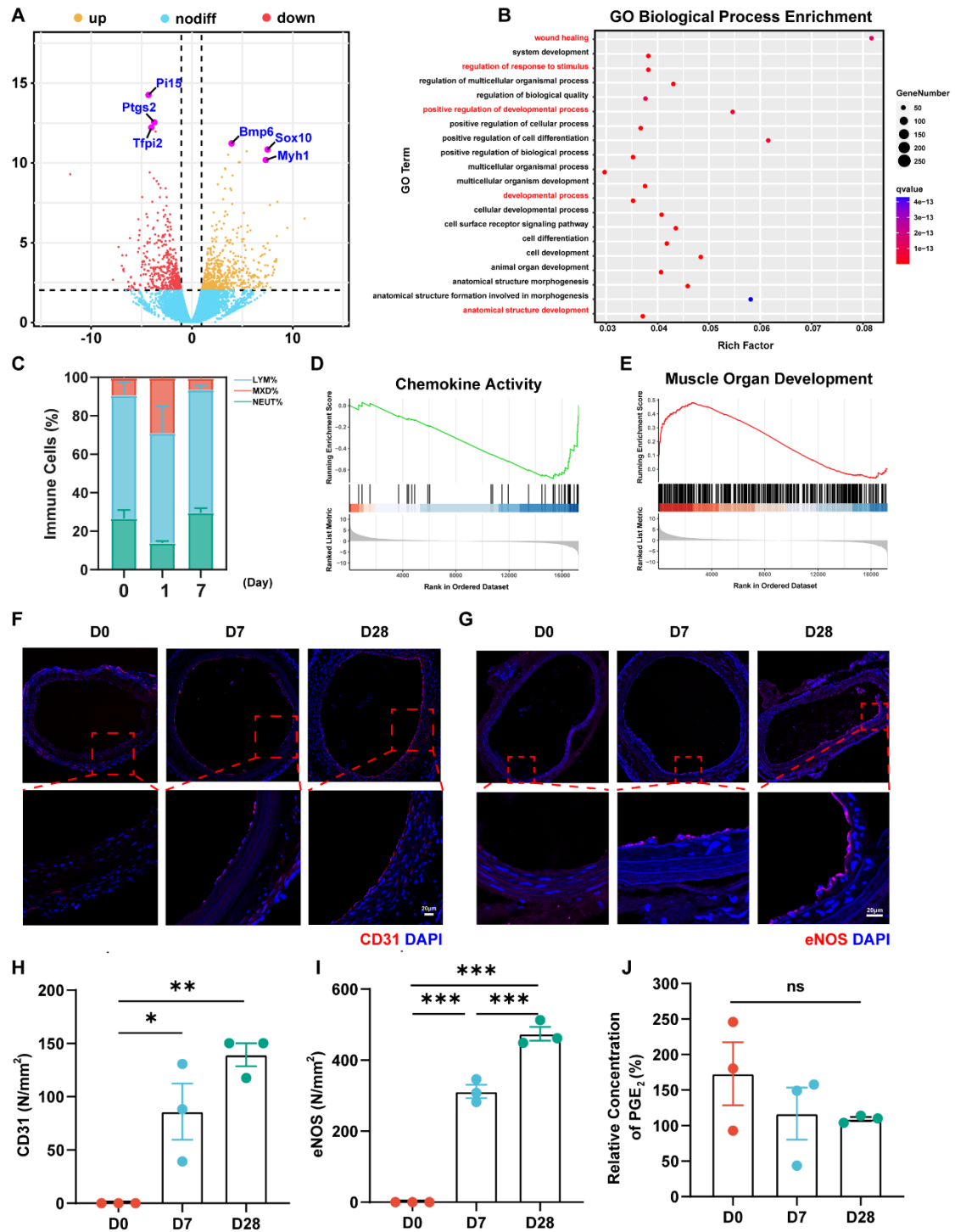


Figure 7. Reconstruction of the recanalized vessels treated with US responsive theranostic platform. A) Volcano plots showing the identified upregulated and downregulated genes a day and a week after the combination therapy ($|\text{fold-change}| \geq 1.0$ with q value < 0.05). $n=3$. B) GO biological process analysis of genes significantly changed between the two groups ($|\text{fold-change}| \geq 1.0$ with P value < 0.05). C) Changes of immune cells after combination therapy. GSEA analysis of D)

chemokine activity and E) muscle organ development. F) Immunofluorescence staining of CD31 and G) eNOS. The number of H) CD31 and I) eNOS. n=3. J) The relative concentration of PGE₂. n=3. Mean $\pm$ SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001.

Comment 4: For the experiments evaluating the breakdown on NETs, you should compare with a control such as DNase.

Response: Thanks for your valuable comment. We have added DNase I as control group both in vitro and in vivo experiments. We have supplemented the results as Figure S8 in the revised manuscript.

Revision: DNase I can disrupt the DNA skeleton and set as a positive control group. The combination therapy group can achieve comparable damage to NETs in both in vitro (Figure S8A and S8B) and in vivo experiments (Figure S8C and S8D) compared with DNase I group. However, DNase I can only increase the reperfusion rate of rat occlusion model to 35.8%(Figure S8E and S8F), indicating that thrombus, as a complex substance, could not be reperfusion only by destroying NETs.

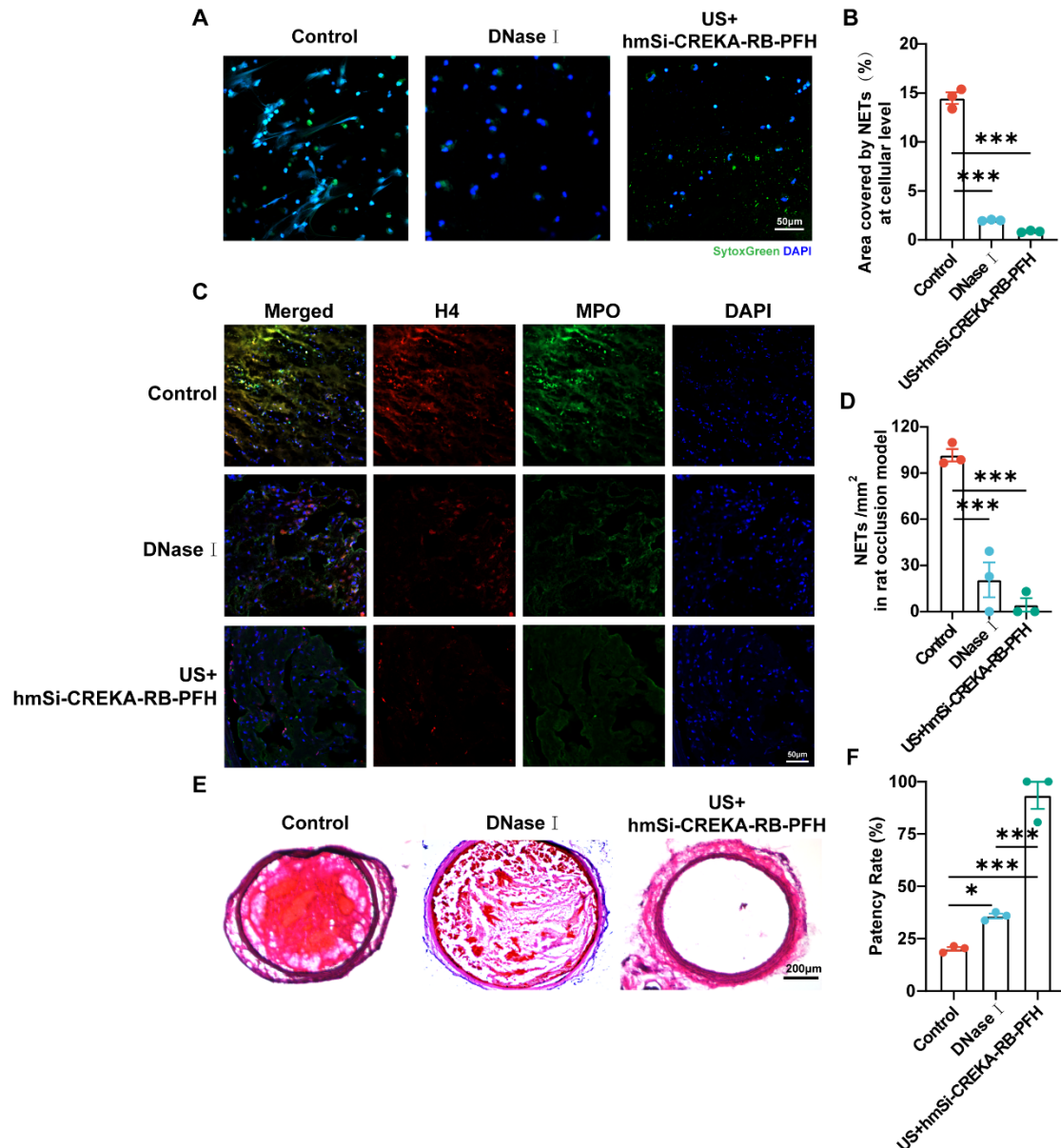


Figure S8. Therapeutic effect of DNase I . A) Immunofluorescence staining of NETs induced in vitro and B) the area covered by NETs after different treatments, mean $\pm$ SEM, n=3. C) Immunofluorescence staining of NETs in rat occlusion model and D) Quantification of NETs/mm² after different treatments, mean $\pm$ SEM, n=3. E) H&E staining and F) its corresponding patency rate, mean $\pm$ SEM, n=3. one-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

Comment 5: It would be of interest to define your agent in the setting of heparin use and antiplatelet use, as this is directly translatable to humans. In other words, within these models, what do the standard agents procedure in terms of patency?

Response: Thank you for the above suggestion. It is true that heparin and antiplatelet are used clinically, however, heparin (anticoagulant) and antiplatelet drugs are mainly used for the prevention of thrombus^{2,3}. tPA is commonly used for the treatment of the advanced thrombus in clinic. In this paper, we had set the tPA treatment group as positive control, the vascular revascularization rate was shown in Figure S14. Given the importance of this comparison, we have strengthened the analysis in the revised manuscript.

Revision: tPA is commonly used for the thrombus treatment in clinic. And we set the tPA drug group as the control and compared it with combined thrombolysis. Statistics revealed that the patency rate gradually increased from the initial 15% to 82% in the combination therapy group (Figure 5D). However, the clinical thrombolytic medications tPA, cannot have the same therapeutic impact, and the vascular patency increased by only 10% (Figure S14). This means in the case of poor clinical drug thrombolysis, SDT combined with mechanical blasting could be an ideal candidate, showing high efficiency of vascular revascularization rate.

Comment 6: From a practical standpoint, what is the depth limit of the ultrasound energy to penetrate to the vessel and activate the nanoparticle agent?

Response: Thanks for your question and we think this is a question worth exploring. As reported, the ultrasound could reach the depth of 10 cm^{4,5}. To further explore the depth limit of ultrasound to activate the sonosensitizers, we tested the ¹O₂ generation ability of the hmSi-CREKA-RB-PFH with different pork thickness under ultrasound irradiation. The results are as follows: The ¹O₂ production decreased with the increase of tissue thickness. The depth of carotid artery tissue in rats was about 1cm, the carotid artery, brachial artery and femoral artery depth in pig were 3 ~ 4 cm, respectively, which was still within the range of effective activation of acoustic sensitizers, providing the feasibility and effectiveness of SDT in vivo.

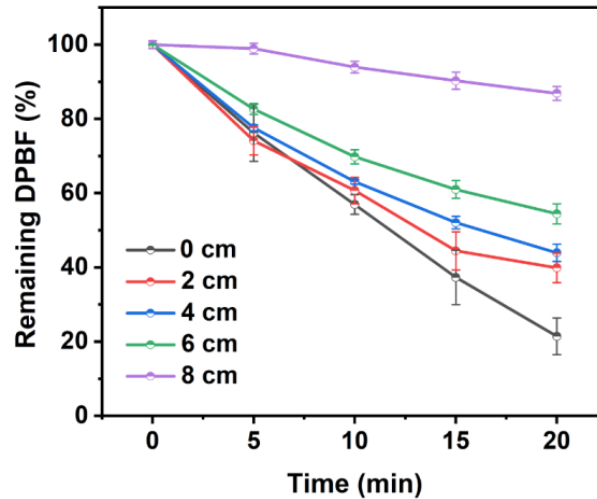


Figure: Relationship between degradation rate of DPBF and time with different pork thickness.

Comment 7: Conceptually, it seems the thrombus is mainly fibrin with platelets, rbc, and less so with NETS -- thus, do you have an agent that mainly 'attacks' the fibrin crosslinking, and not NET digestion?

Response: Thanks for your comment.

(1) We agreed that the proportion of the NETs is less than fibrin, however, it was reported that NETs formation is closely related to the progression of thrombus⁶. NETs is one of the main barrier to prevent thrombolytic drugs from penetrating thrombus⁷. In addition, under the influence of XIIIa, glutamic acid reacted with lysine to form covalent bond (isopeptide bond), transforming unstable fibrin polymers into insoluble and stable fibrin. Therefore, we assumed that the destruction of NETs and isopeptide bonds can further promote the destruction of the fibrin skeleton.

(2) It's worth emphasizing that our treatment can achieve the destruction of NETs, isopeptide bonds and fibrin skeletons through the generation of $^1\text{O}_2$ and gas explosion under ultrasound irradiation. Our experimental results in Figure 2 and Figure 3 confirmed that the combination therapy has a significant effect on the destruction of NETs and isopeptide bonds, respectively.

(3) To verify the comprehensive efficacy, we found the porosity of the clot increased via H&Es staining in Figure 4A. In addition, further fibrin-specific network was

induced to prove that fibrin was indeed damaged (Figure 4F). Changes in peak absorption of fibrin further proved that fibrin was indeed damaged. To analyze the porosity of fibrin more specifically, we further conducted MSB staining to testify the destructive effect on fibrin skeleton. This result was supplemented in Figure S10.

In conclusion, this combination therapy is not only targeted NETs, but also caused significant damage to fibrin.

Revision: Considering that H&E staining can't differentiate fibrin from red blood cells well, we further conducted MSB staining to testify the destructive effect on fibrin skeleton. The decline of fibrin is consistent with HE staining trend and residual fibrin decreased by 16.87% in the combined treatment group compared to the control group (Figure S10B).

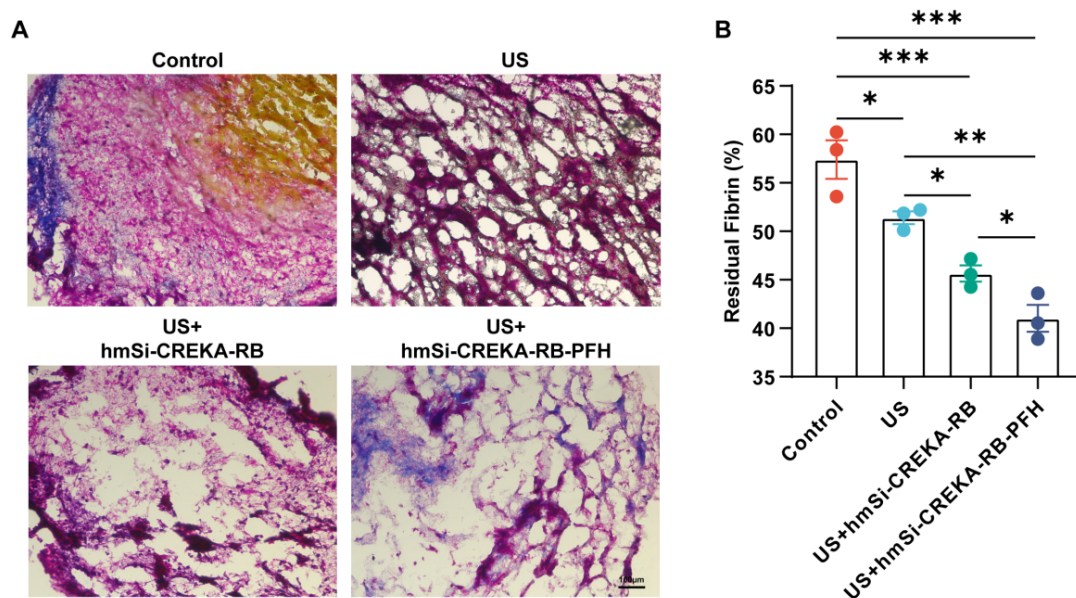


Figure S10. Fibrin porosity of clinical thrombus under different treatment. A) MSB staining and B) the area of residual fibrin, mean $\pm$ SEM, n=3, one-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

Special thanks to you for your good comments. We have tried our best to improve the manuscript and highlighted the revisions in the revised manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Response to reviewer #2

Dear Reviewers,

We would like to thank you for careful reading to our manuscript. Your suggestions and comments have been carefully considered and the manuscript has been revised to address the points you have raised. We hope this revision can make our paper more acceptable.

To facilitate this discussion, we first retype your comments and then present our responses and the corresponding revision to the comments. The relevant literature in the Revision has been annotated in the revised manuscript.

Specific Comments: Title is awkward. Simplify and avoid jargon

Response: Thanks for your comment. We have changed the title into “Ultrasound-responsive theranostic platform for real-time monitoring and efficient thrombolysis in tPA-resistant thrombi” and revised the following specific examples.

Comment 1: Line number: 19-36 Several terms in the abstract are not in general use. Thus, the text is hard to follow. What is meant by “programmed thrombolysis”? Why were TEVGs employed? What is meant by “multifunctional nanoplatform”? Are there more than two functions (diagnosis and therapy)? If not “dual-function submicron particles” may be a more accurate description. Are particles that have a diameter of 200 nanometers considered “nanoparticles”? In my opinion, these are submicron particles. I recommend reserving the term “nanoparticles” for particles on the order of 1 to 10s of nanometers, not 100s of nanometers.

Response: Thank you for your valuable comments and we have revised some of the words and streamlined the abstract to make it easier to understand:

(1) Programmed thrombolysis: We originally meant that our combination therapy can achieve the outside-in thrombolysis by destroying NETs, dipeptides and fibrin

through the generation of $^1\text{O}_2$ and gas explosion. In view of the possible ambiguity, we now replaced “programmed thrombolysis” with “efficient thrombolysis”.

(2) The transplantation failure of small diameter ($\leq 6\text{mm}$) tissue engineered vascular grafts (TEVGs) is often caused by thrombosis after implantation^{8,9}. At present, the lack of effective detection and maintenance method limits its clinical application. Therefore, we investigated the application of this theranostic platform in small diameter TEVGs. Now we have supplemented and improved the description of this part in the revised manuscript.

(3) Multifunctional nanoplatform: We define it as “multifunctional” for the consideration of its function of thrombus-targeting, sonodynamic thrombolysis, mechanical thrombolysis, and ultrasound imaging. Your suggestion classifies the functions of our platform from a larger perspective. We have changed “multifunctional nanoplatform” into “theranostic platform” in the revised manuscript.

(4) Thank you for your advice. We have corrected this statement in the revised manuscript, all the “nanoparticles” were changed into “submicron particles”.

Revision: There is no noninvasive solution for tPA-resistant thrombi while endovascular thrombectomy is the last option. Here, we proposed neutrophil extracellular traps (NETs), ϵ -(γ -glutamyl)lysine isopeptide bonds and fibrin scaffold as keychain in tPA-resistant thrombus, which was confirmed in the thrombi retrieved from stroke patients. A theranostic platform was designed for the combination of sonodynamic and mechanical thrombolysis under the guidance of ultrasonic imaging. Three rounds of 10 min noninvasive treatment led to the breakdown of the keychain, achieving great recanalization rate of 90% in rat occlusion model while tPA only made 10% improvement. RNA sequencing results and endothelium functional tests jointly indicated that the vascular homeostasis can be effectively reconstructed. The noninvasive theranostic capability presented in pig’s lengthy thrombi ($>8\text{mm}$) and thrombosis-susceptible tissue engineered vascular grafts (TEVGs) indicated its clinical transformation prospects. Taken together, this non-invasive theranostic platform provides a new strategy for tPA-resistant thrombi.

Comment 2: Line number: 52 What is mean by “valid time”? The authors should cite a broader clinical literature regarding efficacy of thrombolysis approaches, such as sonothrombolysis.(See for example, <https://doi.org/10.1161/STROKEAHA.120.030960> Stroke. 2021;52:3786–3795, and others)

Response: Thank you for your comment. Valid time refers to the therapeutic time window corresponding to the clinical thrombolytic drugs, that is, the drugs must be applied within a certain time limit to produce the thrombolytic effect. Now we have changed the phrase into “therapeutic time window” in the revised manuscript.

Revision: Sonothrombolysis (tPA coupled with low-intensity ultrasound) was reported to increase the odds of recanalization and favorable functional outcomes, however, these drug-based treatments are still limited by a strict therapeutic time window (generally within 4.5h).

Comment 3: Line number: 569 Reference 11 is incomplete

Response: Thank you for your comment. Now we have replaced a new literature as reference 13 (DOI: 10.1161/STROKEAHA.116.015321) in the revised manuscript.

Comment 4: Line number: 67 Sonodynamic therapy to enhance rt-PA thrombolysis is termed “sonothrombolysis” in the literature. Please cite more relevant work, such as <http://dx.doi.org/10.1016/j.ultrasmedbio.2014.08.018> .

Response: Thank you very much for the comment. The two confusing concepts need to be explained here: the “sonothrombolysis” you mentioned here refers to that ultrasound can enhance the thrombolytic effect of drugs through acoustic cavitation¹⁰. However, sonodynamic therapy (SDT) utilizes ultrasound as the excitation source to trigger sonosensitizers for generating lethal reactive oxygen species (ROS) to kill tumor cells¹¹, in this article, it was used as an innovative method to dissolve the thrombus. The SDT-mediated thrombolysis doesn’t need to be combined with clinical thrombolytic drugs, which are restricted with therapeutic time window and may cause hemorrhage.

Now we have further emphasized the concept of sonodynamic therapy and quoted the corresponding literature (reference 19) in the revised manuscript.

Revision: Ultrasound (US) with high tissue-penetration depth can compensate for the aforementioned drawbacks, in addition to sonothrombolysis, sonodynamic therapy (SDT) refers to the generation of lethal reactive oxygen species (ROS) through sonosensitizers under ultrasound irradiation, which can achieve thrombolysis without the combination of thrombolytic drugs.

Comment 5: Line number:72 The term “Drug-resistant thrombi” needs to be clearly defined in the introduction.

Response: Drug-resistant thrombi refer to certain kind of thrombi that can't be dissolved by thrombolytic drugs (mainly tPA)¹². Considering the inaccuracy of this phrase, we have changed it into “tPA-resistant thrombi” and depicted the preoperative treatment in the revised manuscript to better define the tPA-resistant thrombi.

Revision: To date, as the most commonly used thrombolytic treatment, tissue plasminogen activator (tPA) can lead to fibrinolysis. However, clinical issues still exist owing to the short half-life, poor utilization rate, and high bleeding risk of tPA, which can only dissolve approximately 25% of occluded large arteries and 44% of the retrieved thrombi were still fibrin-dominant. Fewer than 30% of patients have a satisfactory prognosis due to these tPA-resistant thrombi.

Comment 6: Line number:72ff The seminal work by Liebeskind et al. needs to be cited (<https://doi.org/10.1161/STROKEAHA.110.605576> Stroke.2011;42:1237–1243) DOI: 10.1161/STROKEAHA.110.605576

Response: Thank you for your valuable suggestion. We have cited this literature as reference 8 in the revised manuscript.

Revision: tissue plasminogen activator (tPA) can lead to fibrinolysis. However, clinical issues still exist owing to the short half-life, poor utilization rate, and high bleeding risk of tPA, which can only dissolve approximately 25% of occluded large arteries and 44% of the retrieved thrombi were still fibrin-dominant.

Comment 7: Line number:45 “scouring of blood flow” is not an appropriate description in English

Response: Thanks for your comments. Now we have replaced “scouring of blood flow” with “shear stress” in the revised manuscript.

Revision: According to the progression of thrombosis, glutamate and lysine among fibrin monomers will be covalently crosslinked under coagulation factor XIII to produce isopeptide bonds, enabling the thrombus to withstand the impact of shear stress.

Comment 8: Line number:90 “gas-blasting” no clear. Are you referring to acoustic activation of nanodroplets into microbubbles? Or acoustic cavitation? Clarify.

Response: We thank the reviewer for the valuable comments. The gas-blasting here refers to the phase change material PFH transformation from liquid to gas under ultrasound. We have added notes to the revised manuscript.

Revision: Therefore, a theranostic platform that integrates the combination therapy of SDT and gas-blasting (transformation of perfluorohexanes from liquid to gas) with real-time imaging under ultrasound irradiation had been designed.

Comment 9: Line number:94 Thrombolysis with ultrasound exposure alone has been shown to be ineffective, unless the peak rarefactional pressure amplitude is quite high (> 17 MPa) such as pulses used in histotripsy (See for example, Kenneth B Bader et al 2016 Phys. Med. Biol. 61 5253 DOI 10.1088/0031-9155/61/14/5253, and Henley et al, IEEE TRANSACTIONS ON ULTRASONICS, FERROELECTRICS, AND FREQUENCY CONTROL, VOL. 68, NO. 9, SEPTEMBER 2021). It is unclear what is meant by thrombi “soften under the mechanical action of US.” No such evidence of a decrease in Young's modulus of blood clots exposed to US has been demonstrated in the literature.

Response: Thank you for your question. We added a new experiment to illustrate the effect of ultrasound on thrombus. As shown in the Figure, the clinical thrombi were

treated by ultrasound for 30min, and the solution gradually turned red. The structural changes were further observed by scanning electron microscope. The results showed that the surface of thrombus in the ultrasonic group became rough and there were a lot of collapse. The difference of fibrin structure was analyzed by MSB staining, and it was found that after ultrasound, red blood cells decreased and fibrin pores increased. We hypothesized that this might be because the mechanical vibration of ultrasound had some effect on the red blood cells in the thrombus, which caused the destruction or drop of the red blood cells and enlarged the pores of the thrombus. Considering the inaccuracy of the word “soften”, we changed it to “porosity increased” in the revised manuscript.

Revision: The thrombi porosity increased under the mechanical action of US.

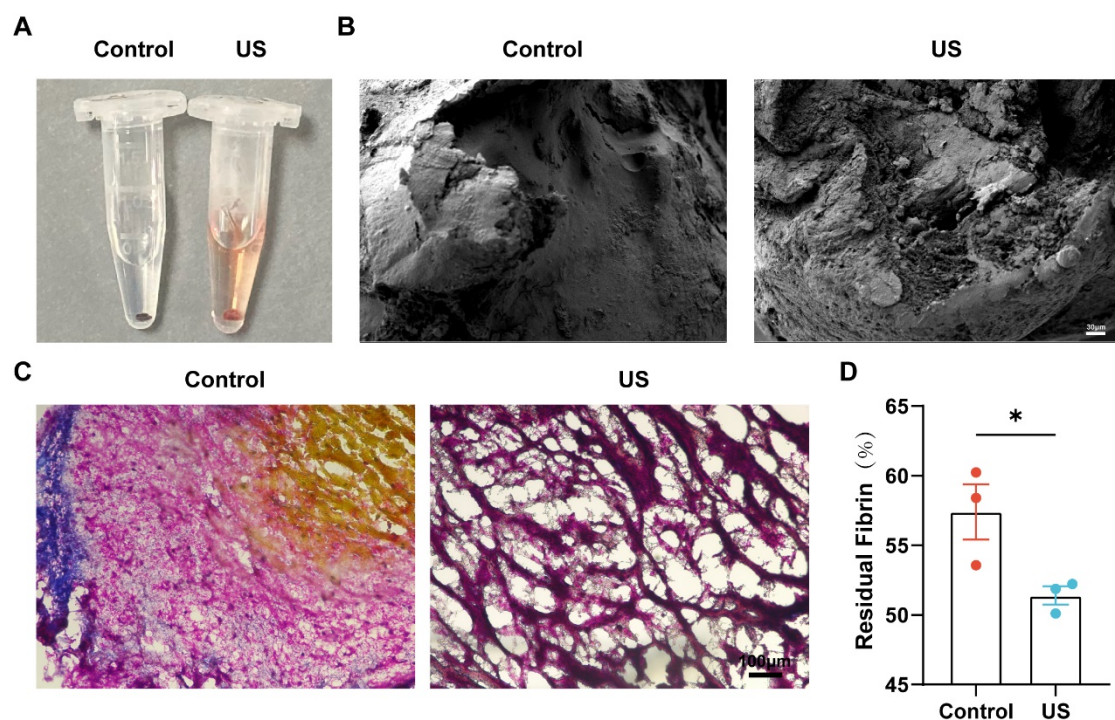


Figure: A) Digital photos, B) SEM and C) MSB staining of clinical thrombi before and after ultrasound. D) Statistical analysis of fibrin porosity. Mean $\pm$ SEM. n=3. Independent samples T-test. *P<0.05, **P<0.01, ***P<0.001.

Comment 10: Line number:94ff Define all acronyms on first usage. What is “RB”? Retinoblastoma protein? Why did you choose this particular therapeutic approach? Overall, the description of your approach is disorganized and the text is hard to follow.

Response: We are sorry to define some acronyms in the supplementary information, which has caused some confusion. RB refers to Rose Bengal, a kind of sonosensitizer. We have supplemented it in its entirety in the revised manuscript.

The reason why we choose this particular therapeutic approach is that there are many tPA-resistant thrombi in clinical practice, so new thrombolysis strategies are urgently needed. While SDT-mediated thrombolysis has shown certain thrombolytic ability in previous studies, we want to further testify whether it is applicable to tPA-resistant thrombi, and if so, what is the underneath possible mechanism. In the revised introduction, we emphasize this treatment method and further explain the reasons for choosing SDT.

Revision: Ultrasound (US) with high tissue-penetration depth can compensate for the aforementioned drawbacks, in addition to sonothrombolysis, sonodynamic therapy (SDT) refers to the generation of lethal reactive oxygen species (ROS) through sonosensitizers under ultrasound irradiation, which can achieve thrombolysis without the combination of thrombolytic drugs.

The sensitizer Rose Bengal (RB) was activated to produce abundant singlet oxygen, which destroyed the NETS and isopeptide bonds.

Comment 11: Line number:94 “Sensibilizer” is not a word in English. Please spell check your manuscript carefully using MS Word.

Response: Thanks for your comment and we have corrected this mistake in the revised manuscript.

Revision: The sensitizer Rose Bengal (RB) was activated to produce abundant singlet oxygen, which destroyed the NETs and isopeptide bonds.

Comment 12: Line number:96 Define PFH. Are these perfluorocarbon nanodroplets? Provide composition details. In general, not enough detail in the synthesis of the submicron particles was provided.

Response: Thank you very much for your comment. We define some acronyms in the supplementary information, which has caused some confusion. PFH refers to

perfluorohexanes. We have defined PFH on first usage in the revised manuscript. Moreover, more synthesis details in the experimental section could be found in supplementary information.

Revision: Theranostics of thrombolysis was aided by mechanical-blasting of phase transition molecules perfluorohexanes (PFH)

Experimental section: Finally, the loading of PFH was referred to the ice bath ultrasonic method reported in the literature. hmSi-CREKA-RB freeze-dried powder was obtained by removing the solvent. 200 mg powder was divided into 600 μ L PFH and placed in a 5 mL sealed bottle. It was subjected to ultrasonic treatment (40KHz, 70 W/cm²) in ice water for 3 min, followed by 3ml of water solution to seal the PFH and store it in the refrigerator at 4°C.

Comment 13: Line number:97 The use of perfluorocarbon nanodroplets to enhance rt-PA thrombolysis is not novel. Previous literature should be cited.

Response: Thank you for your advice. We have cited the related literature (reference 40) in the proper place of the revised manuscript.

Comment 14: Line number:103 What is meant by “transplanted blood vessels”? Clarify.

Response: Thanks for your comment. “transplanted blood vessels” means “tissue engineered vascular grafts”. We have replaced it with detailed description in the revised manuscript.

Revision: The detailed technical procedure of TEVGs preparation and transplantation had been published previously. Briefly, carotid arteries were harvested from SD rats (250-300g), decellularized rat carotid arteries were obtained after incubating with 0.05% trypsin at 37°C for 30min, and stored in a 4°C refrigerator for later use. After vascular transplantation, the thrombosis was determined by Doppler ultrasonography, and nanoparticles (hmSi-CREKA-RB-PFH, 0.2 mL, 5 mg/mL) were injected through tail vein, 10 min ultrasound irradiation (1.0 MHz, 1.5 W/cm²) was performed 1 hour later and repeated for 3 days.

Comment 15: Line number:Fig. 1b: In my opinion, particles that are 200 nm in diameter should be called “submicron” particles, not “nanoparticles”.

Response: Thank you for your valuable advice. We have corrected this statement in the revised manuscript. All the “nanoparticles” were changed to “submicron particles”.

Comment 16: Line number:117-22 There are many undefined acronyms, making the components impossible to discern.

Response: Thank you very much for the comment. We are sorry to define acronyms in the supplementary information, which has caused some confusion. We have defined all acronyms on first usage in the revised manuscript.

Comment 17: Line number:148 Too many undefined acronyms. What is DPBF? What is SOSG?

Response: Thanks for your comment. DPBF refers to 1,3-diphenylisobenzofuran and SOSG refers to Singlet Oxygen Sensor Green. They are both detection probes for the generation of singlet oxygen. We have defined DPBF and SOSG on first usage in the revised manuscript.

Revision: To further explore the sonodynamic characteristic of hmSi-CREKA-RB-PFH, the $^1\text{O}_2$ generation was detected with 1,3-diphenylisobenzofuran (DPBF) (Figure 1F and S2A) and Singlet Oxygen Sensor Green (SOSG) (Figures 1G and S2B).

Comment 18: Line number:155 The term “echo contrast agent” is used in English, not “imaging enhancer agent”.

Response: Thank you very much for the comment. Now we have replaced “imaging enhancer agent” with “echo contrast agent” in the revised manuscript.

Revision: As an echo contrast agent, we further explored vitro imaging under B-mode with different time.

Comment 19: Line number:177-8 The nanoparticles alone were not tested as a control. This is an important limitation of the study.

Response: We thank the reviewer for the comment. Firstly, the submicron particles were supported with RB and PFH by hollow mesoporous silica, in vitro studies, we have demonstrated that submicron particles alone cannot produce $^1\text{O}_2$ (Figure 1F and 1G) or induce phase transition in PFH without ultrasound (Figure S3). Secondly, the biosafety of submicron particles, both at the cellular and animal levels, has also been studied and the results showed no significant toxicity (Figure S4). Therefore, we did not set up an injection submicron particle group in vivo.

Comment 20: Line number:194 Why was the degree of clot retraction no assessed?

Response: Thanks for your suggestion. According to the reference¹³, neutrophils producing intravascular and intraparenchymal neutrophil extracellular traps (NETs) peak at 3–5 days. By immunofluorescence (Figure S12), a large number of NETs were observed on the fourth day after thrombus modeling. tPA thrombolysis was performed on the fourth day, and it was found that the blood vessels could not be reperfused (Figure S14), indicating that the thrombus was tPA insoluble. We think this is a more directly way to judge thrombus tPA-resistant and we use that as a starting point for treatment.

The clot retraction refers to the process whereby activated platelets transduce contractile forces onto the fibrin network of a thrombus, which is considered important for promoting clot stability and maintaining blood vessel patency¹⁴. There is no direct evidence of an association between clot retraction and tPA-resistance according our retrieve literature, so we did not examine this indicator further.

Comment 21: Line number:230 What is meant by “the density and thickness of fibrin skeleton was significantly improved”? How were was fibrin density and fibrin thickness quantified?

Response: Thanks for your comment. According the literatures^{15, 16}, FXIII can, in

principle, alter the structure of the fibrin network, by modulating the density of cross-linking between fibers. Specifically, as FXIII-mediated cross-linking between the α -chains and γ -chains becomes more extensive and complex, the fibers mature as tightly coupled fibril bundles with a smaller diameter and higher protein density, and therefore smaller intrafibrillar pores. In this paper, it is believed that the diameter is thinner because the fibril is more tightly coupled under the mediation of FXIII. We describe that the diameter is larger because more fibers are aggregated compared to a single fiber that is not aggregated. However, based on your comments, we realized that this description was vague and revised it in the manuscript.

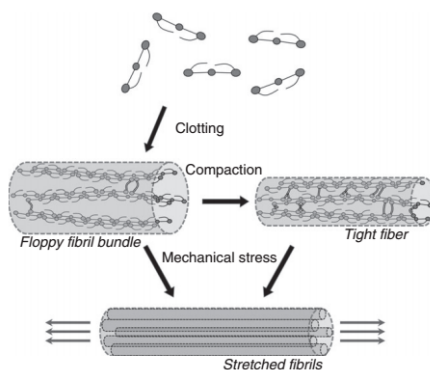


Figure: Structural model of the coupling between protofibrils within fibrin fibers¹⁵

The structural analysis in the presence and absence of FXIII could be quantified by scanning electron microscopy (SEM)¹⁷. In our manuscript, we only described the structural changes before and after the addition of FXIII when fibrin was induced in vitro. After US, SDT and combination therapy, these two factors were not taken as investigation indicators, so there was no further quantitative data was obtained.

Revision: FXIII plays an important role in regulating the structure of fibrin, as shown in Figure 3C, compared with the FXIII-deficient group, the diameter of fibrin became larger, which may be attributed to transverse aggregation between fibrin.

Comment 22: Line number:257 MSB, not H&E is the stain of choice to image fibrin. It is difficult to differentiate fibrin from hemoglobin on H&E (both appear red).

Response: Thank you for the above suggestion. H&E staining is difficult to

differentiate fibrin from hemoglobin. Considering the fibrin-specific ability of MSB staining, we further added the MSB staining on clinical thrombi.

Revision: Considering that H&E staining cannot well distinguish red blood cells and fibrin, we further conducted MSB staining to testify the destructive effect on fibrin skeleton (Figure S10). The experimental results showed that in vitro ultrasound alone can effectively destroy red blood cells in thrombi, and the decline of fibrin is consistent with H&E staining results.

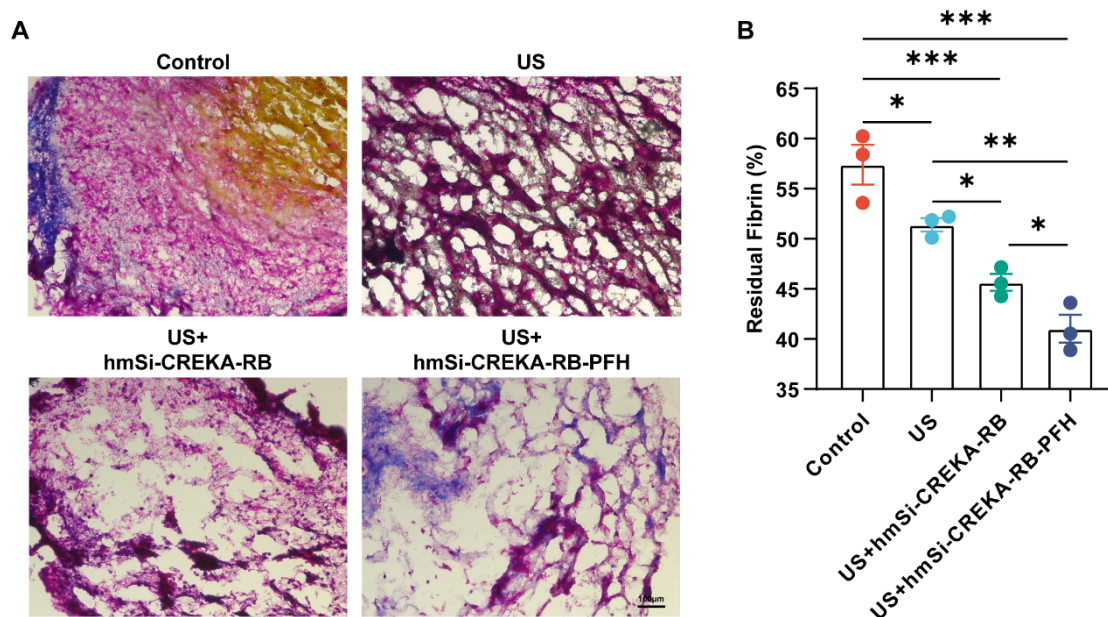


Figure S10. Fibrin porosity of clinical thrombus under different treatment. A) MSB staining and B) the area of residual fibrin, mean $\pm$ SEM, n=3, one-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

Comment 23: Line number:276ff The motivation for flushing the carotid arteries post treatments is not well justified.

Response: Thanks for your comment. It has been previously reported that the formation of isopeptide bonds contributes to the improvement of the anti-shear stress properties of thrombosis¹⁸.Through ex vivo and in vivo experiments we found that the combination therapy can effectively break the isopeptide bonds, this experiment was set up to verify the reduced anti-scouring ability of blood clots after breaking the isopeptide bond.

Comment 24: Line number:305ff The acoustic insonation parameters were not provided in detail, other than the 1 MHz center frequency. See Padilla and ter Haar, Ultrasound in Med. & Biol., Vol. 48, No. 7, 3 pp. 1299 1308, 2022, <https://doi.org/10.1016/j.ultrasmedbio.2022.03.001> for guidance on acoustic parameter reporting.

Response: Thanks for your comment. We provided more detailed treatment parameters according to the instrument specification and revised them in the manuscript.

Revision: The ultrasonic frequency was $1\text{MHz}\pm 5\%$, the pulse period was 1000ms, the duty ratio was 50%, and the power density was $1.5\text{W}/\text{cm}^2$.

Comment 25: Line number:339 TEVGs undefined (Tissue-engineered vascular graft?). No details were provided for this tissue construct.

Response: Thank you for your advice. We have provided the detail information in the revised manuscript.

Revision: Tissue engineered vascular grafts (TEVGs) preparation: Briefly, carotid arteries were harvested from SD rats (250-300g), decellularized rat carotid arteries were obtained after incubating with 0.05% trypsin at 37°C for 30min, and stored in a 4°C refrigerator for later use. After vascular transplantation, the thrombosis was determined by Doppler ultrasonography, and nanoparticles (hmSi-CREKA-RB-PFH, 0.2 mL, 5 mg/mL) were injected through tail vein, 10 min ultrasound irradiation (1.0MHz , $1.5\text{W}/\text{cm}^2$) was performed 1 hour later and repeated for 3 days.

Comment 26: Line number:357-63 GO, KEGG, CSEA acronyms undefined

Response: Thanks for your comment. GO refers to Gene Ontology, KEGG refers to Kyoto Encyclopedia of Genes and Genomes, CSEA refers to Gene Set Enrichment Analysis. CSEA are gene-enrichment analysis of significantly changed (upregulated or down regulated) genes, and GO and KEGG are functional-enrichment analysis of significantly changed genes. Now we have defined GO, KEGG and CSEA in the revised manuscript.

Comment 27: Line number:376 Citation needed for “CCK8 method”.

Response: Thank you for your advice. We have cited the related literature in the revised manuscript (reference 31).

Special thanks to you for your good comments. We have tried our best to improve the manuscript and highlighted the revisions in the revised manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Response to reviewer #3:

Dear Reviewers,

We also appreciate your clear and detailed feedback and hope that the explanation has fully addressed all of your concerns. In the remainder of this letter, we discuss each of your comments individually along with our corresponding responses.

To facilitate this discussion, we first retype your comments and then present our responses and the corresponding revision to the comments. The relevant literature in the Revision has been annotated in the revised manuscript.

Major points:

- Introduction:

Comment 1: the authors present the mechanism of action of their treatment, but the experiments presented don't allow to justify it (at least, they should say that this is a hypothetical mechanism). For example, in the different figures, there is almost never any significant difference between the treatment without and with PFH, and this is not interpreted.

Response: Thanks for your comment and we agree with you that there is little

significant difference between the SDT group and the combination therapy group (without and with PFH). We explain it as follows:

(1) With or without PFH, there was no significant difference between the two groups in vitro, but there was significant difference in vivo. It may be because in vitro experiments, the destruction effect of single factors such as NETs and isopeptidyl bonds was investigated, which had shown an excellent destruction rate in the SDT group, so the data did not change much after combining PFH. However, the in vivo experiment investigated a comprehensive thrombolytic effect. The SDT could not achieve complete vascular recalculation, while the combined mechanical blasting of PFH could further improve the recalculation rate, indicating that although PFH had a poor effect on a single component, on the basis of the SDT destruction of NETs and isopeptidyl bonds, which are two key factors that lead to thrombolysis, PFH can destroy fibrin and promote the overall thrombolytic rate in coordination with SDT. In addition, PFH can also improve the resolution of ultrasound imaging.

(2) The effects of PFH are reflected in two aspects: ① phase-transition under ultrasound irradiation promotes thrombolysis; ② phase-transition improves the resolution of ultrasound imaging of thrombus site. This conclusion can be drawn from the analysis of ultrasonic imaging results of thrombus in pig carotid artery (Figure S20). In addition, the improved contrast of ultrasound imaging was also observed in rat thrombi. Considering the importance of PFH, we have supplemented this result as as Figure S16 in the revised manuscript.

In conclusion, PFH plays an important role in improving the in vivo thrombolysis effect and enhancing the contrast of ultrasonic imaging. Therefore, it's necessary to combined use of PFH.

Revision: In addition, we also showed the ultrasonic imaging of rat thrombus, and the results showed that after 10 minutes of ultrasonic treatment after hmSi-CREKA-PFH injection, the contrast of ultrasonic imaging of the thrombus site was enhanced, indicating the role of PFH in promoting ultrasonic imaging.

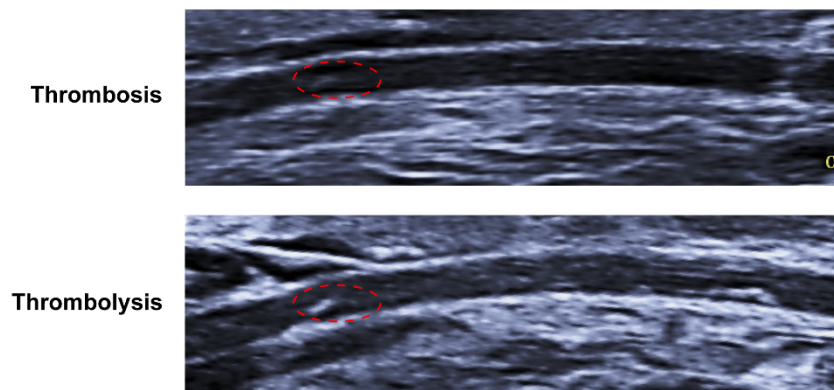


Figure S16: Contrast-enhancement of ultrasound imaging in rat carotid artery.

The ultrasound-mediated physical explosion generated by PFH has been applied in the field of thrombolysis, and previous literature indicated that mechanical action can mainly act on the surface of the thrombi in vitro. Additionally, our experimental results showed that the effect of PFH-blasting alone on NETs and dipeptides is negligible in vivo, suggesting that it needs to be combined with other thrombolytic methods. Sonodynamic therapy has been proven to be able to destroy the fibrin skeleton of thrombi. Experimental results revealed that the generation of singlet oxygen had linear correlation with the destruction of NETs and dipeptides, which is conducive to the dissolution of the fibrin scaffold. Therefore, a combination therapy of SDT and gas-blasting was proposed. However, it was observed in vitro experiments that the addition of PFH didn't enhance the destruction efficacy on single NETs or dipeptides but it can enhance the porosity of thrombi while thrombolysis is a comprehensive process. Similarly, on the basis of the SDT destruction of NETs and isopeptide bonds, two key factors leading to thrombolysis, PFH can promote fibrin degradation while SDT alone can't achieve complete recanalization. Taken together, this kind of programmed thrombolysis can achieve overall destruction of blood clots.

Comment 2: Moreover, the treatment with PFH and without RB has never been tested, which would have been interesting to distinguish the ROS effect and the mechanical action

Response: Thanks for your comment and we really agree with you that the treatment

with PFH and without RB as a control group would be a good way to distinguish the effect of ROS and mechanical blasting.

(1) For the mechanical action of PFH, it has been reported that it can mainly act on the surface of thrombus¹⁹, which could be seen in the reference²⁰ that gas-blasting has a relatively obvious damage to the surface structure of blood clots, while there is no abnormality in the inside of thrombus, indicating it is necessary to be combined with other means to enhance the effect of thrombolysis.

(2) We added US+hmSi-CREKA-PFH without RB as a control group in vivo experiments, immunofluorescence staining results indicated that positive signals of NETs and isopeptide bonds still existed (Figure S15), H&E staining showed that the improvement of vascular patency rate was not obvious, which suggests that mechanical blasting alone is not effective in the treatment of thrombus, so it is not included as a separate group in this manuscript.

This is consistent with literature reports. Since the mechanical blasting effect of PFH mainly occurs on the surface of thrombus. It has no significant destructive effect on isopeptide bonds and NETs, which are two single components, mainly reflected in the synergistic effect with SDT, and the mechanical effect can enhance the overall thrombolytic effect.

Revision: In vivo experiments were conducted to investigate the mechanical blasting thrombolytic effect of PFH (Figure S15). Immunofluorescence results indicated that there were still obvious NETs, isopeptide bonds and fibrin, and the vascular patency rate was less than 40%, indicating that the effect of mechanical blasting alone was not ideal.

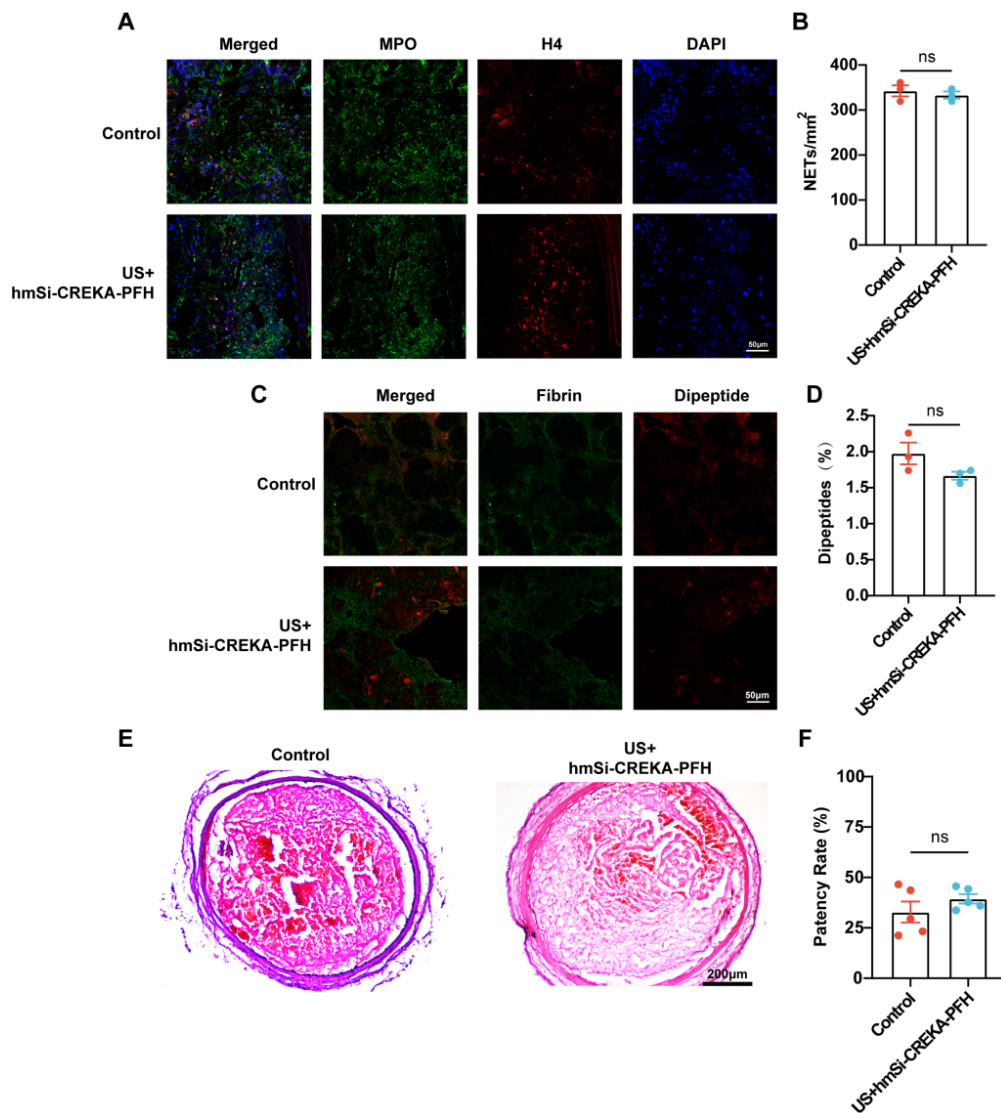


Figure S15. Therapeutic effect of US + hmSi-CREKA-PFH. A) Immunofluorescence staining of NETs in control and US+hmSi-CREKA-PFH groups in rat occlusion model. B) Statistics of NETs per unit area. C) Immunofluorescence staining of Dipeptides bond in control and US+hmSi-CREKA-PFH groups in rat occlusion model. D) Statistics of Dipeptides bond. E) H&E staining of the treated-vessels and F) the corresponding patency rate. mean $\pm$ SEM. n=5, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001.

Comment 3: Furthermore, since barely any exploration of mechanism of action exist, claims about how these objects work can only be hypothetical; this is especially true for lines 93-98.

- Lack of experiment or experiment persistence:

Response: Thank you for your comment. To discuss the mechanism, we mainly explain from the following two points:

Regarding the mechanism of treatment method: Based on literature reports and our experimental data, it can be shown that ① singlet oxygen could be generated by SDT processes. RB is a common sensitizer and two probes, DPBF and SOSG, were used to detect the formation of singlet oxygen under ultrasonic conditions (Figure 2F and 2G). The above results confirmed that RB can indeed produce singlet oxygen under ultrasonic action. ②PFH can undergo phase transformation under ultrasonic stimulation^{21, 22}. And our result in Figure S3 shows that the number and volume of bubbles on the bottle wall change significantly after different ultrasonic time. In summary, we believe that the mechanism of thrombolysis based on SDT generation of singlet oxygen and phase change blasting is clear.

Regarding the mechanism of target action: It is suggested that NETs, isopeptide bonds and fibrin are the key reasons leading to tPA insolubility of thrombus, and disruption of these three factors contributes to thrombus lysis. We added two experiments: ①We tested the relationship between the relative concentration of singlet oxygen and the retention rate of NETs and isopeptide bonds through the elisa experiment, and the results indicated that the percentage of both NETs and isopeptide bonds decreased with the increase of the amount of singlet oxygen produced (Figure S7); ② A new positive control experiment on the destruction of NETs and isopeptide bonds was added : NETs treated with DNase, isopeptide bonds treated with A2LD1 and fibrin treated with tPA. The results showed that the ability of the combination therapy to destroy NETs and isopeptide bonds was close to the effect of commonly used enzymes (Figure S9). These two experiments further demonstrate that SDT has destructive effects on NETs and isopeptide bonds during the production of singlet oxygen.

Revision: In order to further verify the relationship between the generation of singlet oxygen and the destruction of NETs, we further fitted the dose-effect relationship between the two. The experimental results showed that with the extension of ultrasound time, the content of singlet oxygen increased linearly (Figure S7A and S7B)

while the content of NETs decreased linearly (Figure S7C), indicating that singlet oxygen can specifically destroy NETs.

The dose-effect relationship between $^1\text{O}_2$ and the content of dipeptides were similar to that of $^1\text{O}_2$ –NETs (Figure S7D).

Experimental part: Firstly, the UV absorption spectra of DPBF with different concentrations were tested, and the relationship between concentration and absorbance at 417 nm was established. Then hmSi-CREKA-RB with a certain RB absorption value ($A_{564\text{ nm}}=1.77$) was selected and mixed with DPBF to test the absorbance value of DPBF at 417 nm after different ultrasonic time, and the relative amount of singlet oxygen generated was calculated by the absorbance change value of DPBF ($^1\text{O}_2$: DPBF=1:1, only the amount of $^1\text{O}_2$ that reacts with DPBF could be reflected, so it is defined as the relative concentration of singlet oxygen). Finally, NETs and isopeptide bonds were induced in vitro, and a certain concentration of hmSi-CREKA-RB ($A_{564\text{ nm}}=1.77$) was added. After different ultrasonic times, the content of NETs and isopeptide bonds were quantified by ELISA: ① samples of NETs were added into 96-well plates coated with mouse anti-MPO antibody (1:100, Proteintech, 66177-1), followed by incubation with rabbit anti-histone H4 (citrulline 3) (1:200, Millipore, 3517971). ② samples of isopeptide bonds were added into 96-well plates coated with rabbit anti-N epsilon gamma glutamyl Lysine antibody (1:200, abcam, ab424); After labelling with peroxidase (1:1000, ZSGB-Bio, ZB-2301), the absorbance at 405nm was measured by Microplate Reader. The relationship between the relative concentration of singlet oxygen and the content of NETS or isopeptide bonds were performed on Oringin Software.

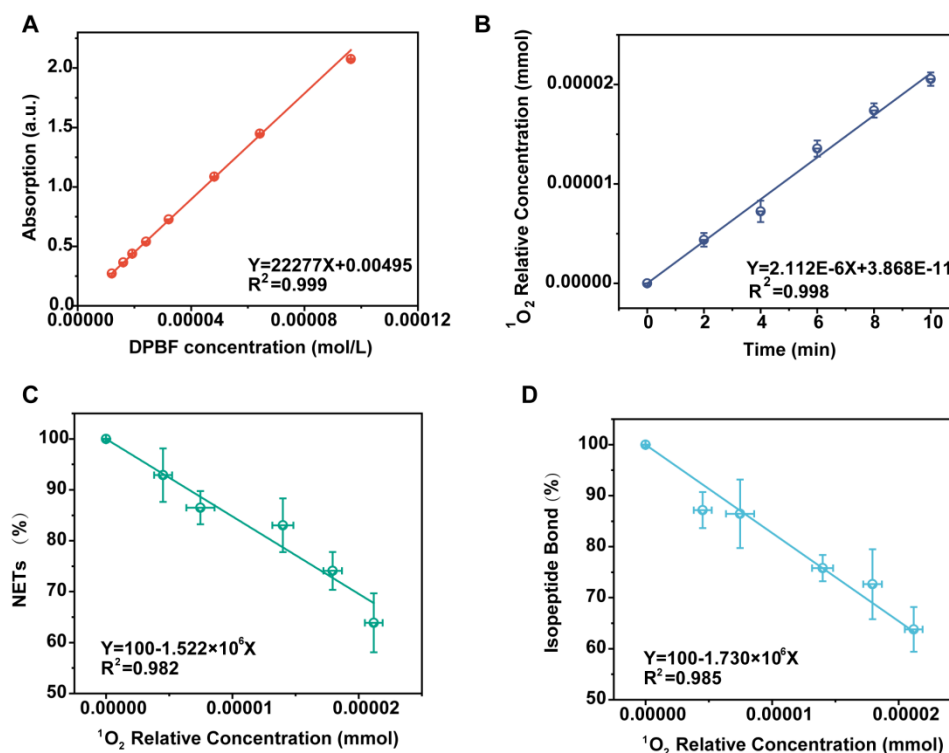


Figure S7. Dose-effect relationship between $^1\text{O}_2$ and the content of NETs and dipeptide bonds. A) Standard curves of DPBF with different concentrations. B) The generation of $^1\text{O}_2$ along with ultrasonic time. The content changes of C) NETs and D) isopeptide bonds under SDT therapy.

To demonstrate that the degradation of NETs and isopeptide bonds contributes to fibrinolysis, complexes containing fibrin and NETs was induced simultaneously in vitro. The existence of NETs can resist the tPA-mediated degradation of fibrin, the combination of DNase I can promote the fibrinolysis (Figure S9A and S9B), suggesting that the destruction of NETs contributes to the degradation of fibrin. Similarly, A2LD1 is an enzyme that specifically breaks the isopeptide bonds, the structure of fibrin loosened and the pores became larger when A2LD1 was used alone. The effectiveness of disrupting the isopeptide bond on fibrin degradation was demonstrated by the significantly improved fibrin degradation when A2LD1 and tPA were combined (Figure S9C and S9D).

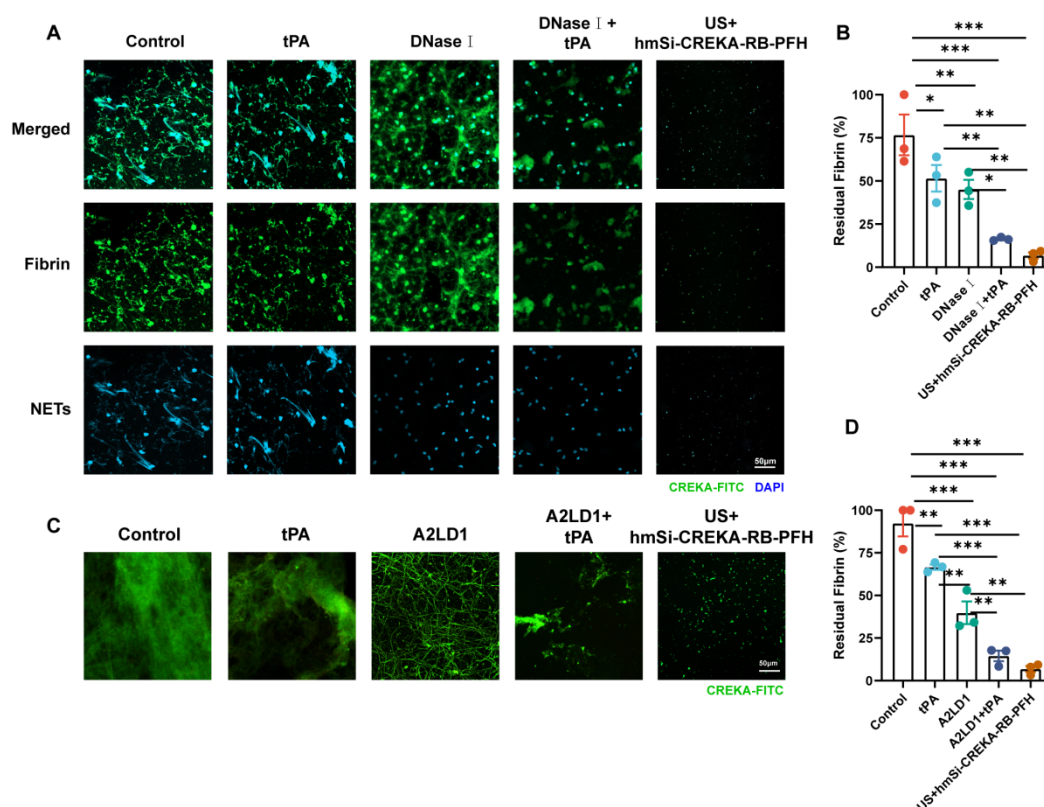


Figure S9. Relationship between disruption of NETs and isopeptide bonds and promotion of fibrin degradation. A) Complex of NETs and fibrin under different treatments and B) the residual fibrin after treatments, mean $\pm$ SEM, n=3. C) Fibrin network under different treatments and D) the residual fibrin after treatments, mean $\pm$ SEM, n=3. one-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

Comment 4: TEM images are not enough to assess size, and the quantification method of size (total and shell) by TEM is not given. DLS size measurements are only given in the supplementary but without mention of solvent, whether these are intensity, volume or number distributions. Solvent and pH for Zeta potential are not mentioned, nor the explanation of variations. A more thorough size, shape and physico-chemical analysis is necessary.

Response: Thank you for your comment. The addition of these details helps to make our manuscript more completely. We used image-J software to count the diameter and shell thickness of 100 particles according to the TEM and calculate the arithmetic

average. As for the test details of DLS and Zeta potentials, we have added them in the revised manuscript.

Revision: Both DLS size and Zeta potentials were measured in aqueous solution and the concentration was 1 mg/mL. The test temperature of the instrument was set at 25°C, each sample was tested five times.

Comment 5: It lacks an experiment that proves the link between NETs / isopeptide bonds degradation and fibrin degradation, which is claimed by the authors. Indeed, they set up experiments to show the efficacy of their combined therapy on NETs, isopeptide bonds and fibrin separately but they didn't prove that NETs and isopeptide bonds degradation increases the lysis of the fibrin network and thus the thrombus.

Response: Thank you very much and your comments are very important for our manuscript. We added a positive control group to demonstrate that disruption of NETs / isopeptide bonds contributes to fibrin degradation, we have added the results in the revised manuscript.

Revision: To demonstrate that the degradation of NETs and isopeptide bonds contributes to fibrinolysis, complexes containing fibrin and NETs was induced simultaneously in vitro. The existence of NETs can resist the tPA-mediated degradation of fibrin, the combination of DNase I can promote the fibrinolysis (Figure S9A and S9B), suggesting that the destruction of NETs contributes to the degradation of fibrin. Similarly, A2LD1 is an enzyme that specifically breaks the isopeptide bonds, the structure of fibrin loosened and the pores became larger when A2LD1 was used alone. The effectiveness of disrupting the isopeptide bond on fibrin degradation was demonstrated by the significantly improved fibrin degradation when A2LD1 and tPA were combined (Figure S9C and S9D).

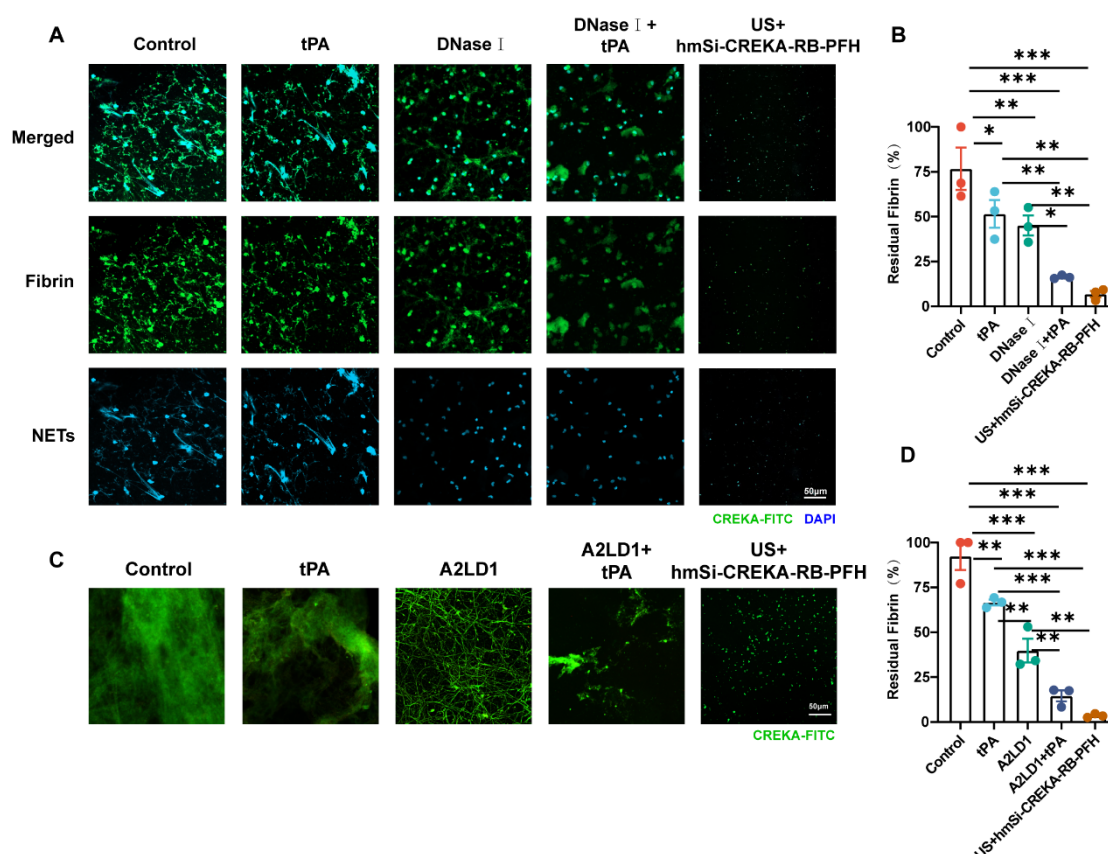


Figure S9. Relationship between disruption of NETS and isopeptide bonds and promotion of fibrin degradation. A) Complex of NETs and fibrin under different treatments and B) the residual fibrin after treatments, mean $\pm$ SEM, n=3. C) Fibrin network under different treatments and D) the residual fibrin after treatments, mean $\pm$ SEM, n=3. one-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

Comment 6: In addition, the liquid-to-gas transformation of PFH under US has not been tested in the article.

Response: Thanks for your comment. It is a pity that this result was easily overlooked as it was presented in the supplementary information (Figure S3).

Comment 7: Part 2.7 (Biosafety assessment), there is no study of the biosafety of the nanoparticles under US stimulation

♣ Notably, it would be expected that safety and cytotoxicity experiments be performed and presented BEFORE switching to in vivo experiments, including big mammals.

Response: (1) The biosafety of nanoparticles (without ultrasound) has been studied in Figure S4, and results such as cell viability, HE staining have demonstrated negligible toxicity of single particles. However, RB will generate singlet oxygen under ultrasound irradiation, which is a highly toxic reactive oxygen species. There have been many reports on sonodynamic therapy for tumors^{23, 24}. Therefore, the high biological toxicity of sonosensitizers under ultrasound can be determined. In this manuscript, it is this highly toxic singlet oxygen that is used for thrombolysis.

(2) The biosafety of nanoparticles (with ultrasound) on tissues surrounding blood clots has also been studied. Firstly, the modification of CREKA can target the fibrin, and the singlet oxygen can only be produced at the thrombi site under the excitation of ultrasound. Moreover, according to literature²⁵, the lifetime of singlet oxygen in aqueous solvent is only 3.5 μ s, so its effective diffusion distance is very short (no more than 155 nm), so it will not cause damage to the surrounding normal tissues.

(3) RNA sequencing results revealed that after recanalization, the inflammatory level of blood vessels slowly subsides and endothelial cells can slowly rebuild over time. To further verify the long-term effectiveness of this therapy, we tested the function of recanalized blood vessels (Figure 7), the experimental results indicated that vascular endothelium can also slowly rebuild after recanalization, and blood vessels can produce nitric oxide and prostaglandins.

Revision: Given the importance of endothelial cells (ECs) in maintaining the long-term patency of the vessels, the morphology of ECs and its biological functions were tested. Almost no ECs were observed at the completion of treatment due to the damage brought by thrombosis. ECs with poor continuity and large gaps were observed after 7 days of reperfusion. After 28 days, there was a distinct layer of ECs with tight connections (Figure 7F). The number of endothelial cells per unit area increased with time (Figure 7H). As an important enzyme in cardiovascular system, endothelial nitric oxide synthase (eNOS) can catalyze the generation of nitric oxide (NO) to regulate

vasomotion and the display of eNOS showed a consistent reconstruction trend with the ECs (Figure 7G and 7I). In addition to endothelial cells, other types of cells, especially invasive inflammatory cells, can produce prostaglandins E₂ (PGE₂) , which is conducive to the regeneration of various organ systems after injury. At the beginning, the concentration of PGE₂ was about 1.8 times higher compared with normal blood vessels, which may be attributed to the upregulation of COX-2 expression during inflammation stage. The relative concentration of PGE₂ decreased over time, which was consistent with the subside of inflammation indicated by Bulk RNA sequencing. After 28 days, the PGE₂ production was close to normal blood vessels (Figure 7J).

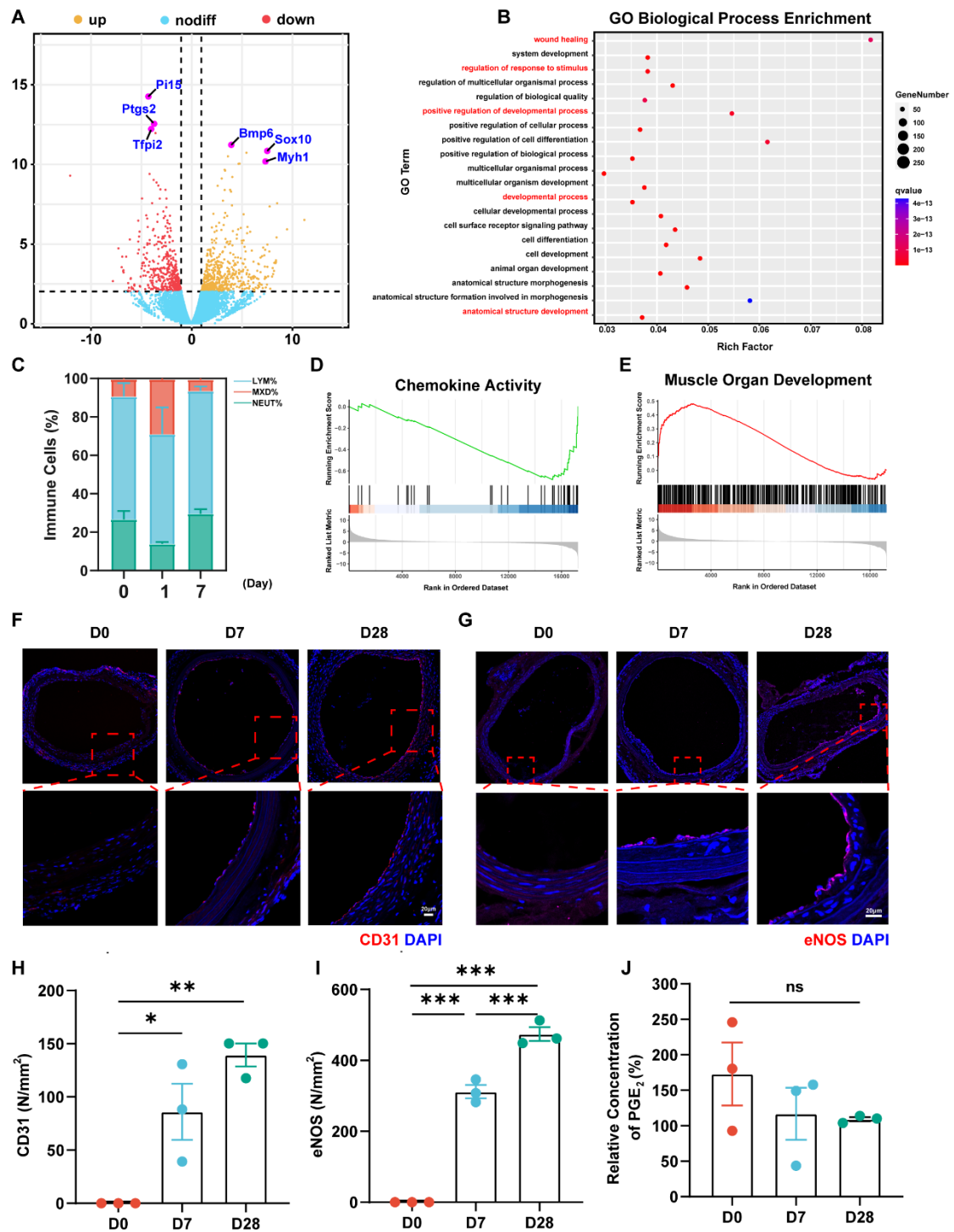


Figure 7. Reconstruction of the recanalized vessels treated with US responsive theranostic platform. A) Volcano plots showing the identified upregulated and downregulated genes a day and a week after the combination therapy ($|\text{fold-change}| \geq 1.0$ with q value < 0.05). $n=3$. B) GO biological process analysis of genes significantly changed between the two groups ($|\text{fold-change}| \geq 1.0$ with P value < 0.05). C) Changes of immune cells after combination therapy. GSEA analysis of D)

chemokine activity and E) muscle organ development. F) Immunofluorescence staining of CD31 and G) eNOS. The number of H) CD31 and I) eNOS. n=3. J) The relative concentration of PGE₂. n=3. Mean $\pm$ SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001.

Comment 8: o Line 371-373 / Figure S11: the maximum dose tested is 400 μ g/mL for hemolysis study, but in vivo experiments were done at 5 mg/mL. Furthermore, the authors make conclusions on this figure, but no statistical test was performed.

Response: Thank you for your comment. The hemolysis experimental results revealed that even at a high dose of 400 μ g/mL, and the nanoparticle won't cause severe hemolysis. The references about thrombolysis based on nanomaterials tend to give drugs according to body weight in the range of 4 to 10 mg/kg^{19, 20, 29}. In our manuscript, the SD rats of approximately 200 g and nanoparticles (0.2 mL, 5 mg/mL) were given through an intravenous injection on the tail vein. That's 5 mg/kg and within the range of 4 to 10 mg/kg. In attention, the blood volume of a rat (200 g) is about 12-15 mL, which is equivalent to 66-83 μ g/mL after blood dilution, and is within the test range of hemolysis experiments.

We have added a statistical test for the results of the hemolysis experiment in Figure S4C.

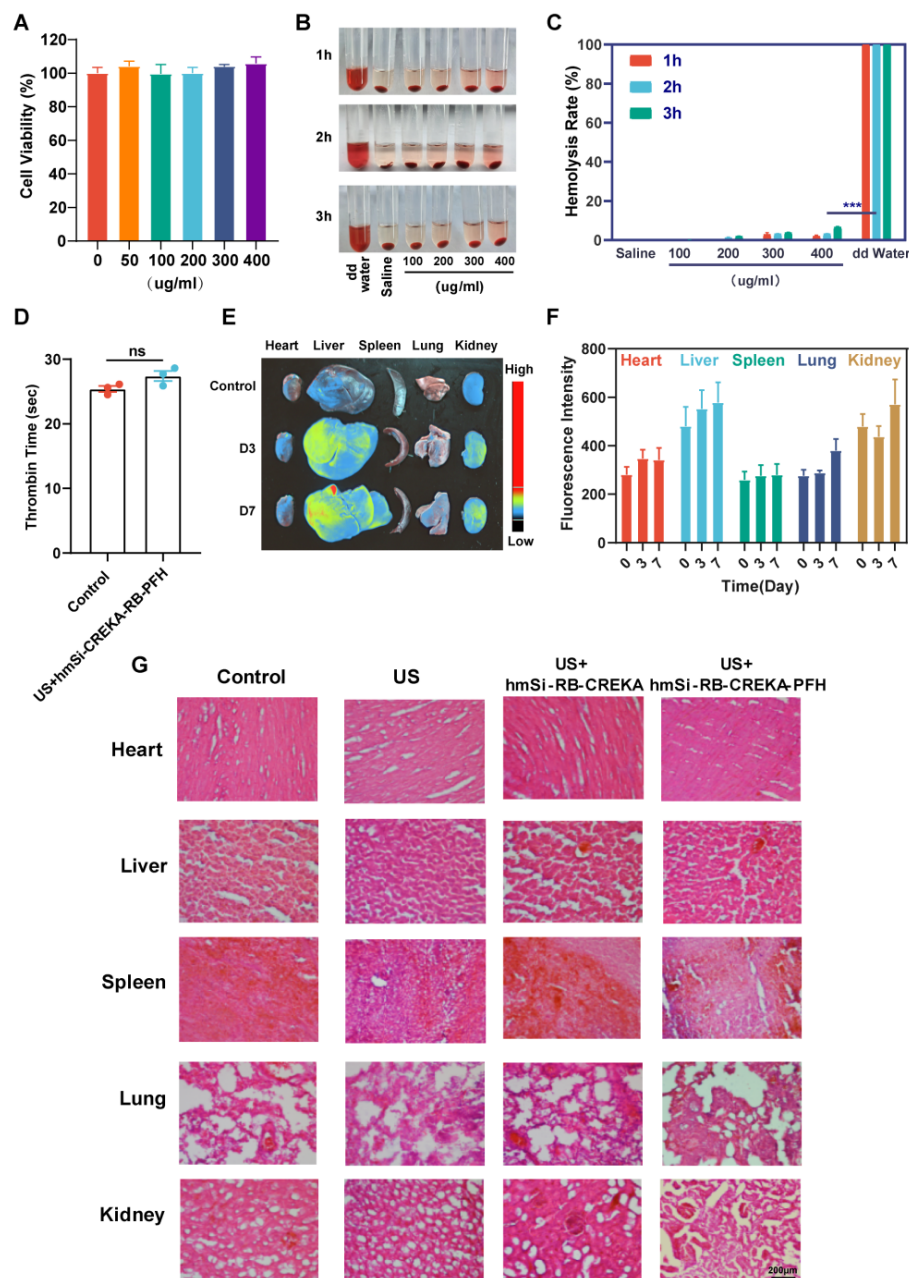


Figure S4. Biosafety of hmSi-CREKA-RB-PFH. A) Cytotoxicity of hmSi-CREKA-RB-PFH with different concentrations through CCK8 detection kit. B) Photographs and C) the hemolysis rate of RBC after incubated with distilled water (positive control), saline (negative control), and hmSi-CREKA-RB-PFH of different concentrations for 1, 2, and 3 h, respectively. mean $\pm$ SEM, n=3, one-way ANOVA analysis. *P<0.05, **P<0.01, ***P<0.001. D) Change of thrombin time after administration for 3 days. mean $\pm$ SEM, n=3. one-way ANOVA, ns(P>0.05). E) Fluorescence image and F) fluorescence intensity of major organs with

hmSi-CREKA(fitc)-RB-PFH injection for 3 and 7 days. G) H&E staining of major organs after combination therapy.

Comment 9: In the in vivo study on pigs, n = 1

Response: Thanks for your comment. Considering the timeliness of supplementary experiments and comment responses, we took the femoral artery thrombosis model of pigs as the representative and expanded the sample size (n=3) to demonstrate the efficacy of the combination therapy proposed in this manuscript. We replaced the results in Figure 8 in the revised manuscript.

Revision: We further constructed occlusion models in the femoral artery (Figure 8A) in pigs. Obvious thrombosis can be observed (Figure 8B) and hmSi-CREKA-RB-PFH was injected through the ear vein and then combination therapy was given. Doppler ultrasonography showed that the blood flow signal of healthy blood vessels vanished after thrombus formation, nearly half of the lumen is blocked (Figure 8C), the blood-flow velocity increased from 45.85 cm/s to 99.53 cm/s as the blood vessel became narrower. After giving the same treatment for 30 min, the blood flow signal recovered, and the flow velocity decreased to 46.22 cm/s (Figure 8D), approaching the velocity in normal blood vessels. Blood samples in normal, thrombosis, and thrombolysis periods were collected for D-dimer detection. There was no significant difference in the contents of D-dimer between the normal and thrombosis groups. After thrombolysis, the D-dimer contents increased sharply to 20 times (Figure 8E), indicating that the thrombus was effectively dissolved. The results of H&E staining also showed that the degree of vascular occlusion was significantly improved (Figure 8F), raising from 17.47% to 84.69% (Figure 8G). Immunofluorescence staining presented a large number of isopeptide bonds in the control group. However, the fluorescence of this crosslinks in the treatment group decreased, and fibrin was observed to be enriched in the position where the isopeptide bond was not yet been destroyed, which further suggests the key role of isopeptide bonds in maintaining the stability of the thrombus (Figure S20). In addition to the excellent recanalization effect, we witnessed the contrast enhancement induced by the transition of PFH in

common carotid artery, the morphology of the thrombus became clearer over time (Figure S21), indicating the feasibility of hmSi-CREKA-RB-PFH submicron particles as an enhanced contrast agent for real-time monitoring.

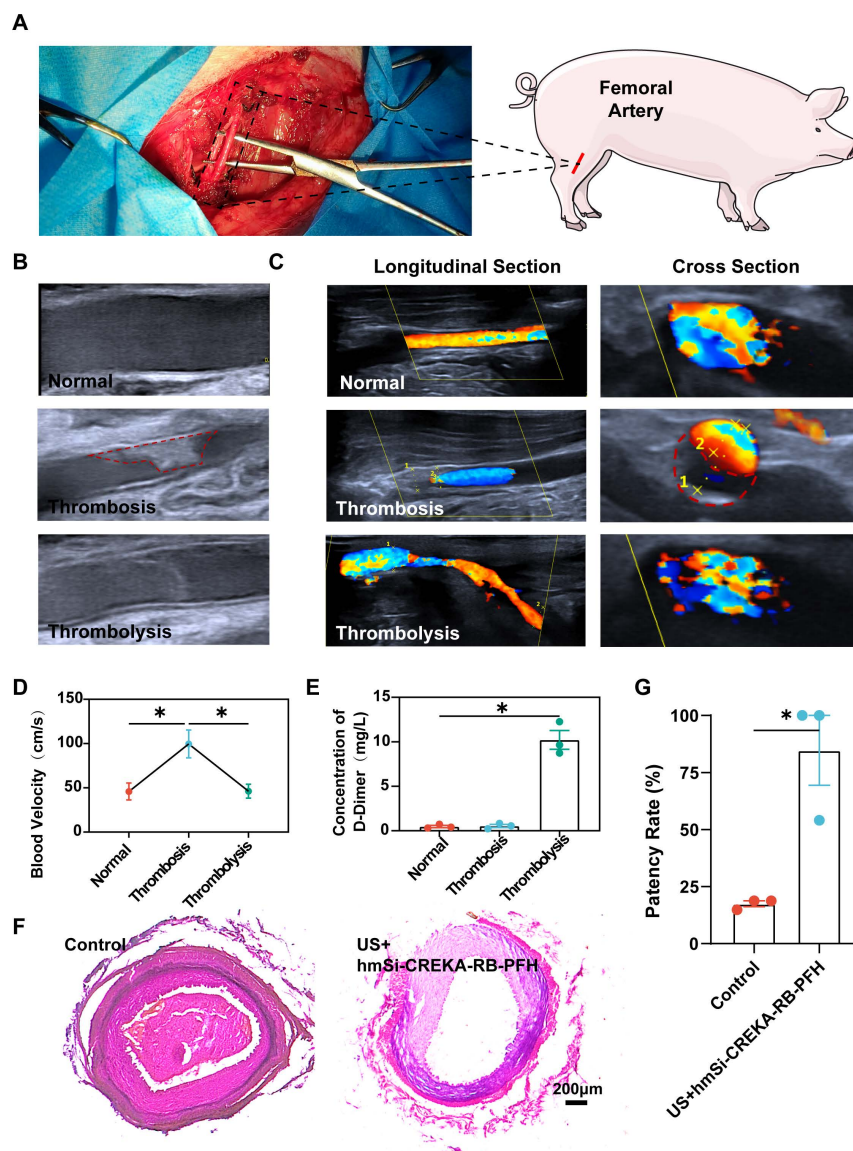


Figure 8. Therapeutic effect of US responsive theranostic platform in pigs. A) Digital photos of the exposed femoral artery. Real-time B) US(B-mode) and C) Doppler ultrasound of thrombosis and thrombolysis in a carotid artery. D) Detection of real-time blood flow velocity. n=3. E) Changes of D-Dimer before and after treatment. n=3. Mean±SEM, paired T test. *P<0.05, **P<0.01, ***P<0.001. F) Histopathology staining and G) the corresponding patency rate. Mean ± SEM. n=3. Independent samples T-test. *P<0.05, **P<0.01, ***P<0.001.

Comment 10: Figure 6 / Paragraph 2.6: The purpose of the study on thrombi induced in TEVGs is not clear. There is no explanation about the procedure of vascular grafting and about its treatment after thrombus induction, neither in the Materials & Methods nor in the article

Response: We thank the reviewer for this comment. Our team has mature small diameter tissue engineered vascular grafts transplantation technology, and the relevant articles have been published^{8, 9, 30}. We have added the purpose and procedure in the revised manuscript.

Revision: The clinical application of tissue engineered vascular grafts (TEVGs) is limited due to the lack of effective integrated thrombolytic strategies for diagnosis and treatment. We hope to further verify the effect of this therapy on the thrombus of the graft.

Experimental part: The detailed technical procedure of TEVGs preparation and transplantation had been published previously. Briefly, carotid arteries were harvested from SD rats (250 ±20 g), decellularized rat carotid arteries were obtained after incubating with 0.05% trypsin at 37°C for 30min, and stored in a 4°C refrigerator for later use. After vascular transplantation, the thrombosis was determined by Doppler ultrasonography, and nanoparticles (hmSi-CREKA-RB-PFH, 0.2 mL, 5 mg/mL) were injected through tail vein, 10 min ultrasound irradiation (1.0 MHz, 1.5 W/cm²) was performed 1 hour later and repeated for 3 days.

Comment 11: The explanation of immunofluorescence markers used here is not given, nor the interpretation of their presence/absence in staining. In particular, SytoxGreen is used to stain nucleic acid: what are the authors trying to see when co-staining with Hoechst) (supposedly, in Fig. 2C)?

Response: Due to the formation of NETs accompanying the death of neutrophils, named NETosis. Sytoxgreen can specifically bind to the nucleic acid of dead cells and it was used to characterize NETs in previous works. Since the main structure of NETs is DNA, we co-stained sytoxgreen with hoechst to better characterize this

extracellular DNA skeleton. Regarding the use of sytoxgreen, we have added relevant literature (reference 9 in supplementary information). The information about all antibodies could be found in the supplementary information.

Comment 12: Quantitative analysis of images is not explained: how do the authors quantify the NET area (fig. 2B)

Response: The nuclear numbers of MPO-positive and Cit3H4-positive in the traced area were determined in 0.102/mm² regions. This quantification method is supported by literature, which we have cited in the revised manuscript (reference 8 in supplementary information).

Comment 13: How are dipeptides stained in Fig. 3A?

Response: Dipeptides refers to N-epsilon-gamma-glutamyl-Lysine bond. This bond is a unique covalent bond found between mono-fibrin mediated by transglutaminase (coagulation factor XIII). It's specific antibody was available (abcam, ab424) and was stained according to routine immunofluorescence steps. The following primary antibodies were used: rabbit anti-N epsilon gamma glutamyl Lysine antibody (1:200, abcam, ab424), and mouse anti-fibrinogen (1:100, abcam, ab34269). After two nights' incubation at 4°C, the secondary antibodies used were as follows: Alexa Fluor 488 goat anti-mouse IgG (1:1000, Invitrogen), Alexa Fluor 568 goat anti-rabbit IgG (1:1000, Invitrogen). Staining was visualized with a confocal microscope (ZEISS LSM 800).

Comment 14: Line 179 / figure 2B, they conclude that the combined treatment significantly destroyed NETs but there is no significant difference between US only and US+hmSi-CREKA-RB-PFH, and they don't mention that it can be the ultrasounds that degrade NETs and not the combined treatment. This happens several times in the article, and would suggest that US only are responsible for the in vitro results.

Response: For the issue of no significant difference between the US alone group (US)

and the SDT group (US + hmSi-CREKA-RB) and the combination therapy group (US + hmSi-CREKA-RB-PFH) in vitro experiments, we consider that it may be due to the thermal effect of ultrasound itself leading to the destruction of NETs and isopeptide bonds. In order to eliminate the influence of thermal effects, we have made improvements to the experimental equipment and conducted new experiments.

By adding flowing water to the beaker between the ultrasonic probe and the processed sample, the accumulation of heat is avoided through water flow, and the effect of solution temperature rise is eliminated as the following schematic diagram.

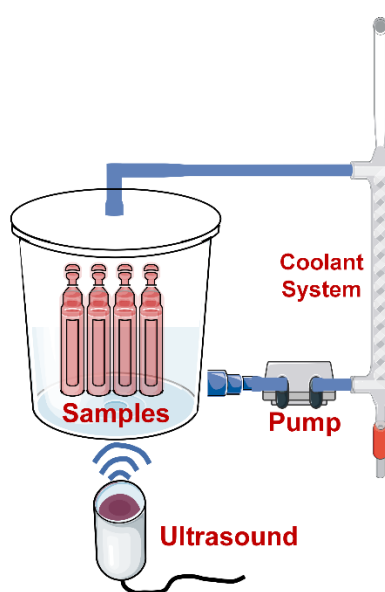
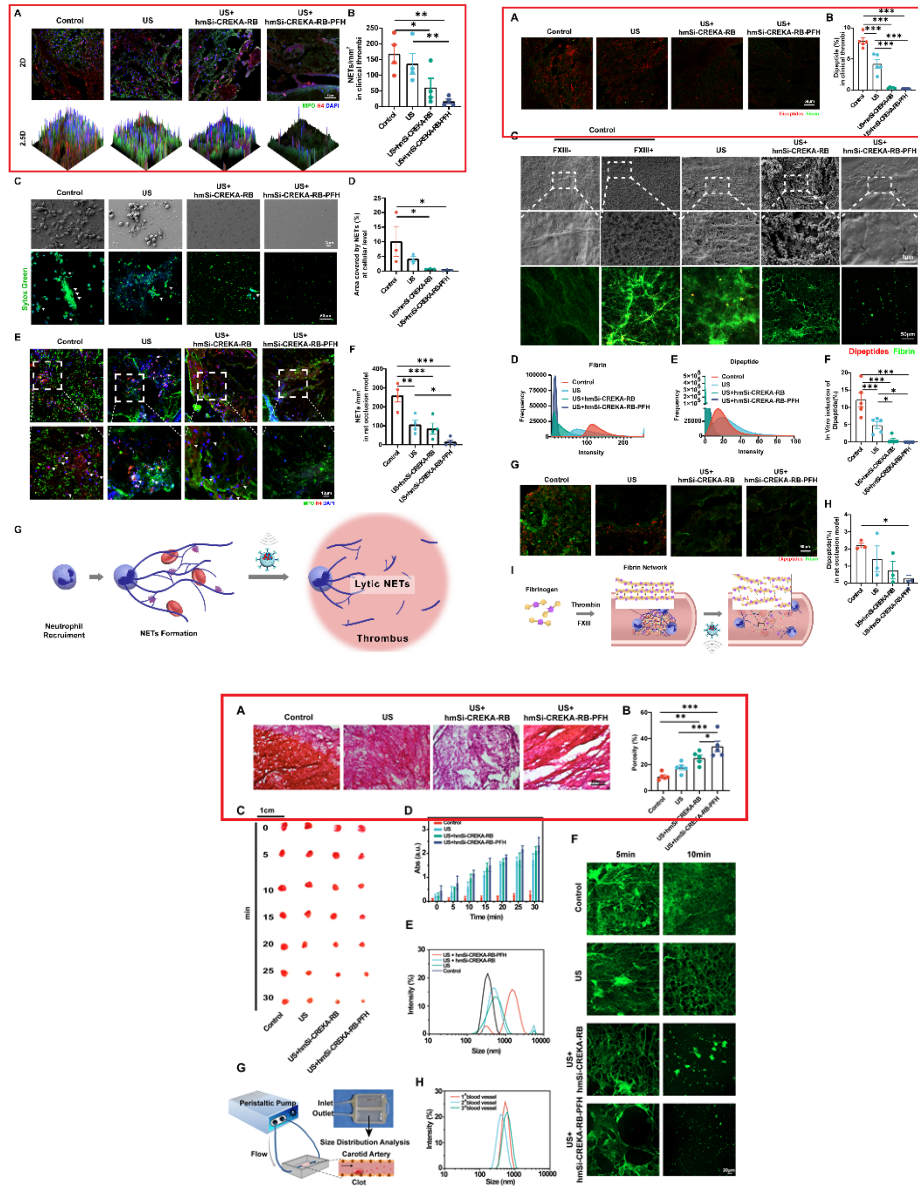


Figure: Schematic diagram of the device for ultrasound therapy

After improvement, it was found that there were significant decrease in positive signals between the US group and the combination therapy group. New statistical data and corresponding replacements (marked with red rectangles) were made in the revised text.



Comment 15: Fig. S4: Nothing can be deduced from the images. There is a clear overexposition and unspecific staining with the MPO channel (which also questions other results in the manuscript). The magnification is not enough to see anything, let alone a NET (where are the cells, where is the NET?) Side-by-side monochannel images are necessary as good practice. The lack of control (non-induction of NETs) is critical here.

Response: Thank you for your comment. This staining method refers to the literature and the stained images in our manuscript are similar to the literature^{31, 32}. We have added monochannel images of both non-induction and induction group and replaced

it in the revised manuscript (Figure S5).

Revision :

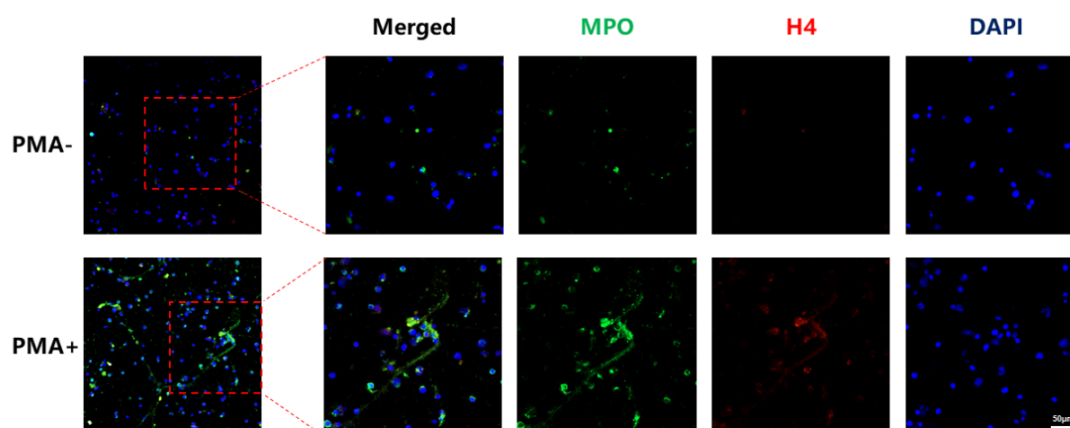


Figure S5: NETs formation in vitro. Neutrophils were treated with PMA to induce NETs and the immunofluorescence staining of NETs.

Comment 16: Line 235-236 / figure 3F: there is no significant difference between US+hmSi-CREKA-RB and US+hmSi-CREKA-RB-PFH. Thus, it is false that “The proportion of isopeptide bonds had dropped from 12.22% to 0.02% when PFH blasting was added (Figure 3F)”. The drop corresponds to the control compared to the combined therapy

Response: Thanks for your comment. 12.22% and 0.02% are the proportion of isopeptide bond in control and combined treatment group, respectively. This is not a comparison between group US+hmSi-CREKA-RB and US+hmSi-CREKA-RB-PFH. We have made improvements to the descriptive language for a clearer understanding.

Revision: Compared with the control group, after combined treatment, the proportion of isopeptide bonds had dropped from 12.22% to 0.02%.

Comment 17: Line 301-303: the specific targeting ability of CREKA has not been demonstrated because there is not the condition hmSi-RB-PFH (without CREKA) as control of passive targeting

Response: Thanks for your valuable comment. We added a new control group (Figure

S13) and randomly divided the rats induced by thrombus into three groups: Control, hmSi-RB-PFH and hmSi-CREKA-RB-PFH (CREKA was labeled with FITC), 1 hour after tail vein injection, take blood vessels for sectioning and observe the fluorescence of the thrombus site. Fluorescence was observed only when injected with CREKA-modified nanoparticles, indicating the targeting ability of CREKA.

Revision:

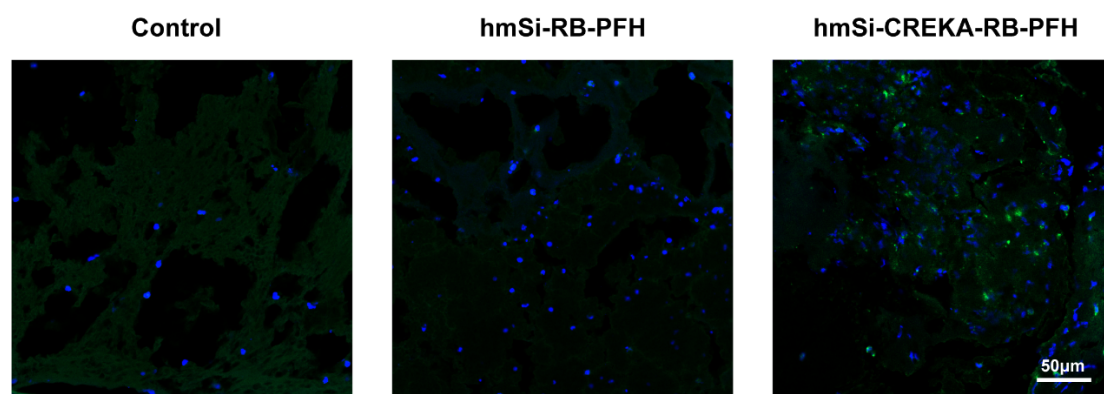


Figure S13. The thrombus-targeting ability of hmSi-CREKA(fitc)-RB-PFH.

Comment 18: Line 420-423: in figure 8D, there is no error bar, no statistical tests, ... The conclusion is hasty, they can't conclude that the thrombus was fully dissolved because there is no control of complete thrombolysis

Response: Thanks for your comment. Considering the timeliness of supplementary experiments and comment responses, we took the femoral artery thrombosis model of pigs as the representative and expanded the sample size (n=3) to demonstrate the efficacy of the combination therapy proposed in this manuscript. The D-Dimer concentration, blood velocity, H&E staining and vascular patency rate before and after thrombolysis was added. We replaced the results in Figure 8 in the revised manuscript.

Revision: We further constructed occlusion models in the femoral artery (Figure 8A) in pigs. Obvious thrombosis can be observed (Figure 8B) and hmSi-CREKA-RB-PFH was injected through the ear vein and then combination therapy was given. Doppler ultrasonography showed that the blood flow signal of healthy blood vessels vanished after thrombus formation, nearly half of the lumen is blocked (Figure 8C), the

blood-flow velocity increased from 45.85 cm/s to 99.53 cm/s as the blood vessel became narrower. After giving the same treatment for 30 min, the blood flow signal recovered, and the flow velocity decreased to 46.22 cm/s (Figure 8D), approaching the velocity in normal blood vessels. Blood samples in normal, thrombosis, and thrombolysis periods were collected for D-dimer detection. There was no significant difference in the contents of D-dimer between the normal and thrombosis groups. After thrombolysis, the D-dimer contents increased sharply to 20 times (Figure 8E), indicating that the thrombus was effectively dissolved. The results of H&E staining also showed that the degree of vascular occlusion was significantly improved (Figure 8F), raising from 17.47% to 84.69% (Figure 8G). Immunofluorescence staining presented a large number of isopeptide bonds in the control group. However, the fluorescence of this crosslinks in the treatment group decreased, and fibrin was observed to be enriched in the position where the isopeptide bond was not yet been destroyed, which further suggests the key role of isopeptide bonds in maintaining the stability of the thrombus (Figure S20). In addition to the excellent recanalization effect, we witnessed the contrast enhancement induced by the transition of PFH in common carotid artery, the morphology of the thrombus became clearer over time (Figure S21), indicating the feasibility of hmSi-CREKA-RB-PFH submicron particles as an enhanced contrast agent for real-time monitoring.

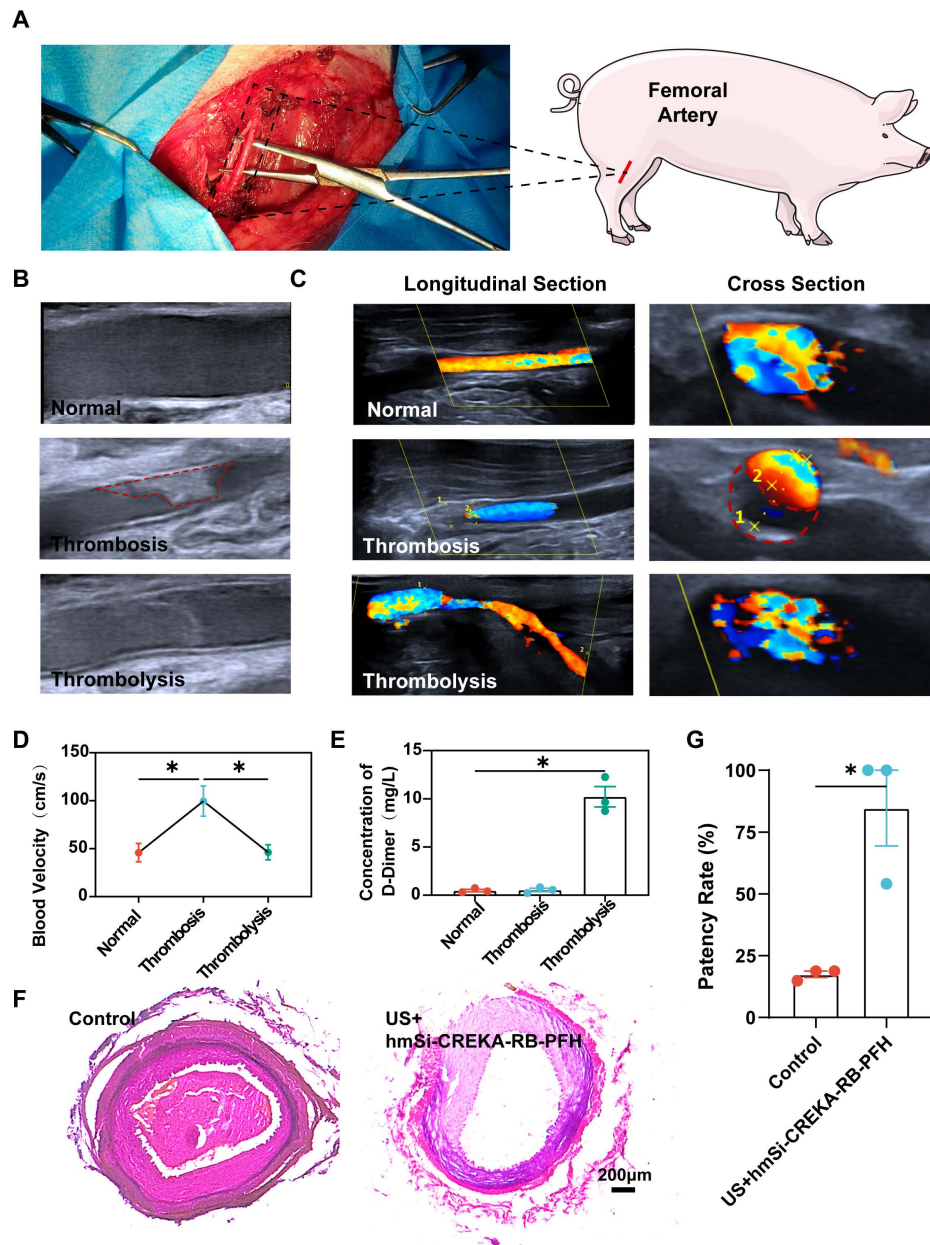


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Comment 19: Figure 5: Since no imaging of the PFH signal enhancement has been done in vivo without thrombus, it can't be said that the change in ultrasound signal is due to PFH bubbles or only to blood reperfusion due to the combined treatment

Response: Thanks for your comment. Experimental results revealed that the loading of phase-transition material PFH can improve the resolution of ultrasound imaging in vitro (Figure S3B). In the pig occlusion model, we further found that the morphology of thrombus gradually becomes clearer under ultrasound irradiation (Figure S20). The Doppler detection indicated that the blood vessels have not yet achieved reperfusion, therefore, changes in contrast due to blood perfusion can be excluded (Figure S20). We further supplemented ultrasound imaging of thrombus in rats (carotid thrombus not completely occluded): hmSi-CREKA-PFH was injected, and improved contrast of ultrasonic imaging was also observed, indicating PFH can enhance the resolution of ultrasound imaging (Figure S16).

Revision: In vivo experiments of US+hmsi-CREKA-PFH were conducted to investigate the mechanical blasting thrombolytic effect of PFH. Immunofluorescence results indicated that there were still obvious NETs (Figure S15A), isopeptide bonds (Figure S15B), and the vascular patency rate was less than 40% (Figure S15C). Although the therapeutic effect of mechanical blasting alone was not ideal, it was found that the contrast of ultrasound imaging was enhanced (Figure S16), indicating the necessity of PFH combination.

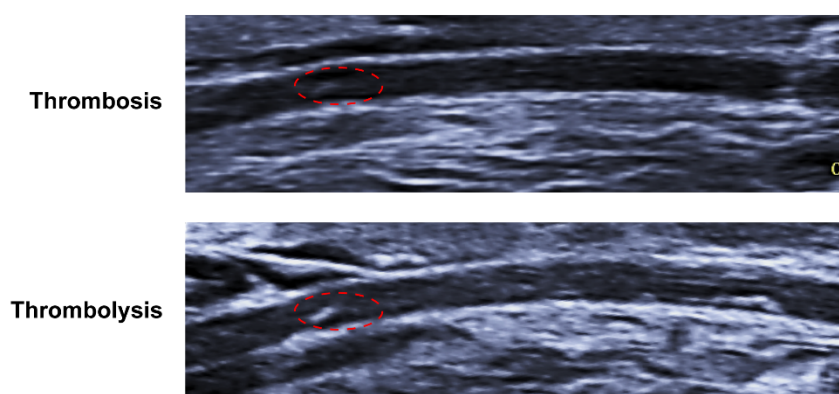


Figure S16: Contrast-enhancement of ultrasound imaging in rat carotid artery.

Comment 20: Fig. 2C: no cells are apparent in SEM images for US with NPs: are they all dead (thus cytotoxicity issues) or are no cell present (then obviously no NET can be formed)?

Response: Thank you for your question and we think this is a question worth exploring. From the SEM results of the control group, it was confirmed that a large number of cells existed on the fragments before treatment and NETs were successfully induced. Why are there fewer cells in the last three visual fields? We hypothesized that this might be due to the mechanical vibration of the ultrasound that caused the cells to fall off. To test this hypothesis, we measured the number of shed cells in the solution after treatment and determined the destruction of NETs between shed cells by fluorescence staining. We analyzed the additional experimental data in the revised manuscript.

Revision: Less cells were observed in the visual field after treatment. Considering the ultrasound's mechanical vibration may cause the detach of NETs, we analyzed the treatment solution thereafter and discovered a significant amount of shed cells in the combination therapy group (Figure S6A). Additional immunofluorescence labeling revealed that the shed cells were still attached to undamaged NETs in the US group. The dispersed state of the shed cells in SDT group and the combination group suggested that the degradation of NETs aggravates the detach of the cells since no complete NETs were seen (Figure S6B)

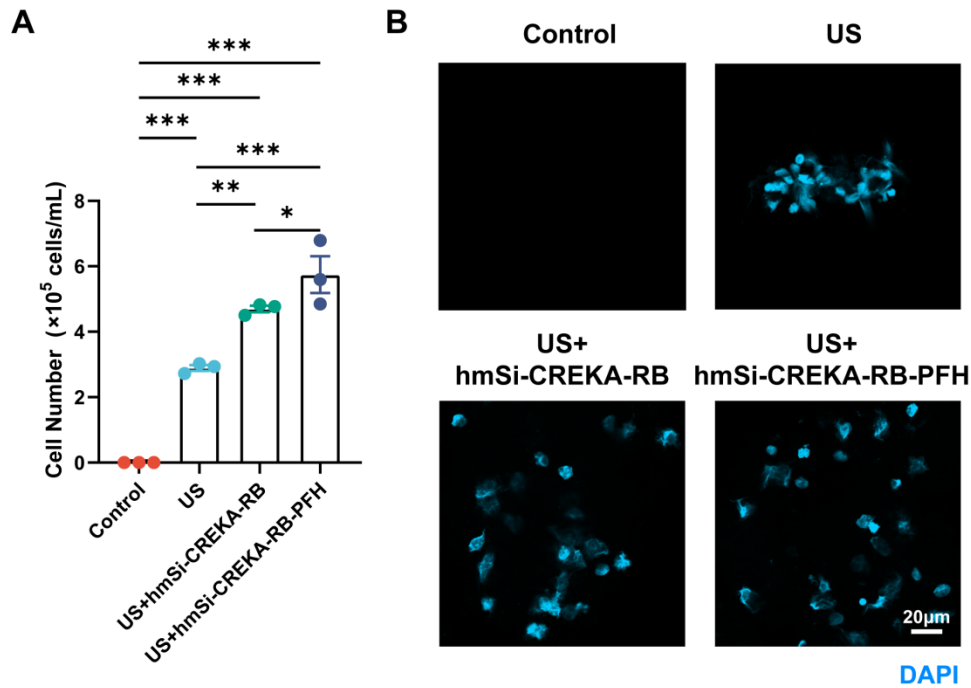


Figure S6. Examination of the shed cells from NETs. A) Cell counting of the solution after different treatments. mean $\pm$ SEM, n=3, one-way ANOVA, *P<0.05, **P<0.01, ***P<0.001. B) Immunofluorescence staining of the shed cells.

Comment 21: Figure 2E: same issues: no more apparent nuclei in the combined treatment conditions. What are the cell types that are present in the control group and not anymore in the treatment group (no more nuclei)? Are they neutrophils? If yes, why are they all dead?

Response: Figure 2E showed the staining results of blood vessel sections after in vivo treatment. In this section, we statistically referenced literature³² to co-locate three colors as positive signals. With the enhancement of treatment methods, the degree of damage to NETs increases, resulting in a gradual decrease in NETs at the site of thrombosis.

(1) With the enhancement of treatment, the degree of thrombus dissolution increased. This results in a decrease in the volume of the thrombus and a consequent decrease in the number of observable nuclei.

(2) It should be noted that this process not only destroys NETs, but also involves the destruction of cells such as white blood cells, red blood cells, and platelets trapped in

them, resulting in a decrease in observed nuclei. The disappeared nuclei are not all neutrophils, as DAPI staining cannot distinguish specific cell types. This result can be analyzed together with Figure 4A, and through HE staining, it can be seen that the porosity of the thrombus increases, which is also related to the destruction and decomposition of cells trapped in the thrombus.

In summary, the observed reduction of nuclei is the result of the gradual dissolution of the thrombus and the destruction of the cells trapped in the thrombus. However, this damage is limited to the targeted thrombus and has no effect on normal tissue cells around blood vessels.

Minor points:

- Introduction:

Comment 22: the authors justified the use of sonodynamic therapy by the fact that PTT induces fragments larger than 10 μm but the reference of this statement claims this without any reference. In addition, they did not present how singlet oxygen produced by their therapy are supposed to destroy the NETS and isopeptide bond (at least, they should present the literature on this topic). Also, the authors designed CREKA-functionalized nanoparticles, but they didn't explain how this peptide targets the thrombus. The different components of the nanoparticles are not presented either.

Response: Thank you for your valuable comments. We should indeed cite relevant literature to support this view. These are cited in the revised manuscript and described briefly here:

(1) Exposure to photothermal heating would lyse the non-covalent interactions in the thrombus, resulting in loosening and downsizing of the clot³³. However, PTT might cause severe complications because the small blood vessels are possibly embolized by photothermal thrombolysis fragments, which are usually larger than 10 μm ²⁹.

(2) Singlet oxygen is a typical reactive oxygen species, which presence in cells is associated with oxidative stress. Strand breaks and base damage in the form of 8-oxo-7,8-dihydroguanine have been identified as consequences of singlet oxygen-induced damage³⁴. The main component of NETs is DNA, so we think that

singlet oxygen can destroy NETs. In addition, it has been reported that singlet oxygen can destroy fibrin skeleton²⁹. We cited relevant documents in the revised manuscript. We also established the relationship between ultrasonic time and the relative amount of singlet oxygen production at specific ultrasonic power ($1.5\text{W}/\text{cm}^2$) in Figure S9. The results show that there is a good linear dependence between the relative concentration of singlet oxygen and the percentage remaining after the destruction of NETs and isopeptide bonds, demonstrated the destruction of NETs and isopeptide bonds by $^1\text{O}_2$.

(3) CREKA, a pentapeptide with the sequence, Cys–Arg–Glu–Lys–Ala, is known to specifically target fibrin in various diseases, including metastatic cancer, renal fibrosis, and thrombosis^{35, 36}. Therefore, we modified it on the surface of hmSi to achieve the aggregation of nanoparticles to thrombus.

(4) The submicron particles are based on hollow mesoporous silica as the carrier, modified CREKA to achieve thrombus targeting. RB is loaded as the sound sensitizer, and RB can react with oxygen to produce singlet oxygen under ultrasound. As a phase transition molecule, PFH can be transformed from liquid to gas under ultrasound, resulting in mechanical blasting thrombolysis and improving the resolution of ultrasonic imaging. In summary, the design of the submicron material enables ultrasound imaging enhancement and combined thrombolysis.

Comment 23: Certain words are used seemingly to increase complexity but are not justified or relevant: “spatiotemporal thrombolytic method” for instance.

Response: “Spatiotemporal thrombolytic method” means that the treatment can be controlled in time and space due to the laser as excitation source in photothermal therapy. The term “Spatiotemporal” is derived from the references^{37, 38}. In view of the difficulty and complexity of the entry, we have removed this term in the revised manuscript.

Revision: Singh et al. discovered that photothermal therapy (PTT) can be used as a noninvasive thrombolytic method.

Comment 24: The claim that NET and isopeptide bonds are contributor to drug resistance is a conjecture: it might have to do with mechanical strength, secondary networks, etc. but not drug resistance.

Response: Drug-resistant here differs from the define in the field of antibiotic. Drug-resistant thrombi refers to certain kind of thrombi that can't be dissolved by thrombolytic drugs (mainly tPA)¹². Considering the inaccuracy of this phrase, we have changed it into "tPA-resistant thrombi".

(1) It was reported that NETs formation is closely related to the progression of thrombus⁶. NET is one of the main barrier to prevent thrombolytic drugs from penetrating thrombus⁷. In addition, under the influence of XIIIa, glutamic acid reacted with lysine to form covalent bond (isopeptide bond), transforming unstable fibrin polymers into insoluble and stable fibrin³⁹. Therefore, we considered NETs, dipeptides and fibrin as the key factors for thrombolysis.

(2) We added a positive control group to demonstrate that disruption of NETs / isopeptide bonds contributes to fibrin degradation, we have added the results in the revised manuscript.

Revision: To demonstrate that the degradation of NETs and isopeptide bonds contributes to fibrinolysis, complexes containing fibrin and NETs was induced simultaneously in vitro. The existence of NETs can resist the tPA-mediated degradation of fibrin, the combination of DNase I can promote the fibrinolysis(Figure S9A and S9B), suggesting that the destruction of NETs contributes to the degradation of fibrin. Similarly, A2LD1 is an enzyme that specifically breaks the isopeptide bonds, the structure of fibrin loosened and the pores became larger when A2LD1 was used alone. The effectiveness of disrupting the isopeptide bond on fibrin degradation was demonstrated by the significantly improved fibrin degradation when A2LD1 and tPA were combined (Figure S9C and S9D).

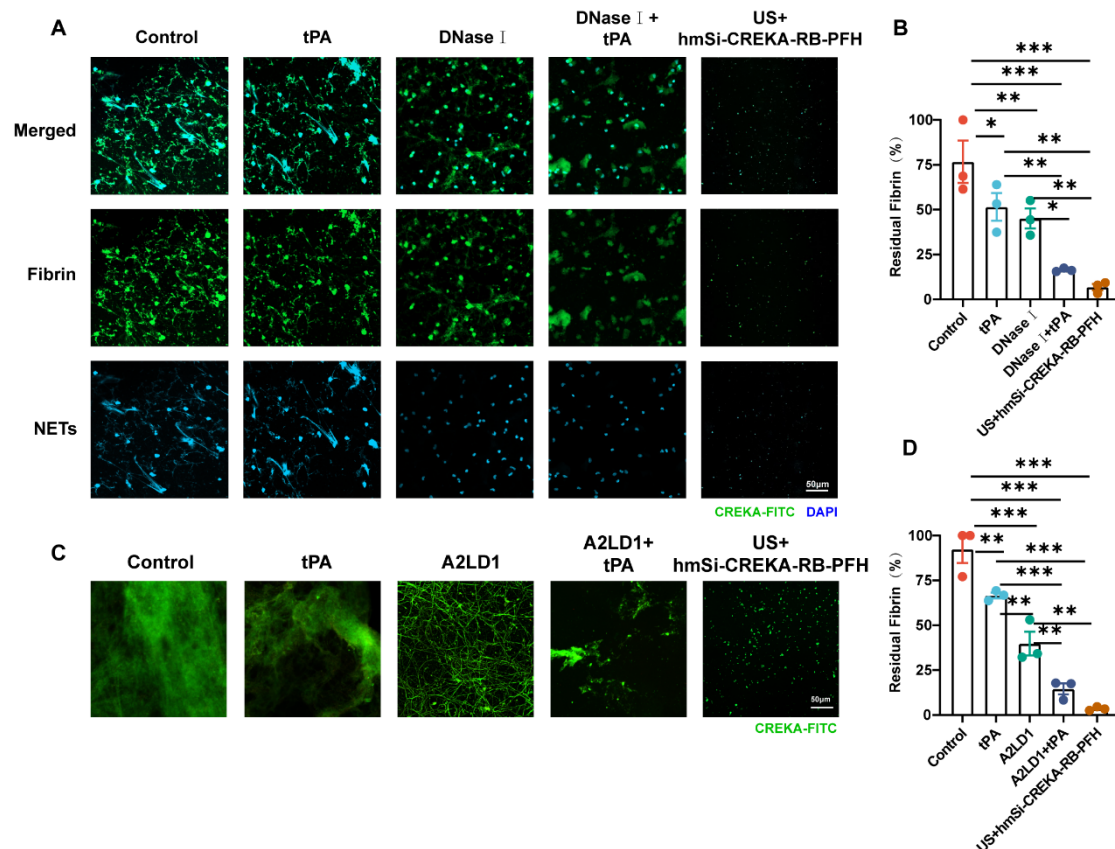


Figure S9. Relationship between disruption of NETs and isopeptide bonds and promotion of fibrin degradation. A) Complex of NETs and fibrin under different treatments and B) the residual fibrin after treatments, mean $\pm$ SEM, n=3. C) Fibrin network under different treatments and D) the residual fibrin after treatments, mean $\pm$ SEM, n=3. one-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

Comment 25: Regarding the organization of the results of the article, the authors made the choice not to separate in vitro and in vivo experiments, which is surprising but not irrelevant. However, the order of the different parts is sometimes not very persistent like the parts on tissue-engineered vascular grafts and biosafety between the therapeutic efficacy tests on rats and those on pigs. Furthermore, the experiments of RNA sequencing have been added in the biosafety part whereas these tests are focused on inflammation and don't seem to be very connected to this part. Finally, some figures should be in the article instead of the supplementary data, for example the figure S8 that compares the authors' treatment with tPA or the figure S11 on blood compatibility that is the main element of the part on biosafety.

Also, the organization of the Materials & Methods does not follow always the one of the articles (example: Characterization before Synthesis whereas it's the opposite in the article).

Response: Thanks for your comment.

(1) From Figure 2 to Figure 4, we put together the clinical thrombus, in vitro induction, and in vivo treatment together, with the destruction target as the object of distinction. The three figures examined the destruction of NETs, isopeptide bonds and fibrin, respectively.

(2) The purpose of this manuscript is to explore a new method to treatment of clinical drug tPA-resistant thrombus. According to previous literature⁴⁰, the thrombus induced by FeCl₃ was most similar to clinical thrombus. Therefore, we first constructed the thrombus induced by FeCl₃ and investigated the vascular recanalization effect from multiple indicators (Figure 5).

To verify the universality of this diagnostic platform, we investigated its effect on TEVGs thrombosis. And to promote translational research, we tested some clinically relevant indicators in the thrombus of pig. Considered that only changed model to verify the effect, we did not design the same test as FeCl₃ induced thrombus. But in all our models, Doppler ultrasound, HE staining and their corresponding statistical analyses were unified.

(3) Biosafety-related experiments, such as cytotoxicity test, hemolysis test, organ fluorescence and HE staining, were divided into a separate part and adjusted to the latter part of the preparation and characterization of nanomaterials to make them more logical sequence.

The experiments of RNA sequencing, reflected the changes of vascular inflammation over time, indicating that the combination therapy would not cause irreversible damage to peripheral blood vessels. In addition, we supplemented vascular endothelial cell staining experiments and functional verification in Figure 7, the experimental results indicated that vascular endothelium can also slowly rebuild after recanalization, and blood vessels can produce nitric oxide and prostaglandins. We treat these two parts as a unit and set the subheading to “Long-term monitoring of

recanalized –vessels”.

(4) We agree that the tPA treatment as positive control group is a very important result. However, in this paper, we mainly emphasize the potential of combination therapy in thrombolysis. tPA is also used to verify that the constructed thrombus model is tPA-resistant. Considering that the data of this group is not as rich as Figure 5, we still put it in the supplementary figure, but strengthen the analysis of the results.

In short, we re-examined the sequence of articles and adjusted and optimized it to make it more logical.

Comment 26: Some acronyms like RB, DPBF or PFH are not explained in the article

Response: Thank you very much for the comment. We are sorry to define some acronyms in the Supplementary Information, which has caused some confusion. We have defined all acronyms on first usage in the revised manuscript.

Revision: Meanwhile, the sensitizer Rose Bengal (RB) were activated to produce abundant singlet oxygen, which destroyed the NETs and isopeptide bonds. Theranostics of thrombolysis was aided by mechanical-blasting of phase transition molecules perfluorohexanes (PFH).

To further explore the sonodynamic characteristic of hmSi-CREKA-RB-PFH, the $^1\text{O}_2$ generation was detected with 1,3-diphenylisobenzofuran (DPBF) in Figure 1F and S2A, and Singlet Oxygen Sensor Green (SOSG) in Figures 1G and S2B.

Comment 27: Line 140: Partial analysis. Explain more in detail why DLS and ELS measurements confirm the “stepwise construction”. Nanoparticles are less positive when CREKA is added, is it because CREKA is negatively charged? Why are there Zeta changes when RB and PFH are added considering that, in Scheme 1, they seem to be incorporated inside the particles and not on their surface?

Response: Thanks to your comments, we further refined the DLS and Zeta changes for each component during the preparation process and analyzed the results in the revised manuscript.

Revision: Additionally, the hydrodynamic size and zeta potentials (Figure S1) were measured, the hydrodynamic size were increased from 218 to 230, 271 and 247 nm after connected with CREKA, RB and PFH, respectively. There is an insignificant decrease maybe due to the strongly hydrophobic about PFH. As for zeta potential, it changed from 33.0 to 25.4, -8.5 and 25.3 mV, due to the electronegativity of CREKA and RB, Zeta potential shifts to negative charge.

RB and PFH are more loaded inside the carrier, but the possibility of surface adhesion cannot be ruled out. Therefore, potential changes occur when RB and PFH are loaded. Which indirectly proved that the corresponding components were embedded successfully.

Comment 28: Line 148-151: The relationship between $^1\text{O}_2$ generation and DPBF & SOSG evolutions are not explained, neither in Materials & Methods nor in the article.

Response: Thank you very much for the comment. The basis of $^1\text{O}_2$ detection by DPBF, is their reaction with $^1\text{O}_2$ to form an endoperoxide via a [4+2]-cycloaddition, and the resulting loss of the extended π -electron system and its characteristic spectroscopic properties. DPBF forms endoperoxide 1 which then decomposes to 1,2-dibenzoylbenzene (DBB) and the absorbance of DPBF at 410 nm decreases as it reacts with $^1\text{O}_2$ into DBB^{41, 42}.

The fluorescence probe Singlet Oxygen Sensor Green reagent (SOSG), can react with $^1\text{O}_2$ to produce SOSG endoperoxides (SOSG-EP) that are strongly fluorescence. With a broad excitation peak around 510 nm, the fluorescence of SOSG-EP has the emission peak at 525-536 nm^{43, 44}. We have cited relevant references in the revised manuscript (references 3-6 in supplementary information).

Special thanks to you for your good comments. We have tried our best to improve the manuscript and highlighted the revisions in the revised manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Response to reviewer #4:

Dear Reviewers,

We appreciate your clear and detailed feedback and hope that the explanation has fully addressed all of your concerns. In the remainder of this letter, we discuss each of your comments individually along with our corresponding responses.

To facilitate this discussion, we first retype your comments and then present our responses and the corresponding revision to the comments. The relevant literature in the Revision has been annotated in the revised manuscript.

Major comments

Comment 1: Using CREKA to target thrombus and ultrasound to induce PFH thrombolysis have been reported (ACS Nano. 2019 Mar 26;13(3):3387-3403). Here in this manuscript, RB and PFH are combined, and CREKA-RB is used as a control. Why not use CREKA-PFH as a control?

Response: Thank you for your comment.

(1) For the mechanical action of PFH, it has been reported that it mainly act on the surface of thrombus¹⁹, which could be seen in the reference²⁰ that gas-blasting has a relatively obvious damage to the surface structure of blood clots, while there is no abnormality in the inside of thrombus.

(2) We added US + hmSi-CREKA-PFH as the control group in vivo treatment, immunofluorescence staining results indicated that there were still more positive signals of NETs and isopeptide bonds (Figure S15). HE staining showed that the improvement of vascular patency rate was not obvious, which suggests that mechanical blasting alone is not effective in the treatment of thrombus, so it is not included as a separate group in this manuscript.

This is consistent with literature reports. Since the mechanical blasting effect of PFH mainly occurs on the surface of thrombus. It has no significant destructive effect on

isopeptide bonds and NETs, which are two single components, mainly reflected in the synergistic effect with SDT, and the mechanical effect can enhance the overall thrombolytic effect. And we have cited the literature (reference 29) in the revised manuscript.

Revision: In vivo experiments were conducted to investigate the mechanical blasting thrombolytic effect of PFH (Figure S15). Immunofluorescence results indicated that there were still obvious NETs, isopeptide bonds and fibrin, and the vascular patency rate was less than 40%, indicating that the effect of mechanical blasting alone was not ideal.

The ultrasound-mediated physical explosion generated by PFH has been applied in the field of thrombolysis, and previous literature indicated that mechanical action can mainly act on the surface of the thrombi in vitro. Additionally, our experimental results showed that the effect of PFH-blasting alone on NETs and dipeptides is negligible in vivo, suggesting that it needs to be combined with other thrombolytic methods. Sonodynamic therapy has been proven to be able to destroy the fibrin skeleton of thrombi. Experimental results revealed that the generation of singlet oxygen had linear correlation with the destruction of NETs and dipeptides, which is conducive to the dissolution of the fibrin scaffold. Therefore, a combination therapy of SDT and gas-blasting was proposed. However, it was observed in vitro experiments that the addition of PFH didn't enhance the destruction efficacy on single NETs or dipeptides but it can enhance the porosity of thrombi while thrombolysis is a comprehensive process. Similarly, on the basis of the SDT destruction of NETs and isopeptide bonds, two key factors leading to thrombolysis, PFH can promote fibrin degradation while SDT alone can't achieve complete recanalization. Taken together, this kind of programmed thrombolysis can achieve overall destruction of blood clots.

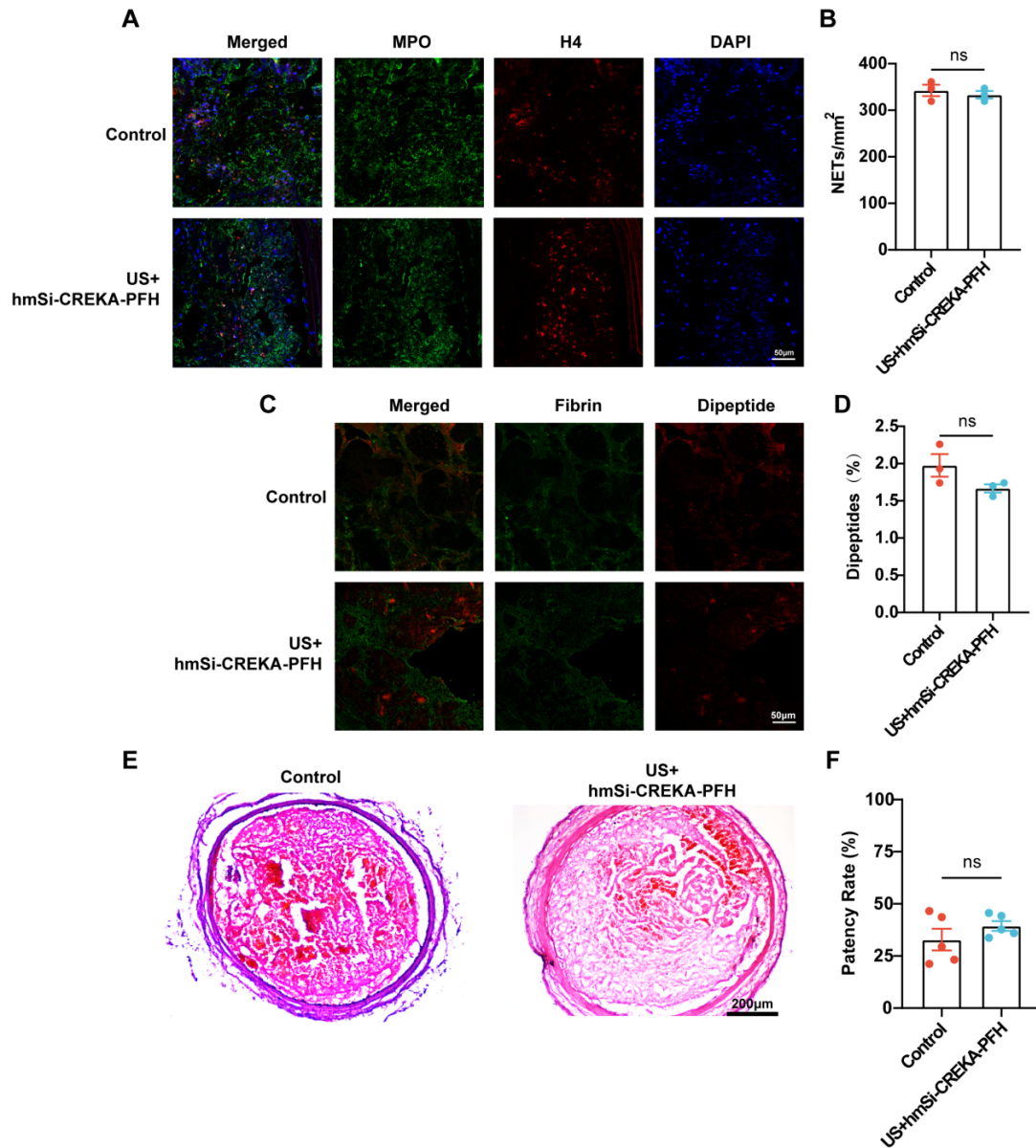


Figure S15. Therapeutic effect of US + hmSi-CREKA-PFH. A) Immunofluorescence staining of NETs in control and US+hmSi-CREKA-PFH groups in rat occlusion model. B) Statistics of NETs per unit area. C) Immunofluorescence staining of Dipeptides bond in control and US+hmSi-CREKA-PFH groups in rat occlusion model. D) Statistics of Dipeptides bond. E) H&E staining of the treated-vessels and F) the corresponding patency rate. mean $\pm$ SEM. n=5, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001.

Comment 2: RB, as a photosensitizer, is also a commonly used method in vascular injury thrombus model. Why should it be used for thrombolysis, will it induce

apoptosis and local inflammatory reaction, and long-term detection of blood vessels after thrombolysis? Although the author has sequenced the common transcriptome of vascular tissue in the US+hmSi-CREKA-RB-PFH group 1 and 7 days after thrombolysis, it cannot be explained whether the use of RB has a significant impact on the safety of blood vessels.

Response: Thanks for your comment and we think this is a question worth exploration. There are many tPA-resistant thrombi in clinical practice, so a new thrombolytic strategy is urgently needed. Sonodynamic thrombolysis has been reported to have certain therapeutic effect on thrombus^{45, 46}, but it is not clear whether it is applicable for tPA-resistant thrombus. Therefore, we have studied the universality of this therapy through in vitro and in vivo experiments. We think the singlet oxygen's damage to normal tissue around blood vessels is extremely limited. Firstly, the modification of CREKA can target the fibrin, and the singlet oxygen can only be produced at the thrombi site under the excitation of ultrasound. Moreover, according to literature²⁵, the lifetime of singlet oxygen in aqueous solvent is only 3.5 μ s, so its effective diffusion distance is very short (no more than 155 nm), so it will not cause damage to the surrounding normal tissues. Secondly, besides the RNA sequencing demonstrated the safety of our therapy, we have further supplemented with functional experiments of the recanalized vessels by detecting the endothelium morphology at 0, 7, and 28 days after reperfusion, and their ability to produce prostaglandins (Figure 7). We have added this result in the supplementary information and depicted it in the revised manuscript.

Result analysis: Given the importance of endothelial cells (ECs) in maintaining the longterm patency of the vessels, the morphology of ECs and its biological functions were tested. Almost no ECs were observed at the completion of treatment due to the damage brought by thrombosis. ECs with poor continuity and large gaps were observed after 7 days of reperfusion. After 28 days, there was a distinct layer of ECs with tight connections (Figure 7F). The number of endothelial cells per unit area increased with time (Figure 7H). As an important enzyme in cardiovascular system,

endothelial nitric oxide synthase (eNOS) can catalyze the generation of nitric oxide (NO) to regulate vasomotion and the display of eNOS showed a consistent reconstruction trend with the ECs (Figure 7G and 7I). In addition to endothelial cells, other types of cells, especially invasive inflammatory cells, can produce prostaglandins E₂ (PGE₂) , which is conducive to the regeneration of various organ systems after injury. At the beginning, the concentration of PGE₂ was about 1.8 times higher compared with normal blood vessels, which may be attributed to the upregulation of COX-2 expression during inflammation stage. The relative concentration of PGE₂ decreased over time, which was consistent with the subside of inflammation indicated by Bulk RNA sequencing. After 28 days, the PGE₂ production was close to normal blood vessels (Figure 7J)

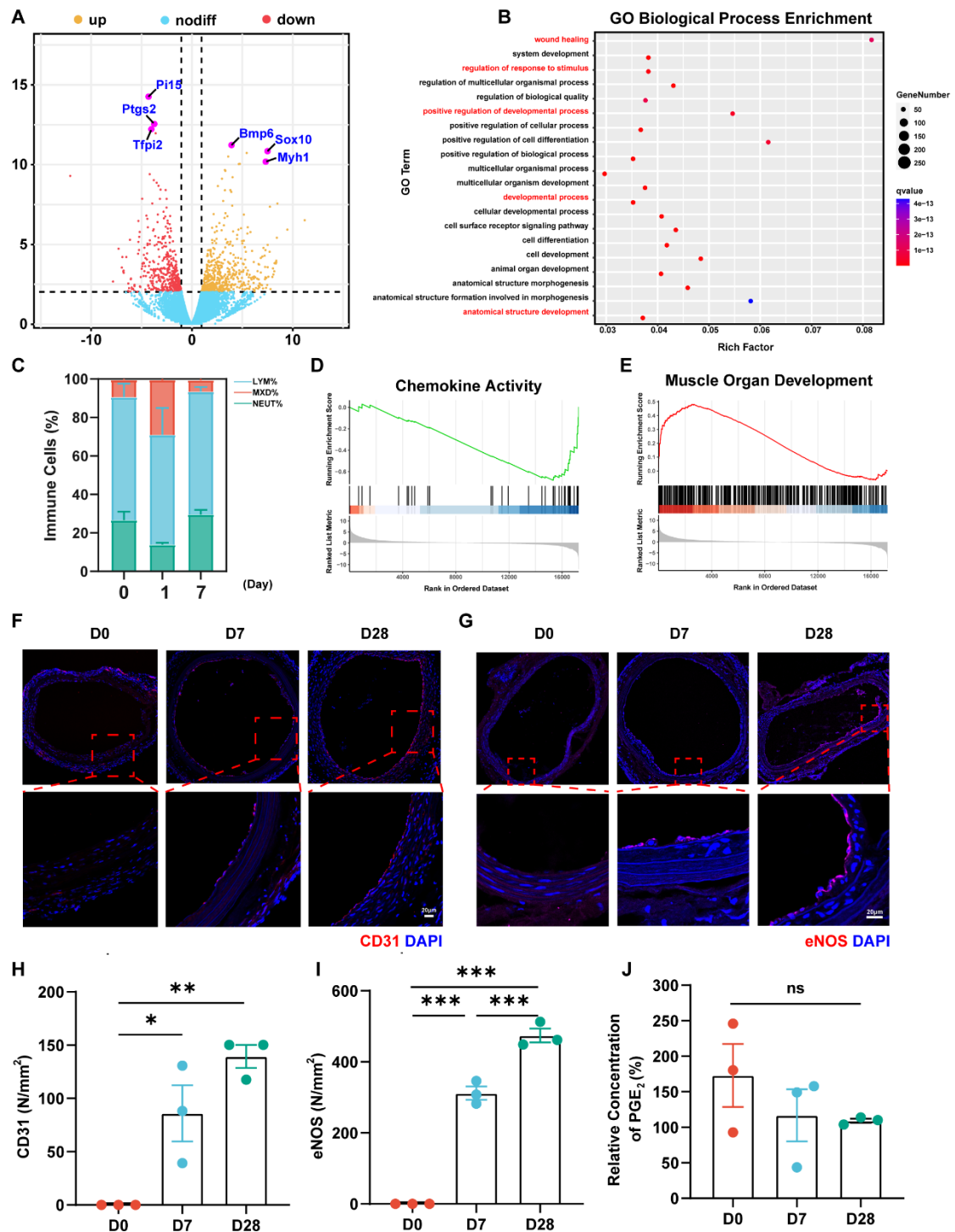


Figure 7. Reconstruction of the recanalized vessels treated with US responsive theranostic platform. A) Volcano plots showing the identified upregulated and downregulated genes a day and a week after the combination therapy ($|\text{fold-change}| \geq 1.0$ with q value < 0.05). $n=3$. B) GO biological process analysis of genes significantly changed between the two groups ($|\text{fold-change}| \geq 1.0$ with P value < 0.05). C) Changes of immune cells after combination therapy. GSEA analysis of D)

chemokine activity and E) muscle organ development. F) Immunofluorescence staining of CD31 and G) eNOS. The number of H) CD31 and I) eNOS. n=3. J) The relative concentration of PGE₂. n=3. Mean $\pm$ SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001.

Comment 3: RB is not only a photosensitizer, but also a fluorescent dye. If RB reaction is incomplete, or RB in the blood continues to reach the thrombolytic site after ultrasonic treatment, will it affect the late staining and cause false positive results?

Response: Thanks for your comment. RB has obvious absorption in the range of 500-600 nm, and the emission peak is about 580nm⁴⁷. The fluorescence absorption and emission peaks of the secondary antibodies used are 578 and 603 nm, respectively. In the red channel of confocal fluorescence imaging, 568 nm was used as the excitation light source to collect fluorescence emission signals. As a fluorescent dye, RB may affect red light channels. We designed the following experiment to exclude the effect of RB: clinical thrombus and hmSi-CREKA-RB-PFH were incubated at 37°C for two hours, then washed three times with PBS buffer, and confocal fluorescence imaging was performed for thrombus section. The fluorescence signal of the green light channel proved that the nanoparticles were successfully targeted to the thrombus site, but there was no fluorescence signal in the red light channel, indicating that RB itself, although a fluorescent molecule, would not interfere with the results of immunofluorescence staining.

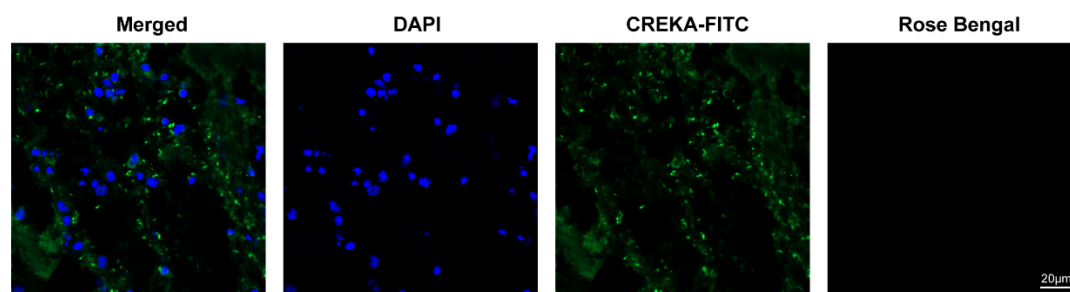


Figure: Confocal image of clinical thrombus after co-incubation with hmSi-CREKA-RB-PFH.

Comment 4: How to control the concentration of injected nanoparticles and the amount of nanoparticles that can reach the thrombus site and play an effective role? What is the retention and clearance time of nanoparticles in other organs?

Response: Thank you for your comment.

(1) The injection concentration of nanoparticles was referred to previous literature reports^{19, 20, 29}, The references on thrombolysis based on nanomaterials tends to give drugs according to body weight in the range of 4 to 10 mg/kg. In our manuscript, the SD rats of approximately 200 g and nanoparticles (0.2 mL, 5 mg/mL) were given through an intravenous injection on the tail vein. That's 5 mg/kg and in the range of 4 to 10 mg/kg.

(2) Fluorescence imaging was used to measure the accumulation of nanoparticles in other organs after injection. As shown in Figure S4E and F, the intensity of fluorescence signal observed in heart, liver, spleen, lung and kidney after injection for 3 and 7 days was closed to the autofluorescence in the control group (without injection of nanomaterials), indicating that the nanoparticles were less absorbed by other organs and cleared quickly. HE staining results in Figure S4G also indicated there was no obvious tissue toxicity.

Comment 5: In the in vitro cytotoxicity assay, it is impossible to predict the toxicity of nanoparticles to local cells only with co-cultured nano-particles and HUVEC without the application of ultrasound.

Response: Thanks for your comment. There are two considerations for the setup of this experiment:

Firstly, in order to investigate the biosafety of nanoparticles, we incubated them with HUVEC, and tested cell viability after co-culture for a period of time. The cytotoxicity of the nanomaterial under ultrasound stimulation was obvious, because the prepared particles will produce singlet oxygen, which has been confirmed in our in vitro experiment (Figure 1F and G), and it was also proved to be cytotoxic in previous studies and used for tumor therapy^{48, 49}. In this manuscript, we use highly toxic reactive oxygen species ($^1\text{O}_2$) to destroy blood clots.

Secondly, in this manuscript, we designed NETs and isopeptic bonds as important targets of thrombolysis, rather than cells trapped in thrombus. We mainly observed the destruction effect of NETs and isopeptides on the formation of singlet oxygen and PFH phase change mechanical blasting by nanoparticles stimulated with ultrasound. Due to the targeted action of CREKA, singlet oxygen destroys only the components in the thrombus. There are no significant side effects on perivascular tissue cells due to its limited lifespan and diffusion distance (<3.5 us, 155 nm), which can also be seen from endothelial remodeling (Figure 7).

To sum up, to investigate the biosafety of the nanomaterials, we think combining ultrasonic stimulation is not required when they are incubated with HUVEC.

Comment 6: In Figure 4E, why are the particles produced after thrombolysis in US+hmSi-CREKA-RB-PFH group larger than those in the other three groups, and the size is mostly greater than 1000 nm? However, Figure 4G and 4H show that the particle size after thrombolysis is less than 1000 nm.

Response: Thanks for your comment. The objects and execution details of the two tests are different and need further explanation here. In Figure 4E, we studied in vitro induced blood clots and tested the particle size of dissolved thrombus after different treatments. In the US + hmSi-CREKA-RB-PFH group, due to the mechanical effect of the PFH phase change blasting, which produced some small pieces directly off the edge of the thrombus, the diameter of the particles in the solution was larger than in the other groups and the size is mostly greater than 1000 nm but less than 3000 nm.

While in Figure 4G and 4H, we took the vessels that were not completely unobstructed after combined treatment (US + hmSi-CREKA-RB-PFH group) as the research object, and designed in vitro scour experiment to investigate whether residual thrombus would cause the blockage of distal small vessels under the effect shear stress. The size of fragments from residual thrombus scour by shear stress is tested here and particle size is less than 1000 nm.

Although the size of the fragments produced by the two measure groups is different, they are far less than 10 μm . It is not easy to cause secondary blockage of distal small

vessels²⁹. We have reinforced the analysis of these two results in the revised manuscript.

Revision: In the US + hmSi-CREKA-RB-PFH group, due to the mechanical effect of the PFH blasting, which produced some small pieces directly off the edge of the thrombus, the diameter of the particles in the solution was larger than in the other groups and the size is mostly greater than 1000 nm but less than 3000 nm (Figure 4E).

Comment 7: In general, if the animal has no other special conditions (such as continuous inflammatory reaction), the thrombus induced by FeCL3 will be gradually absorbed. Therefore, the thrombus still completely occluded after 30 days shown by the author in Figure S9 is puzzling. The thrombus that is not absorbed after 30 days will become more compact. Does the author detect the amount of nanoparticles entering the thrombus at this time?

Response: Thanks for your comment and we think this is a question worth exploring. Whether the thrombus could gradually be absorbed maybe depend on the size of the clot. In our experiment, obvious vascular blockage was still observed after 30 days. We further studied the targeting performance of 30 days old thrombus with nanoparticles. One hour after caudal vein injection, the blood vessel section was taken and obvious fluorescence from nanoparticles was observed at the thrombus site, while no fluorescence signal was observed in the control group, indicating that effective targeting could still be achieved for thrombus formed one month ago. And we depicted it in the revised manuscript.

Revision: For old thrombus, we again investigated the targeting effect of nanoparticles. It was found that there was still an obvious fluorescence signal, indicating that the targeting effect of CREKA is still effective even after a long time of formation.

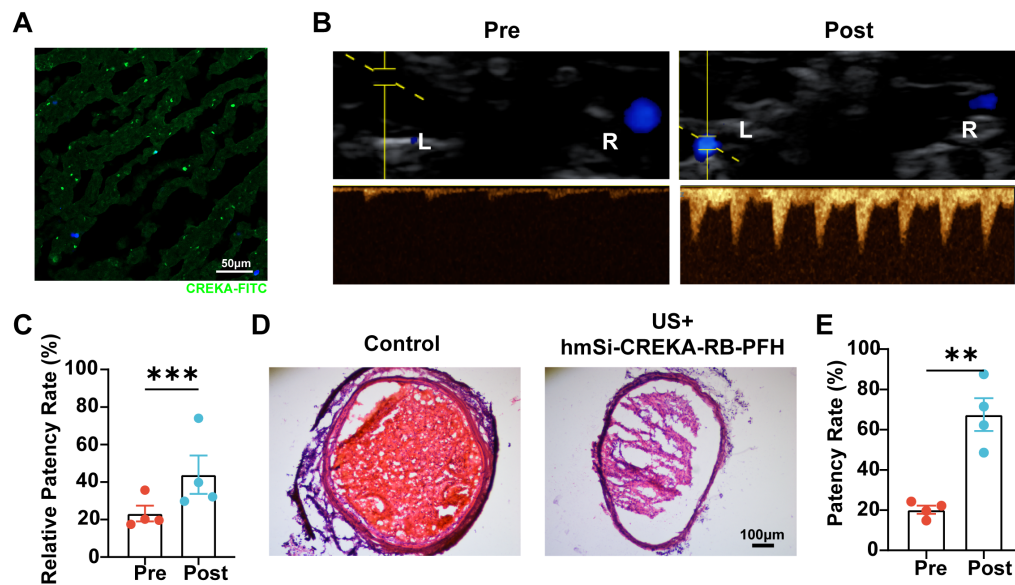


Figure S18. The therapeutic effect of US responsive theranostic platform in rat old thrombi. A) Targeting ability of hmSi-CREKA-RB-PFH to old thrombi. B) Real time Doppler ultrasound of combination therapy in one-month occlusion model and C) relative patency rate. mean $\pm$ SEM. n = 4. Paired t-test. *P<0.05, **P<0.01, ***P<0.001. D) H&E staining and E) the corresponding patency rate. mean $\pm$ SEM. n=4, Independent samples t test, *P<0.05, **P<0.01, ***P<0.001.

Comment 8: Figure 8, n=1 ?

Response: Thanks for your comment. Considering the timeliness of supplementary experiments and comment responses, we took the femoral artery thrombosis model of pigs as the representative and expanded the sample size (n=3) to demonstrate the efficacy of the combination therapy proposed in this manuscript. We replaced the results in Figure 8 in the revised manuscript.

Revision: We further constructed occlusion models in the femoral artery (Figure 8A) in pigs. Obvious thrombosis can be observed (Figure 8B) and hmSi-CREKA-RB-PFH was injected through the ear vein and then combination therapy was given. Doppler ultrasonography showed that the blood flow signal of healthy blood vessels vanished after thrombus formation, nearly half of the lumen is blocked (Figure 8C), the blood-flow velocity increased from 45.85 cm/s to 99.53 cm/s as the blood vessel

became narrower. After giving the same treatment for 30 min, the blood flow signal recovered, and the flow velocity decreased to 46.22 cm/s (Figure 8D), approaching the velocity in normal blood vessels. Blood samples in normal, thrombosis, and thrombolysis periods were collected for D-dimer detection. There was no significant difference in the contents of D-dimer between the normal and thrombosis groups. After thrombolysis, the D-dimer contents increased sharply to 20 times (Figure 8E), indicating that the thrombus was effectively dissolved. The results of H&E staining also showed that the degree of vascular occlusion was significantly improved (Figure 8F), raising from 17.47% to 84.69% (Figure 8G). Immunofluorescence staining presented a large number of isopeptide bonds in the control group. However, the fluorescence of this crosslinks in the treatment group decreased, and fibrin was observed to be enriched in the position where the isopeptide bond was not yet been destroyed, which further suggests the key role of isopeptide bonds in maintaining the stability of the thrombus (Figure S20). In addition to the excellent recanalization effect, we witnessed the contrast enhancement induced by the transition of PFH in common carotid artery, the morphology of the thrombus became clearer over time (Figure S21), indicating the feasibility of hmSi-CREKA-RB-PFH submicron particles as an enhanced contrast agent for real-time monitoring.

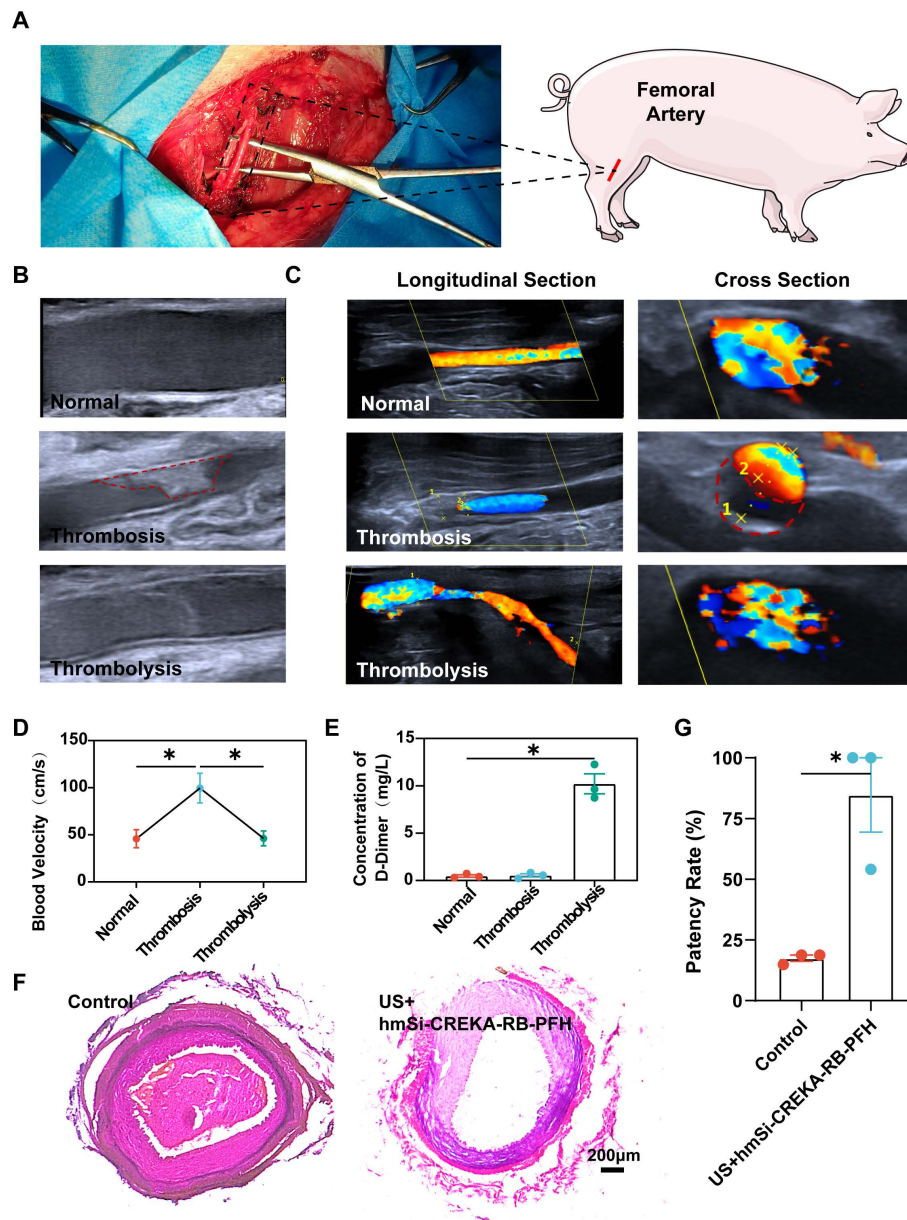


Figure 8. Therapeutic effect of US responsive theranostic platform in pigs. A) Digital photos of the exposed femoral artery. Real-time B) US(B-mode) and C) Doppler ultrasound of thrombosis and thrombolysis in a carotid artery. D) Detection of real-time blood flow velocity. $n=3$. E) Changes of D-Dimer before and after treatment. $n=3$. Mean $\pm$ SEM, paired T test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. F) Histopathology staining and G) the corresponding patency rate. Mean $\pm$ SEM. $n=3$. Independent samples T-test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Comment 9: It is suggested that the author should try to unify the results of animal experiments, such as blood vessel recanalization rate, serum D-dimer, FDP level, and

vascular pathological sections.

Response: Thanks for your comment and your suggestions help to unify our manuscripts. The purpose of this manuscript is to explore a new method to treatment of clinical drug tPA-resistant thrombus. According to previous literature⁴⁰, in the thrombus model induced by FeCl₃, the thrombus component was most similar to clinical thrombus. Therefore, we first constructed the thrombus induced by FeCl₃ and investigated the vascular recanalization effect from multiple indicators such as Doppler ultrasound, laser speckle imaging, DSA and histopathology (Figure 5).

To verify the universality of this diagnostic platform, we investigated its effect on VEGFs thrombosis. And in order to promote translational research, we tested some clinically relevant indicators in the thrombus of pig. Considered that only changed model to verify the effect, we did not design the same test as FeCl₃ induced thrombus. But in all our models, Doppler ultrasound, HE staining and their corresponding statistical analyses were unified.

Minor

Comment 10: Dipeptide antibody information is missing.

Response: Thanks for your comment. The antibody information is in the supplementary information of “Immunofluorescence” part, which may be easily overlooked.

Comment 11: In Figure 2E, high magnificent figures do not match the low magnificent figures.

Response: Thanks for the comment from reviewer. The incompatible of figures at different multiples due to the operation of image-rotation during typesetting. We have now changed the image direction in the revised manuscript.

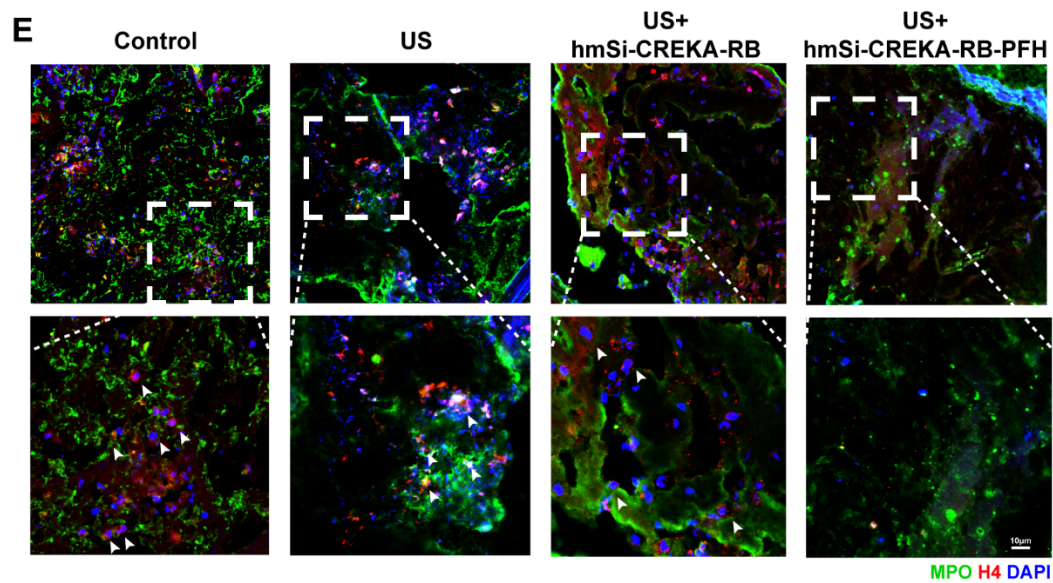


Figure 2E: Immunostaining of NETs in rat carotid artery thrombus in different groups. Arrows point to NETs (DNA colocalized with Myeloperoxidase and Cit3H4).

Comment 12: The same problem in Figure S5A, day 3, and S5B, day 4.

Response: Thank you for the comment. The pictures were not obtained in same device (fluorescence microscopic and confocal microscopic). We have now deleted the images captured by fluorescence microscopic in the revised manuscript.

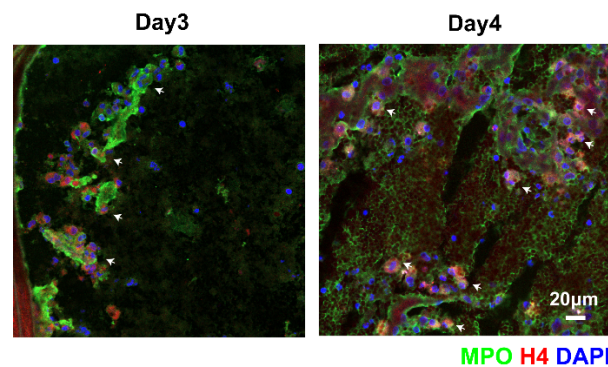


Figure S12. NETs formation in vivo. The immunofluorescence staining of NETs in rat common carotid artery at 3 and 4 days after thrombosis.

Special thanks to you for your good comments. We have tried our best to improve the manuscript and highlighted the revisions in the revised manuscript. These changes will not influence the content and framework of the paper. We appreciate for

Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Thank you for the prompt and thorough response to revisions. I have a few additional queries:

1. In figure S17 the results of the experimental treatment are shown in a rat ferric chloride model. How long after thrombosis was the treatment performed? The number treated appears very low – n=3? Is this correct? Most thrombolysis/pharmacologic studies employ n=8-10. How was the patency rate identified? Was this a percentage luminal occlusion by duplex?
2. Re: “stroke patients received IV thrombolysis within 4.5hrs ... AND endovascular thrombectomy” – I believe the AND must be a typo. Generally patients receive: systemic IV thrombolysis or mechanical thrombectomy. Percutaneous intervention following systematic IV thrombolysis is contraindicated. I wonder if the authors meant to say “OR”? Or if the IV thrombolysis was referring to catheter directed thrombolysis?
3. With regards to additional data regarding endothelial function and continuity in the pig model, I appreciate that it is difficult to obtain large animal data and the attempt to gain this information in the rat model. However, it appears as though the data shown is a time course of the same treatment? Is there data comparing the parameters of re-endothelialization, PGE2 and eNOS in control v. experimental treatment?
4. Thank you for the additional data provided in figure S8. What was the dosage of DNAase I and how was it chosen – can the authors provide rationale? How was it delivered?
5. I disagree with the authors statement that heparin is used for primarily the prophylaxis against venous thrombosis. The cornerstone of therapy for venous thrombosis according to all national and international guidelines is anticoagulation. I treat many patients a year with DVT and all receive anticoagulation: very few receive thrombus reduction procedures, in accordance with the current standards of care. Thrombolysis is performed only in a small minority of patients with proximal DVT who are thought to perhaps benefit from reduction in long term limb morbidity. Even those who undergo thrombus reduction are all still subsequently treated with anticoagulation for a minimum of 3 months to reduce risk of thrombus extension, recurrence and embolization. It would be important to show at late time points that this new strategy, v. anticoagulation alone, or anticoagulation after thrombolysis offers a benefit for it to be relevant clinically. In large clinical trials, even though it is possible for instance to reduce thrombus burden early with endovascular techniques, this does not always translate into a meaningful clinical benefit (e.g. ATTRACT trial, femoropopliteal cohort).
6. Can this technology be adapted > 10cm in depth? Indications for thrombus reduction are currently restricted to the iliofemoral segments of the veins, most of which are deeper than 10cm at maximum depth in a normal adult, due to the curvature of the pelvis.
7. How was color thresholding performed for MSB fibrin staining?

Reviewer #2 (Remarks to the Author):

The present paper examines the use of sonodynamic therapy treatment (silica particles complexed with fibrin targeting peptide combined with ultrasound) of tpa resistant thrombi.

Overall comment, the manuscript is poorly written and the experiments poorly explained. It is extremely difficult to follow what exactly was done and makes evaluation of this technology difficult.

Major issues:

1) Characterization: The characterization of the system may not be complete as cytotoxicity evaluate for 24 hours is inadequate and does not include whether the parcels themselves are inflammatory and lead to foreign body response.

How was hemolysis measured--statement like hemolysis could not be observed is not helpful--how was it measured?

Measurement of thrombin time is an old measure of coagulation. Was there an effect on platelet aggregation or activation?

The statement on biocompatibility is not accurate. No long term toxicity study was conducted in vivo.

2) section 2.2--how were the thrombi exposed to the different groups? This is never made clear in the results. if the thrombi were from different patients how so we know if there were dissimilar to begin with? What types of thrombi, from where?

Treatment of NETS is mentioned but how were the NETS treated in what setting?

What is the control for the involve experiment? Groups are not clearly mentioned.

Reviewer #3 (Remarks to the Author):

The authors have diligently answered most of our questions. This has significantly improved the quality of this manuscript, including supplementary experiments and controls that clearly enhance the scientific demonstration in the proposed article. However, several major unanswered comments remain that must be answered to allow for a successful publication. These are, following our initial comments:

- Comment 1/6: Regarding the liquid-to-gas transition of PFH, a picture of 5 Eppendorf tubes (Fig. S3A) is not sufficient to assess that gas was indeed formed (a simple agitation by hand could produce such bubbles): this was not overlooked but deemed not enough. A quantitative method would be desired, which might include quantitative assessment of Fig. S3B.
- Comment 4: Since image analysis can be heavily dependent on the analysis protocol used, the authors should state their protocol extensively: did they measure by hand the distances, for each particle? Did they resort to image treatment and automation? The distribution curves are needed to check whether the number of analyzed particles is high enough to provide a fine estimation of size distribution: it is also needed as distribution width is often paramount to characterize nanoparticles. Moreover, the PDI for DLS measurements was also not provided, nor the intensity distribution. Finally, the salinity of buffers used for Zêta potential was not given, even though it is generally known that salinity has a strong impact on measured Zêta potential (see <https://www.materials-talks.com/zeta-potential-in-salt-solution-or-any->

other-ions/ for instance).

- Comment 7: Regarding safety of the objects, since the authors recognize the toxicity of the particles under US excitation, other experiments should be made to prove the non-toxicity to surrounding tissues. A coculture of endothelial cells with separated NPs (with a Transwell device for instance) under activation could be a good way to check that no toxicity is exerted over a minimal distance of a few microns to millimeters since they estimate the diffusion of singlet oxygen to less than 1 micron. Moreover, the data of recanalized blood vessels, although apparently showing slow rebuild, first demonstrates high damage and inflammation overall. Without a control to prove that this is not due to NPs, this could prove too high damage to be viably used; thus controls with recanalization without the use of NPs (through rtPA or surgery) is needed to confirm that, as authors claim, the damages are due to recanalization and not the toxicity of activated NPs. One could also imagine activating the NPs without presence of thrombi and check for resulting damages.
- Comments 11/12/13: Since image analysis can be heavily dependent on the analysis protocol use, the authors should state their protocol extensively: the sentence “The nuclear numbers of MPO-positive and Cit3H4-positive in the traced area were determined in 0.102/mm² regions” is not clear. How were positive areas defined (manual thresholding?). A clear image analysis protocol is needed.
- Comment 15: Why are NETs not stained by DAPI? This is surprising as NETs DNA should be stained by both SytoxGreen and DAPI.
- Comment 17: One image is not enough to demonstrate anything. The authors need to show quantitative data with relevant replicate number N.

Minor points:

- Additional comment to the RNA sequencing: can the authors give the baseline that was used to measure relative expressions changes?
- Comment 13: Nowhere are the dipeptides defined as N-epsilon-gamma-glutamyl-Lysine bond (which are only evoked once in the abstract). They should be defined in the “Detailed analysis of the composition...” paragraph of the intro.
- Comment 14: The proposed modification by authors is satisfactory. However, this additional setup is not explained neither in the methods nor the manuscript. It should be added, especially since the resulting conclusion is important: thus the authors should comment in the text about how this setup allowed them to show that heat alone with US could destroy bonds, but that a more physiological setup with flow could show that the NPs were necessary in that case for thrombolysis.
- Comment 18: the “D-dimer” is never introduced: is it the isopeptide bond degradation product? How is measured? And why did the authors remove the FDP data?
- Finally, language quality is not good enough, with as pointed out by another reviewer instances of non-idiomatic expressions that could be generated by automatic translators or text generators. Thus, the authors must proofread their manuscript with the help of a native English speaker to use appropriate expressions. Examples in the introduction include: “lengthy thrombi”, “According to the progression of thrombosis”, in-depth research based on tPA-resistant”, “Along with time, the recanalized”, “liquid to gaseity”, “due to the strongly hydrophobic about PFH”, “loaded. Which indirectly “...
- The introduction mentions “To date, as the most commonly used thrombolytic treatment, tissue plasminogen activator (tPA) can lead to fibrinolysis.” tPA always aims for fibrinolysis (due to its mechanism of action) and then acts as a thrombolytic. Rephrase.
- Fig. S4D: What is “thrombin time”? Please explain.

Reviewer #4 (Remarks to the Author):

In this manuscript authors adequately addressed important as well as minor concerns previously raised by editing the text and images as recommended and by performing additional experiments.

Of note, they now include the use of US + hmSi-CREKA-PFH treatment as in vivo control in the rat occlusion model, thereby increasing the rigor of the study. These results demonstrate that US + hmSi-CREKA-PFH treatment did not improve lumen patency compared to challenged control animals and establishes more definitely the improved efficacy of US + hmSi-CREKA-RB-PFH.

Next, they deepen RNA sequencing analysis to investigate immune and endothelial cell responses to the US + hmSi-CREKA-RB-PFH at 1 and 7 days after treatment and additionally show that the vasculature recover at 28 days. Additional analysis of VE-Cadherin staining for EC junction stability (or dextran perfusion) and perivascular/smooth muscle cell counts would improve this data in establishing complete recovery of the vasculature.

Rigor is additionally improved by including n=3 animals in the pig occlusion animal model so that blood velocity, D-dimer and lumen patency could be analyzed statistically.

Response to the Review Comments

Reviewer #1 (Remarks to the Author):

Thank you for the prompt and thorough response to revisions. I have a few additional queries:

Response to reviewer 1:

We would like to thank you very much for your recognition. The suggestions of the reviewer were very helpful to us. We have revised the manuscript very carefully as follows and hope it is more suitable for publication in *Nature Communications*.

Comment 1. In figure S17 the results of the experimental treatment are shown in a rat ferric chloride model. How long after thrombosis was the treatment performed? The number treated appears very low – n=3? Is this correct? Most thrombolysis/pharmacologic studies employ n=8-10. How was the patency rate identified? Was this a percentage luminal occlusion by duplex?

Response: Thanks for your question. Your suggestions are helpful to improve our manuscript more clearly.

① Figure S17 is about the changes in inferior vena cava (IVC) thrombus before and after treatment. The modeling and treatment of IVC thrombosis were consistent with the conditions of carotid artery thrombosis in rats: Starting on the fourth day after thrombosis and three consecutive days of treatment was given. Which referred to the time of NETs formation reported in the literature (*Nature Communications* 11, no. 1 (2020). <https://doi.org/10.1038/s41467-020-16191-y>), and also matched the results observed by our experiment.

② We further expanded most of the sample size to n=8, and updated the corresponding data in the revised manuscript. We have integrated all the revised statistical charts and marked them with the corresponding chart numbers in the manuscript, which were clearly displayed in the following figure.

③ There are two concepts related to patency rate in this manuscript: “relative patency rate” is the ratio of blood flow velocity of the occluded side to the normal opposite side;

The “patency rate” means the percentage of luminal occlusion according to H&E staining.

Revision:

① It was reported that NETs peaks at 3-5 days after stroke²⁴, and we found that 4 days after thrombosis, a great amount of NETs appeared (Figure S9), so we chose the fourth day as the starting time of treatment.

②

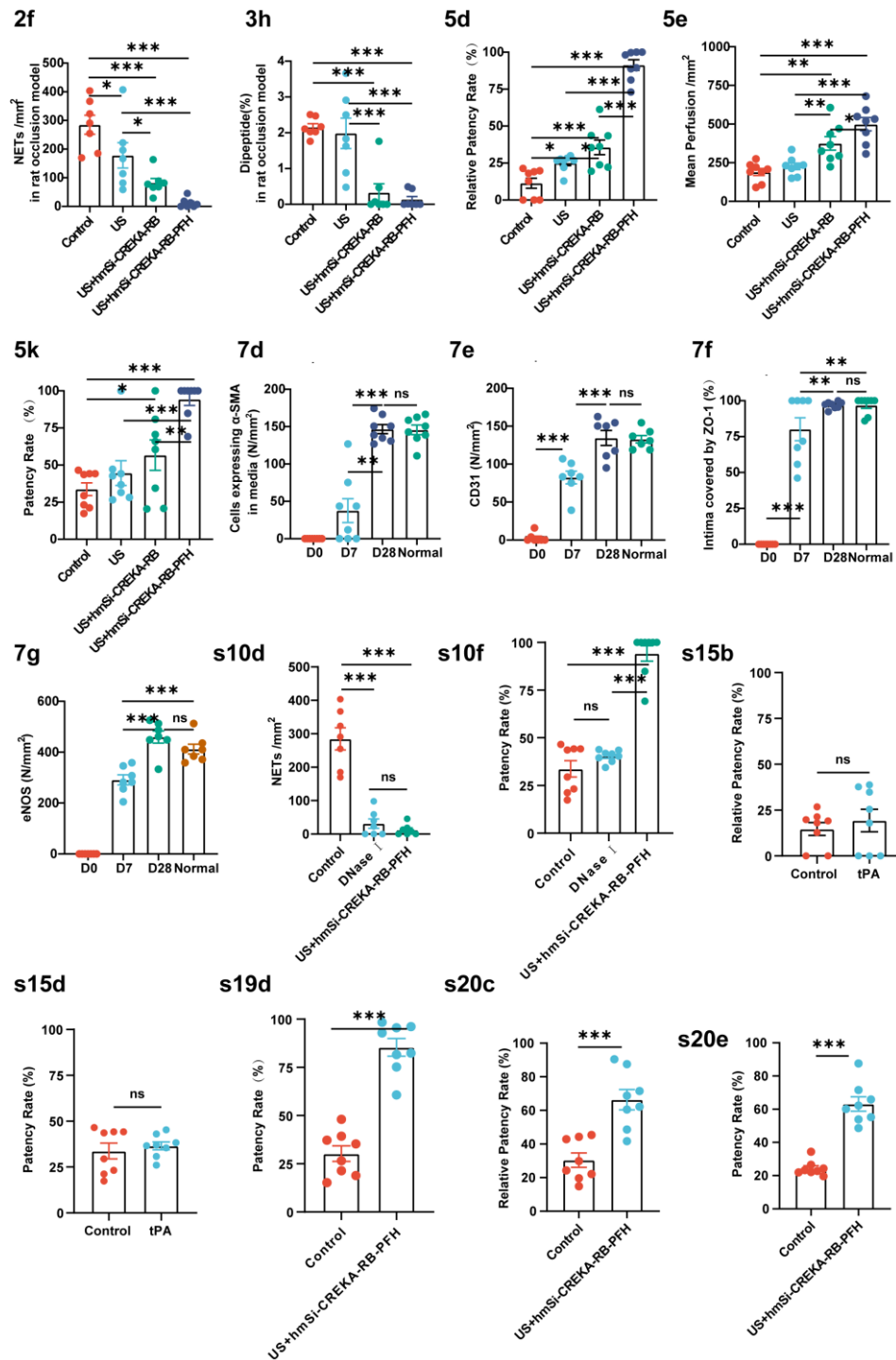


Figure: The integrated revised statistical charts

③ Due to the correlation between blood flow velocity and lumen diameter, we calculated the ratio of blood flow velocity between the occlusive side and the normal control side of same rat, and defined it as the relative patency rate of the blood vessels.

Comment 2. Re: “stroke patients received IV thrombolysis within 4.5hrs ... AND endovascular thrombectomy” – I believe the AND must be a typo. Generally patients

receive: systemic IV thrombolysis or mechanical thrombectomy. Percutaneous intervention following systematic IV thrombolysis is contraindicated. I wonder if the authors meant to say “OR”? Or if the IV thrombolysis was referring to catheter directed thrombolysis?

Response: Thanks for your comment. Perhaps a more detailed explanation is needed here:

① The tPA-resistant thrombi samples used in our manuscript were indeed obtained by surgical thrombectomy after tPA treatment but failed to dissolve. Although tPA promotes degradation of fibrin in the occluding thrombus, but endovascular treatment is not always available, the rate of early arterial recanalization after tPA administration is low (*Stroke* 50, no. 11 (2019): 3228-37. <https://doi.org/10.1161/strokeaha.119.026848>).

② According to the literature, successful reperfusion was more frequent with combination of intravenous thrombolysis and mechanical thrombectomy (*The Lancet* 400, no. 10346 (2022): 76-78. [https://doi.org/10.1016/s0140-6736\(22\)01286-7](https://doi.org/10.1016/s0140-6736(22)01286-7)). And European Stroke Organisation–European Society for Minimally Invasive Neurological Therapy expedited recommendation on indication for intravenous thrombolysis before mechanical thrombectomy in patients with acute ischaemic stroke and anterior circulation large vessel occlusion (*European Stroke Journal* 7, no. 1 (2022): I-XXVI. <https://doi.org/10.1177/23969873221076968>). The description in our manuscript may not be clear and we have been revised it.

Revision: The clinical thrombi were obtained from acute ischemia stroke (AIS) patients who have failed intravenous thrombolysis within the therapeutic time window (4.5h), and underwent endovascular thrombectomy within 24 hours from symptom onset.

Comment 3. With regards to additional data regarding endothelial function and continuity in the pig model, I appreciate that it is difficult to obtain large animal data and the attempt to gain this information in the rat model. However, it is appears as though the data shown is a time course of the same treatment? Is there data comparing the parameters of re-endothelialization, PGE2 and eNOS in control v. experimental

treatment?

Response: Thanks for your comment. We observed the endothelial reconstitution over time after thrombolysis. As you suggested, the comparison with control group is also meaningful. Therefore, we took the blood vessels of the control group (i.e. no treatment after thrombosis) and characterized the parameters of re-endothelialization at d0, d7, and d28, including staining of CD31, ZO-1 and eNOS (Figure S23). In addition, we added staining of α -SMA related to smooth muscle reconstruction. Considering that PGE2 can be produced by various cells (including inflammatory cells) (*Science* 382, no. 6675 (Dec 8 2023): 1148-55. <https://doi.org/10.1126/science.adi1563>), which means it is not directly related to endothelial cells. We replaced this data with endothelial tight junction protein ZO-1, an important protein for the maintenance of ECs junction stability.

Revision: Given the importance of endothelial cells (ECs) in maintaining the long term patency of the vessels, the morphology of ECs and its biological functions were tested. ZO-1, a tight- junction protein between cells, also indirectly reflects the reconstruction of ECs. There was no reconstruction of α -SMA and ECs in the control group (Figure S23a-S23f). As for recanalized vessels, almost no ECs were observed at the completion of treatment due to the damage brought by thrombosis (Figure 7c and 7e). ECs with poor continuity and large gaps were observed after 7 days of reperfusion. Over time, there was a distinct layer of ECs with tight connections at 28 days (Figure 7f and S23f). The number of endothelial cells per unit area increased with time (Figure 7e), indicating that the unobstructed blood flow is the premise of vascular reconstruction. As an important enzyme in cardiovascular system, endothelial nitric oxide synthase (eNOS) can catalyze the generation of nitric oxide (NO) to regulate vasomotion. The blood vessels after treatment were taken at 0, 7, and 28 days for eNOS immunofluorescence staining, and the display of eNOS showed a consistent reconstruction trend with the ECs (Figure 7g and S23g).

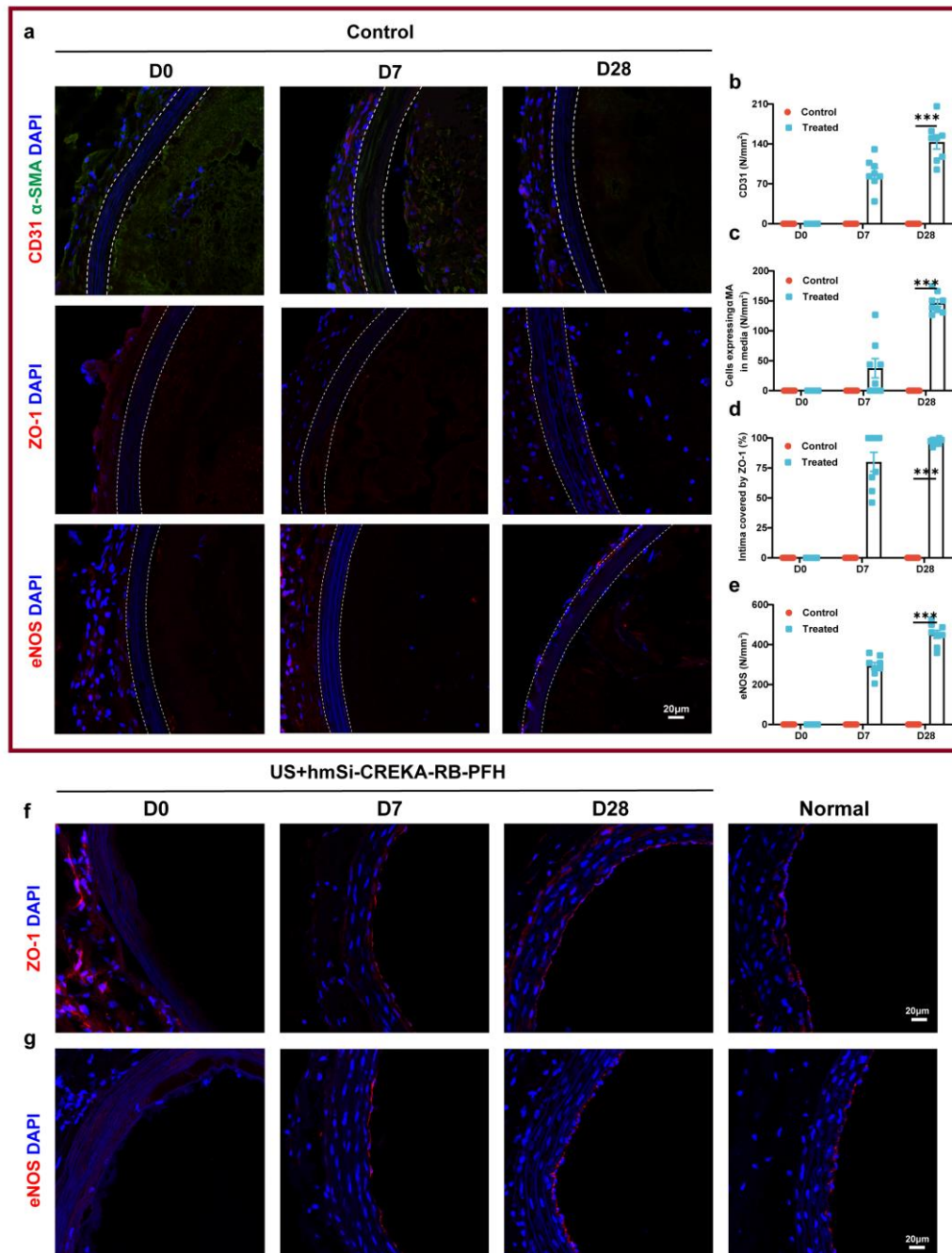


Figure S23. Vascular remodeling. **a** Immunofluorescence staining of CD31, α-SMA, ZO-1 and eNOS in untreated control blood vessels and the quantification of **b** CD31 **c** α-SMA **d** ZO-1 and **e** eNOS. n=8, Mean ± SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001. Immunofluorescence staining of **f** ZO-1 and **g** eNOS in combination treated blood vessels.

Comment 4. Thank you for the additional data provided in figure S8. What was the dosage of DNAase I and how was it chosen – can the authors provide rationale? How

was it delivered?

Response: Thank you for the comments. According to relevant literature, the dosage of DNase I was 100U/mL in vitro (*Stroke* 49, no. 3 (2018): 754-57. <https://doi.org/10.1161/strokeaha.117.019896>; *Proceedings of the National Academy of Sciences* 107, no. 36 (2010): 15880-85. <https://doi.org/10.1073/pnas.1005743107>). As for the in vivo treatment, 1000U DNase I (dispersed in 0.9% sterile saline) was by tail vein injection (*Proceedings of the National Academy of Sciences* 118, no. 28 (2021). <https://doi.org/10.1073/pnas.2100561118>; *Nature Communications* 6, no. 1 (2015). <https://doi.org/10.1038/ncomms7673>). It is worth noting that, in the literature the authors used mice (5-10 weeks), whereas we used a rat thrombus model, so the dosage increased 10 times according to body weight. Details of the experiment have been added to the method section.

Revision: Involving the use of drugs as controls, according to the literature¹²⁻¹⁴, tPA (10mg/kg) or DNase I (1000U) through the tail vein.

Comment 5. I disagree with the authors statement that heparin is used for primarily the prophylaxis against venous thrombosis. The cornerstone of therapy for venous thrombosis according to all national and international guidelines is anticoagulation. I treat many patients a year with DVT and all receive anticoagulation: very few receive thrombus reduction procedures, in accordance with the current standards of care. Thrombolysis is performed only in a small minority of patients with proximal DVT who are thought to perhaps benefit from reduction in long term limb morbidity. Even those who undergo thrombus reduction are all still subsequently treated with anticoagulation for a minimum of 3 months to reduce risk of thrombus extension, recurrence and embolization. It would be important to show at late time points that this new strategy, v. anticoagulation alone, or anticoagulation after thrombolysis offers a benefit for it to be relevant clinically. In large clinical trials, even though it is possible for instance to reduce thrombus burden early with endovascular techniques, this does not always translate into a meaningful clinical benefit (e.g. ATTRACT trial, femoropopliteal cohort).

Response: Thank you for the comment. We agree with you that heparin is primarily used for anticoagulant, including post-thrombolytic use to prevent recurrence of thrombi. We have added some discussion about the combination therapy proposed in this manuscript with anticoagulants such as heparin after thrombolytic therapy, which may be helpful for clinical application.

Revision: At late time points after recanalization, the hematological parameters and Bulk RNA analysis revealed that the recanalization can lead to the subside of thrombosis-induced inflammation. Although the rebuilding of smooth muscle and endothelial cells suggests a positive therapeutic outcome, it takes certain amount of time for vascular reconstruction, indicating the necessity of long-term anticoagulation after thrombolysis. The usage of thrombolysis in combination with anticoagulants, such as heparin, may be promising in clinical for preserving long-term vascular patency and preventing thrombus recurrence.

Comment 6. Can this technology be adapted > 10cm in depth? Indications for thrombus reduction are currently restricted to the iliofemoral segments of the veins, most of which are deeper than 10cm at maximum depth in a normal adult, due to the curvature of the pelvis.

Response: Thank you for the question. We separated 10 cm pork to detect $^1\text{O}_2$, when the power density increased to $2.5\text{W}/\text{cm}^2$, there is no significant improvement. According to the literature, ultrasound at a frequency of 1 MHz has been used safely for therapy at tissue depths of 2.3 to 5 cm (*Nature Biomedical Engineering* 1, no. 8 (2017): 644-53. <https://doi.org/10.1038/s41551-017-0117-6>). In our manuscript, the depth of carotid artery tissue in rats is about 1cm, the carotid artery, brachial artery and femoral artery depth in pig are 3 ~ 4 cm, which were in the range of effective activation of acoustic sensitizers, providing the feasibility and effectiveness of SDT in vivo. The tissue penetration depth will be further improved through optimize intensity, amplitude, focus, beam uniformity and effective radiating of transducer head (*Physiotherapy* 105, no. 1 (2019): 120-25. <https://doi.org/10.1016/j.physio.2017.08.010>; *Rheumatology* 40, no. 12 (2001): 1331-36. <https://doi.org/10.1093/rheumatology/40.12.1331>). For

example, high intensity focused ultrasound (HIFU) is being promoted as a completely noninvasive and extracorporeal method to treat disease and 3D printing in deep tissue(*Science* 382, no. 6675 (Dec 8 2023): 1148-55. <https://doi.org/10.1126/science.ad1563>; *Nature Communications* 14, no. 1 (2023). <https://doi.org/10.1038/s41467-023-40998-0>). Popular HIFU systems (with the frequency about 1MHz) that have been reported in clinical use are listed in Table 1(*World Journal of Clinical Oncology* 2, no. 1 (2011). <https://doi.org/10.5306/wjco.v2.i1.8>). According to the above analysis, we believe it has great potential for clinical application of deep tissue thrombus.

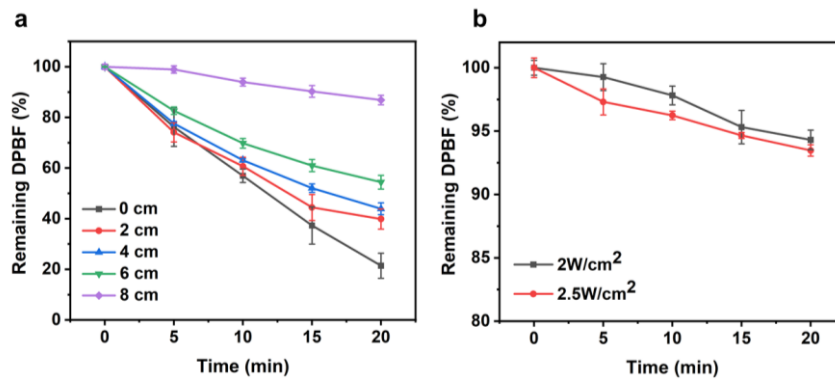


Figure: Relationship between degradation rate of DPBF and time. **a)** 1.5W/cm² with different thickness of pork. **b)** 10 cm pork thickness in 2 W/cm² and 2.5 W/cm².

Table 1 some of popular HIFU systems (with the frequency about 1MHz) that have been reported in clinical use

Model	Manufacturer	Size (mm)	Focal length (mm)	Frequency (MHz)	Focusing method	Imaging guidance	Clinical applications
FEP-BY02	Beijing Yuande Biomedical Engineering Inc, China	OD = 370 ID = 120	255	1	251 elements driven in phase	GE Logiq series ultrasound system	Liver, kidney, breast, pancreatic, bone tumor and uterine fibroid
Model-JC	Chongqing Haifu Tech Ltd, China	D = 120 or 150	90, 130, 160	0.8, 1.6, 3.2	Flat ceramics with acoustic lens	AU3 ultrasound imaging system	Liver, kidney, breast, pancreatic, bone tumor and uterine fibroid
ExAblate 2000	InSightec Ltd, Israel	120	150	0.9, 1.3	Phased array 208 hexagonal elements, 3 cavitation detector	GE Signa 1.5/3.0T MR imaging system	Uterine fibroids, breast tumor, liver cancer, bone metastases, neurosurgery, prostate cancer
Sonalleva	Philips, USA	Not available	Not available	1.2, 1.4	Phased array	Philips Achieva 1.5/3.0T MR imaging system	Uterine fibroids

Comment 7. How was color thresholding performed for MSB fibrin staining?

Response: Thanks for your comments. MSB staining was used to characterize fibrin in thrombus, where yellow represents red blood cells, fibrin is purple or blue, and the fibrin coloring varies due to different formation times according to the instructions of the reagent kit (Solarbio, G2040). The proportion of fibrin was represented by dividing the area except yellow by the total area of the thrombus.

Special thanks to you for your good comments. We have submitted the revised version (with marks in red) and pure version (without marks) in the PDF format of manuscript and Supplementary Information. We have tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the corrections will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Reviewer #2 (Remarks to the Author):

The present paper examines the use of sonodynamic therapy treatment (silica particles complexed with fibrin targeting peptide combined with ultrasound) of tpa resistant thrombi.

Overall comment, the manuscript is poorly written and the experiments poorly explained. It is extremely difficult to follow what exactly was done and makes evaluation of this technology difficult.

Response to reviewer: Thanks very much for your comments. We have carefully reorganized the logic of the manuscript, added more experimental details, and strengthened analysis of the results. We also re-checked and revised the spelling, word using and grammar to make our manuscript more readability.

Major issues:

Comment 1: Characterization: The characterization of the system may not be complete as cytotoxicity evaluate for 24 hours is inadequate and does not include whether the parcels themselves are inflammatory and lead to foreign body response.

How was hemolysis measured--statement like hemolysis could not be observed is not helpful--how was it measured?

Measurement of thrombin time is an old measure of coagulation. Was there an effect on platelet aggregation or activation?

The statement on biocompatibility is not accurate. No long term toxicity study was conducted in vivo.

Response: Thanks for your comment. We have supplemented some experiments for your questions.

① Extending the time of cytotoxicity test, we investigated the survival rate of endothelial cells after 48 and 72 hours of incubation with hmSi-CREKA-RB-PFH.

② In order to investigate whether the injection of hmSi-CREKA-RB-PFH would cause inflammation, we detected the percentage changes of immune cells after three consecutive days of hmSi-CREKA-RB-PFH injection (0.2 mL, 5 mg/mL).

③ The method of hemolysis test has been added to the revised supporting information. Specifically as follows:

Red blood cells (RBCs) were obtained from fresh blood of rats by centrifugation at 3000 rpm for 10 min. Subsequently, hmSi-CREKA-RB-PFH was added into the suspension of RBCs at different concentrations (0, 50, 100, 200, 300, and 400 $\mu\text{g/mL}$). The mixture was incubated in a 5% CO_2 atmosphere at 37 $^{\circ}\text{C}$ for 1, 2, and 3 h, respectively. The absorption values of the collected supernatant were determined at 577 nm with a microplate spectrophotometer (BioTek). The hemolysis ratio of RBCs was calculated using the following formula: $\text{Hemolysis (\%)} = (\text{OD}_{\text{sample-background}} - \text{OD}_{\text{negative control}}) / (\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}}) \times 100\%$. PBS and distilled water were used as the negative and positive controls, respectively. The background absorption was the absorption of nanoparticles at different concentrations.

④ The effect on platelet activation was examined. After three consecutive days of hmSi-CREKA-RB-PFH injection (0.2 mL, 5 mg/mL) and treatment, we took blood at different time (first and seventh days) to extract platelets. Platelet activation rate was marked with CD62p antibody.

⑤ Long term toxicity study in vivo, we added the following experiment: the rats in the last group (hmSi-CREKA-RB-PFH +US) were kept for one month after treatment, and organs were used for HE staining.

Revision:

① In vitro cytotoxicity

The HUVECs were cultured in a DMEM medium at 37 °C and a 5% CO₂ atmosphere. After being planted in 96-well plates (10⁵/well), fresh medium containing hmSi-CREKA-RB-PFH at different concentrations (0, 50, 100, 200, 300, and 400 µg/mL) was added and incubated for 24, 48 and 72h. Following a standard protocol of CCK8 assay, the absorbance was measured at 450 nm by using a microplate spectrophotometer (BioTek).

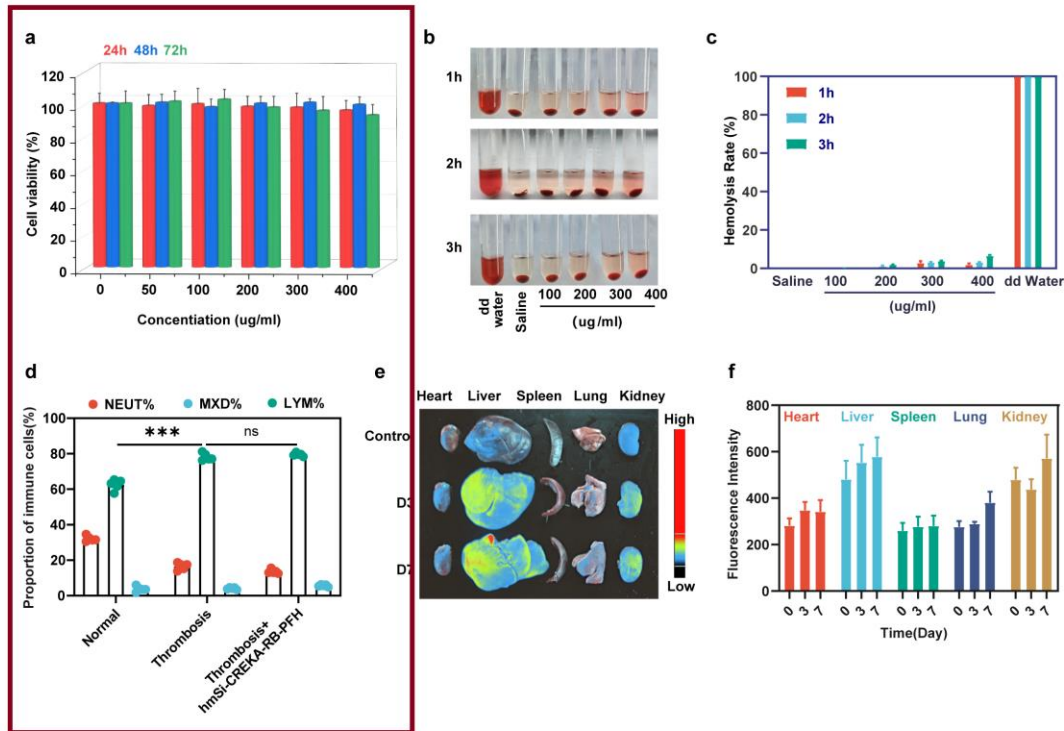


Figure S4. Biosafety of hmSi-CREKA-RB-PFH. **a** Cytotoxicity of hmSi-CREKA-RB-PFH with different concentrations through CCK8 detection kit. **b** Photographs and

c hemolysis rate of RBC after incubated with distilled water (positive control), saline (negative control), and hmSi-CREKA-RB-PFH of different concentrations for 1, 2, and 3 h, respectively. **d** Changes of immune cells after hmSi-CREKA-RB-PFH injection. $n=5$. Mean $\pm$ SEM, one-way ANOVA analysis, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. **e** Fluorescence image and **f** fluorescence intensity of major organs with hmSi-CREKA(FITC)-RB-PFH injection for successive 3 and 7 days.

② Platelet activation and blood inflammatory test

hmSi-CREKA-RB-PFH (0.2 mL, 5 mg/mL) was injected through tail vein consecutive three days. The blood was taken at first and seventh day to extract platelets. EDTA-anticoagulated blood was centrifuged at $500 \times g$ for 20 min to isolate platelets. Platelet-inhibition buffer was prepared as PBS buffer containing 1 mM EDTA, 2 μ M prostaglandin E1 (PGE1, Sigma Aldrich) and protease inhibitor (Beyotime, P1005). The supernatant was collected and mixed with platelet-inhibition buffer at a ratio of 1:1 to prevent platelet activation. Platelets were then collected by centrifugation at 800g for 20 min at room temperature. After discarding the supernatant, the platelets were resuspended in platelet-inhibition buffer, 30 μ l of this dilution was stained with 1 μ l of anti-mouse/rat CD62P-PE (1:30, BioLegend, 148305) for 30min and washed with 1 ml PBS. Platelets from 50 μ l of this last dilution were measured on a BD LSRFortessa™ flow cytometer. EDTA-anticoagulated blood from control group (without treatment) was set as negative group and the platelets stimulated with 5 μ M PMA for 1 min at 37 °C while stirring (900 rpm) was set as positive group.

For the inflammatory response, the blood at the same time point was sent to the clinical laboratory for testing.

③ We further examined blood inflammation after injection of submicron particles, the proportion of lymphocytes increased by 25.1% after thrombosis and remained unchanged after continuous injection of hmSi-CREKA-RB-PFH for three days (Figure S4d), indicating that the early inflammation may attributed to the thrombosis progress, and the submicron particles would not further trigger the inflammatory response.

To further elucidate the long term therapeutic efficacy mediated by our combination therapy, the blood samples and the recanalized vessels one day and one week after treatment were harvested. Hematological parameters showed that the injection of the hmSi-CREKA-RB-PFH and the treatment process did not further exacerbate the inflammatory response caused by thrombosis (Figure S4d and S21a). Although platelets showed slight activation (2.11%) on the first day after treatment, this phenomenon subsided at seventh day after recanalization (Figure S21b), during which no secondary embolism was observed. Histological staining of major organs showed no long term toxicity in the combination therapy group (Figure S21c).

④ For the four different treatment groups, after three days of continuous dosing and treatment, the organs were further fixed in 4% paraformaldehyde and cut into frozen sections for H&E staining. Moreover, for a long term toxicity study, the rats in the fourth group (hmSi-CREKA-RB-PFH +US) were kept for one month after treatment, and organs were used for HE staining.

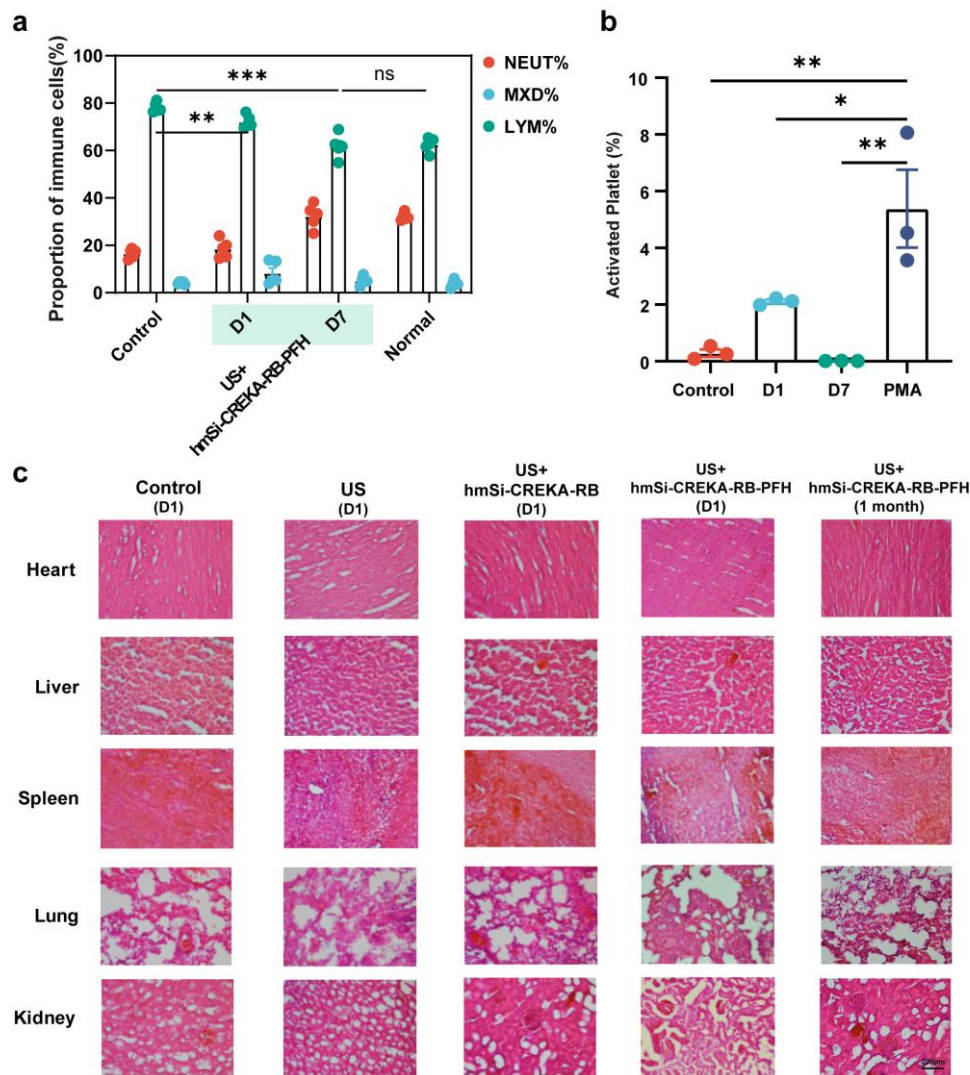


Figure S21. Safety assessment after treatment. **a** Proportion of immune cells in the blood of rats with different treatment (D1 and D7 means the first and seventh day after completing three consecutive days of treatment, respectively). $n=5$. NEUT%, neutrophil%. MXD%, monocyte%. LYM%, lymphocyte%. **b** Platelet activation rate in different time after treated with US+hmSi-CREKA-RB-PFH. $n=3$, Mean $\pm$ SEM, one-way ANOVA analysis, $*P<0.05$, $**P<0.01$, $***P<0.001$. **c** H&E staining of major organs after different treatment

Comment 2: section 2.2--how were the thrombi exposed to the different groups? This is never made clear in the results. if the thrombi were from different patients how so we know if there were dissimilar to begin with? What types of thrombi, from where? Treatment of NETS is mentioned but how were the NETS treated in what setting?

What is the control for the involve experiment? Groups are not clearly mentioned.

Response: Thank you for your comment. We apologize for the difficulty caused by the lack of detail in our writting. We have revised the manuscript carefully.

① The clinical thrombi used in our manuscript were obtained from acute ischemia stroke (AIS) patients who have failed intravenous thrombolysis within the therapeutic time window (4.5h) and underwent endovascular thrombectomy within 24 hours from symptom onset. We divided every clinical thrombus into four parts as evenly as possible and placed it in four groups for in vitro treatment. From this point of view, there was no difference in the blood clots used between the groups.

② For the treatment of NETs, there are three levels: cell induction in vitro, thrombi samples from clinical patients, and treatment of rat thrombus models. There is no specific time to start in vitro treating, we could perform operation after finished in vitro induction or receipt of clinical samples. For thrombus therapy in rats, it started on the fourth day after the thrombus model was constructed. According to literature reports, during all stages of ischemic stroke, neutrophils producing intravascular and intraparenchymal neutrophil extracellular traps (NETs) peak at 3–5 days(*Nature Communications* 11, no. 1 (2020). <https://doi.org/10.1038/s41467-020-16191-y>), and a large number of NETs were indeed observed in our rat thrombus sections on the fourth day. Therefore, we chose the fourth day as the starting.

③ The control group in our manuscript means that nothing has been done. Such as thrombi therapy in rats, the control group is rats with occluded blood vessels without any drug injection or ultrasound treatment, representing a completely negative control group.

Revision:

Thrombi obtained from AIS patients were used for ex-vivo characterization. To rule out the heterogeneity of clinical thrombi, each thrombus was divided into four parts evenly and the groups were set as follows: control, US, US + hmSi-CREKA-RB(SDT), and US+ hmSi-CREKA-RB-PFH (combination therapy of SDT and mechanical-blasting).

It was reported that NETs peaks at 3-5 days after stroke²⁴, and we found that 4 days

after thrombosis, a great amount of NETs appeared (Figure S9), so we chose the fourth day as the starting time of treatment.

Special thanks to you for your good comments. We have submitted the revised version (with marks in red) and pure version (without marks) in the PDF format of manuscript and Supplementary Information. We have tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the corrections will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Reviewer #3 (Remarks to the Author):

The authors have diligently answered most of our questions. This has significantly improved the quality of this manuscript, including supplementary experiments and controls that clearly enhance the scientific demonstration in the proposed article. However, several major unanswered comments remain that must be answered to allow for a successful publication. These are, following our initial comments:

Response to reviewer 3

We would like to thank you for careful attention to our manuscript. Your suggestions and comments have been carefully considered and the manuscript has been revised to address the points you have raised, as shown below. We hope this revision can make our paper more acceptable.

Comment 1: Comment 1/6: Regarding the liquid-to-gas transition of PFH, a picture of 5 Eppendorf tubes (Fig. S3A) is not sufficient to assess that gas was indeed formed (a simple agitation by hand could produce such bubbles): this was not overlooked but

deemed not enough. A quantitative method would be desired, which might include quantitative assessment of Fig. S3B.

Response: Thanks for your comment. We more intuitively presented the formation of bubbles by shooting videos, and added the quantitative statistics on Fig. S3B in the revised manuscript.

Revision: The US mediated phase transition of PFH was also be explored. Digital photos confirmed the US responsive behavior of PFH-capsulated submicron particles, and PFH bubbles gradually generated and be visualized under the US for 20 min (Figure S3a and supporting video 1). PFH can act as an echo contrast agent, the contrast of ultrasound images increased with the duration of US (Figure S3b and S3c).

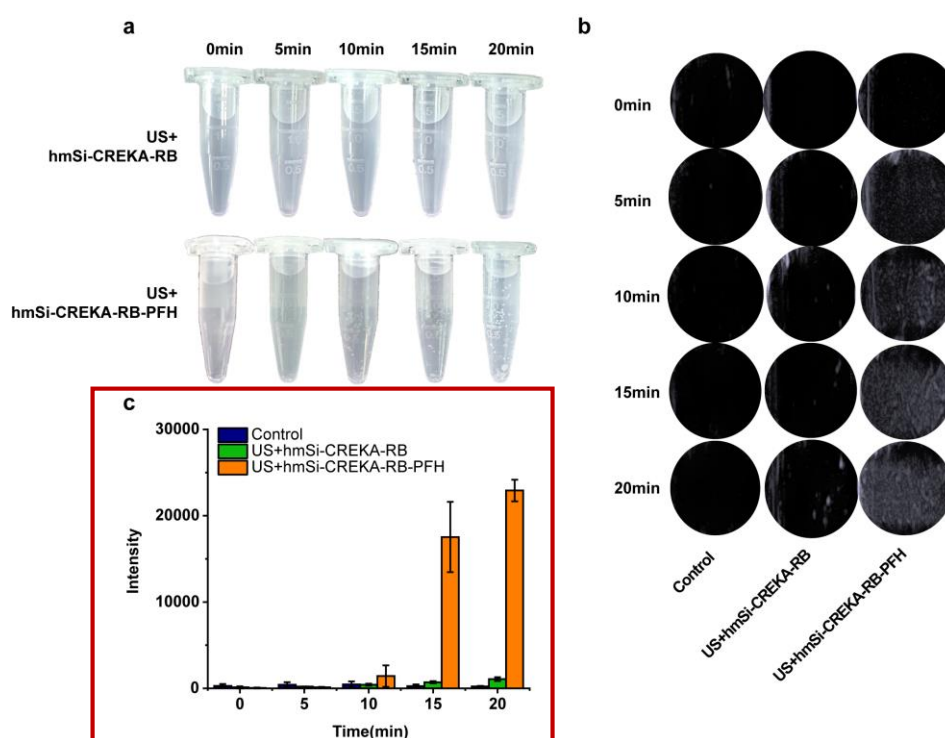


Figure S3. Phase transition of PFH. **a** Digital photos of the US-mediated phase transition. **b** Resolution improvement of hmSi-CREKA-RB-PFH under ultrasound irradiation. **c** Signal of ultrasound images.

Comment 2: Comment 4: Since image analysis can be heavily dependent on the analysis protocol used, the authors should state their protocol extensively: did they measure by hand the distances, for each particle? Did they resort to image treatment and automation? The distribution curves are needed to check whether the number of analyzed particles is high enough to provide a fine estimation of size distribution: it is

also needed as distribution width is often paramount to characterize nanoparticles. Moreover, the PDI for DLS measurements was also not provided, nor the intensity distribution. Finally, the salinity of buffers used for Zêta potential was not given, even though it is generally known that salinity has a strong impact on measured Zêta potential (see <https://www.materials-talks.com/zeta-potential-in-salt-solution-or-any-other-ions/> for instance).

Response: Thanks for your comment. ① We used image-J software to count the diameter and shell thickness according to the TEM. By building a scale, then measured 100 particles and calculate the arithmetic average. We have added the distribution curves in the revised manuscript (Figure S1a and S1b). ② The intensity distribution and PDI for DLS measurements were supplied in Figure S1c. ③ Both DLS size and Zeta potentials were measured in aqueous solution with a concentration 1 mg/mL, so the test data is not affected by salinity.

Revision:

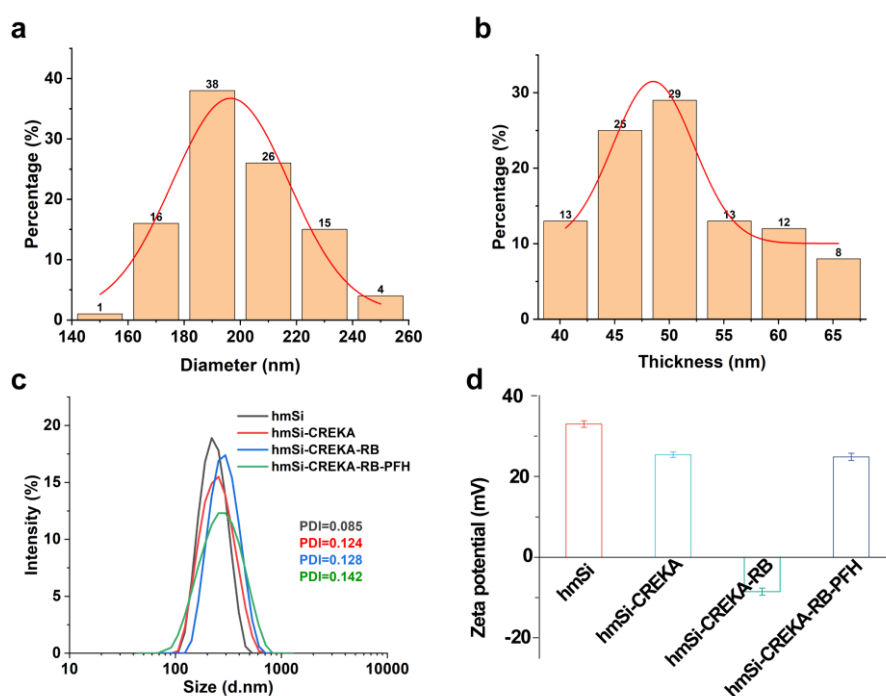


Figure S1. Characterization of hmSi-CREKA-RB-PFH. **a** Diameter and **b** Thickness of hmSi. **c** Intensity distribution of DLS and **d** Zeta potentials of hmSi, hmSi-CREKA, hmSi-CREKA-RB and hmSi-CREKA-RB-PFH.

Comment 3: Comment 7: Regarding safety of the objects, since the authors recognize

the toxicity of the particles under US excitation, other experiments should be made to prove the non-toxicity to surrounding tissues. A coculture of endothelial cells with separated NPs (with a Transwell device for instance) under activation could be a good way to check that no toxicity is exerted over a minimal distance of a few microns to millimeters since they estimate the diffusion of singlet oxygen to less than 1 micron. Moreover, the data of recanalized blood vessels, although apparently showing slow rebuild, first demonstrates high damage and inflammation overall. Without a control to prove that this is not due to NPs, this could prove too high damage to be viably used; thus controls with recanalization without the use of NPs (through rtPA or surgery) is needed to confirm that, as authors claim, the damages are due to recanalization and not the toxicity of activated NPs. One could also imagine activating the NPs without presence of thrombi and check for resulting damages.

Response: Thanks for your comment.

- ① We tried the transwell device to investigate the effect of activated nanoparticles on the survival of endothelial cells, but endothelial cells continuously leak through these small pores under ultrasound, making it difficult to characterize their subsequent cell activity effectively. Therefore, a new alternative method was devised: The endothelial cells were planted on the cell climbing sheets. The nanoparticles dispersed in medium (400ug/mL) were added into the 6-well plate with 3mL per well. Let stand for 3 hours to allow the nanoparticles settle to the bottom naturally. The cell strainer (Biofil, 70 um) equipped with the cell climbing plate was suspended in the 6-Well cell culture plates, and ultrasonic treatment was performed for 5 minutes ($1.5\text{W}/\text{cm}^2$) apart 1cm of pork. At last, the cells were stained with Calcein-AM/PI and plated in laser confocal dish for observation. As shown in the follow figure, when the nanoparticles were activated, the cell survival rate has slightly decreased, but still remained above 98%. Combined with the singlet oxygen lifetime ($3.5\mu\text{s}$) and diffusion distance (no more than 155 nm) mentioned in the literature(*Chem Soc Rev* 39, no. 8 (Aug 2010): 3181-209. <https://doi.org/10.1039/b926014p>), we believe that the activated nanoparticles are negligible toxic to the surrounding tissues.

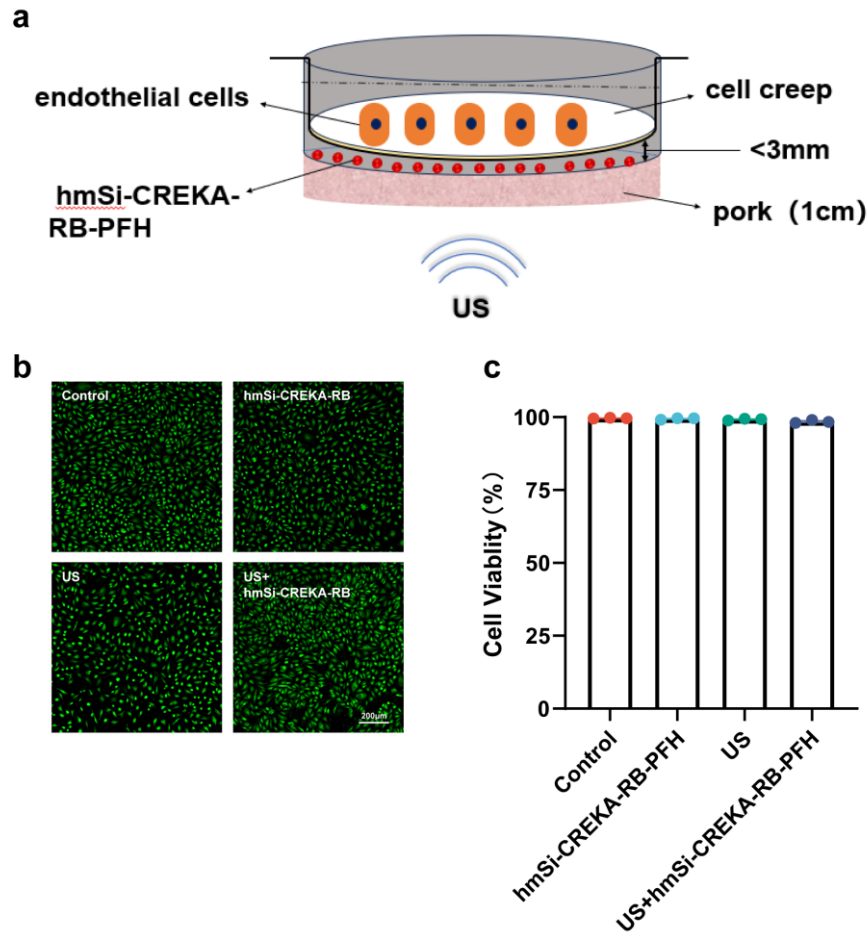


Figure: Cell fluorescence staining used to characterize the effect of activated nanoparticles on endothelial cells. **a** Scheme illustration. **b** Calcein-AM/PI staining and **c** Quantification of cell viability.

② For the initial damage and inflammation, we are inclined to believe that it was caused by coating of ferric chloride and blockage of blood vessels during thrombus modeling, rather than the toxicity of nanoparticles. So we added a blood routine test, and the five groups were: normal rats, ferric chloride induced thrombus group (thrombosis), thrombus group was injected with nanoparticles (0.2mL, 2mg/mL) for three consecutive days (thrombosis +hmSi-CREKA-RB-PFH), and thrombus group performed three days of treatment followed by blood collection on day 1 and day 7 (thrombolysis D1 or D7). All blood samples were tested at the hospital's clinical laboratory.

The blood test results indicated that the process of thrombosis significantly changed the

proportion of immune cells in rats, and further administration of hmSi-CREKA-RB-PFH (0.2mL, 2mg/mL) for three consecutive days didn't exacerbate the inflammatory response. Additionally, the inflammatory response in treated rats subsided a day after recanalization and returned to normal levels after seven days.

Revision:

We further examined blood inflammation after injection of submicron particles, the proportion of lymphocytes increased by 25.1% after thrombosis and remained unchanged after continuous injection of hmSi-CREKA-RB-PFH for three days (Figure S4d), indicating that the early inflammation may attributed to the thrombosis progress, and the submicron particles would not further trigger the inflammatory response.

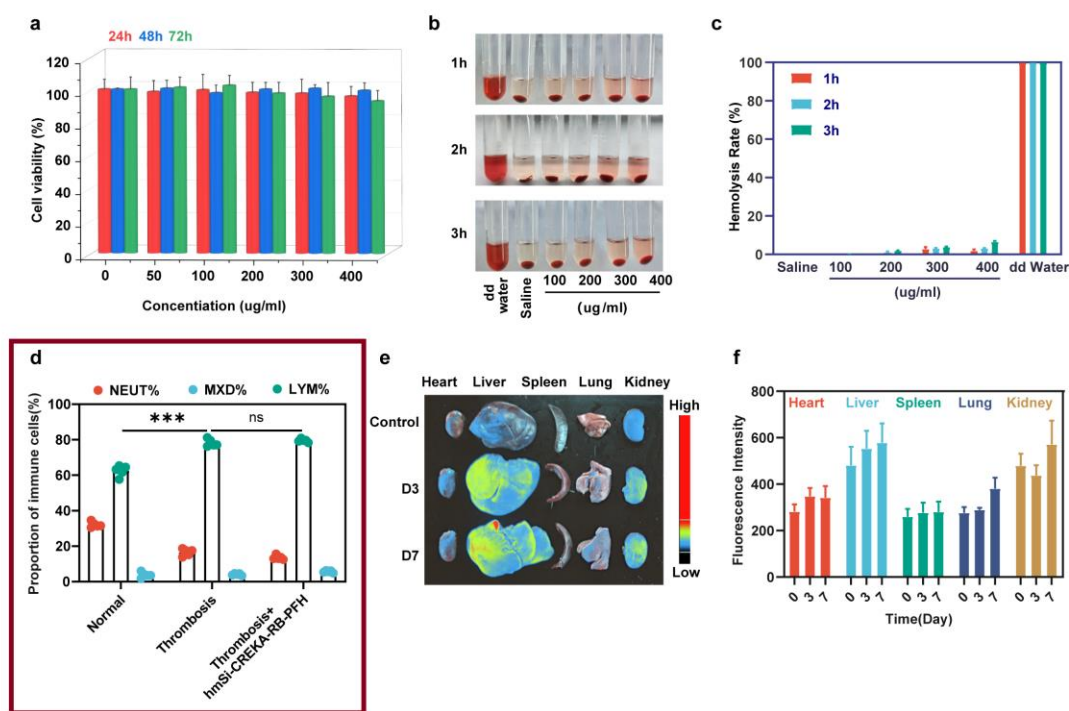


Figure S4. Biosafety of hmSi-CREKA-RB-PFH. **a** Cytotoxicity of hmSi-CREKA-RB-PFH with different concentrations through CCK8 detection kit. **b** Photographs and **c** hemolysis rate of RBC after incubated with distilled water (positive control), saline (negative control), and hmSi-CREKA-RB-PFH of different concentrations for 1, 2, and 3 h, respectively. **d** Changes of immune cells after hmSi-CREKA-RB-PFH injection.

n=5. Mean±SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001. **e** Fluorescence image and **f** fluorescence intensity of major organs with hmSi-CREKA(FITC)-RB-PFH injection for successive 3 and 7 days.

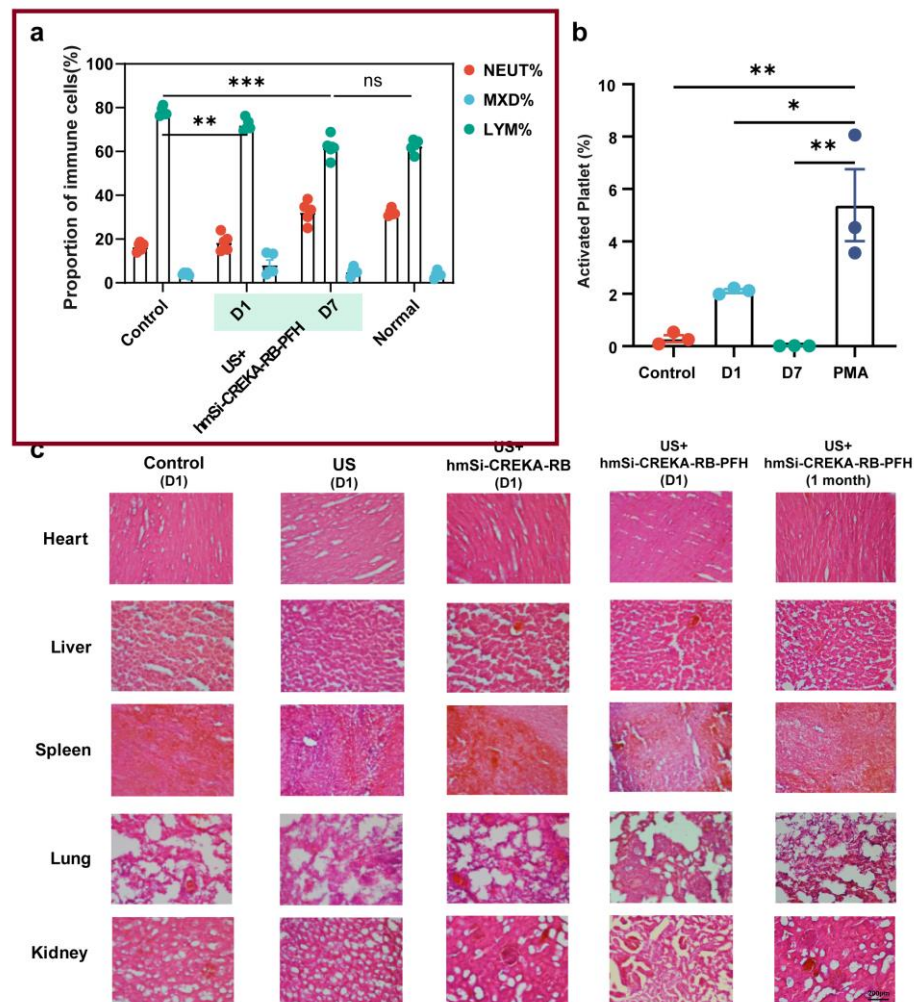


Figure S21. Safety assessment after treatment. **a** Proportion of immune cells in the blood of rats with different treatment (D1 and D7 means the first and seventh day after completing three consecutive days of treatment, respectively). n=5. NEUT%, neutrophil%. MXD%, monocyte%. LYM%, lymphocyte%. **b** Platelet activation rate in different time after treated with US+hmSi-CREKA-RB-PFH. n=3, Mean ± SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001. **c** H&E staining of major organs after different treatment

Comment 4: Comments 11/12/13: Since image analysis can be heavily dependent on

the analysis protocol use, the authors should state their protocol extensively: the sentence “The nuclear numbers of MPO-positive and Cit3H4-positive in the traced area were determined in 0.102/mm2 regions” is not clear. How were positive areas defined (manual thresholding?). A clear image analysis protocol is needed.

Response: Thanks for your suggestion. We have added relevant literature(*Faseb j* 35, no. 9 (Sep 2021): e21835. <https://doi.org/10.1096/fj.202100471RR>) and the detail about analysis protocol in the revised manuscript.

Revision: Images were processed using specialized software packages (ZEN Viewer and ImageJ). The nuclear numbers of MPO-positive and Cit₃H₄-positive in the traced area were determined in 0.102 mm² regions according to the literature. Using 40X confocal images, each with an area of 0.102 mm², we quantified NETs by manually counting the number of MPO positive (green) and Cit₃H₄ positive (red) co-locators.

Comment 5: Comment 15: Why are NETs not stained by DAPI? This is surprising as NETs DNA should be stained by both SytoxGreen and DAPI.

Response: Thanks for your comment. We did stain NETs with both SytoxGreen and DAPI (as shown in following single channel picture), but we are sorry that we did not clearly label it. We have now made modifications to address this issue in the in the revised manuscript.

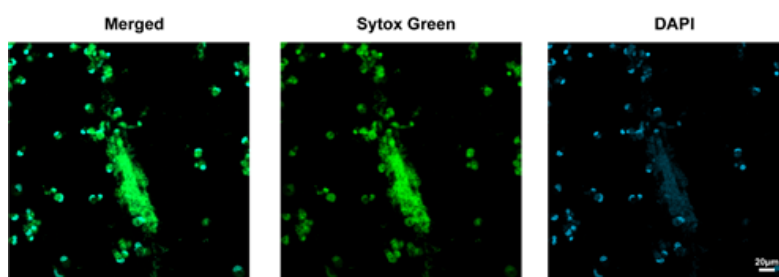


Figure: NETs staining of SytoxGreen and DAPI

Revision:

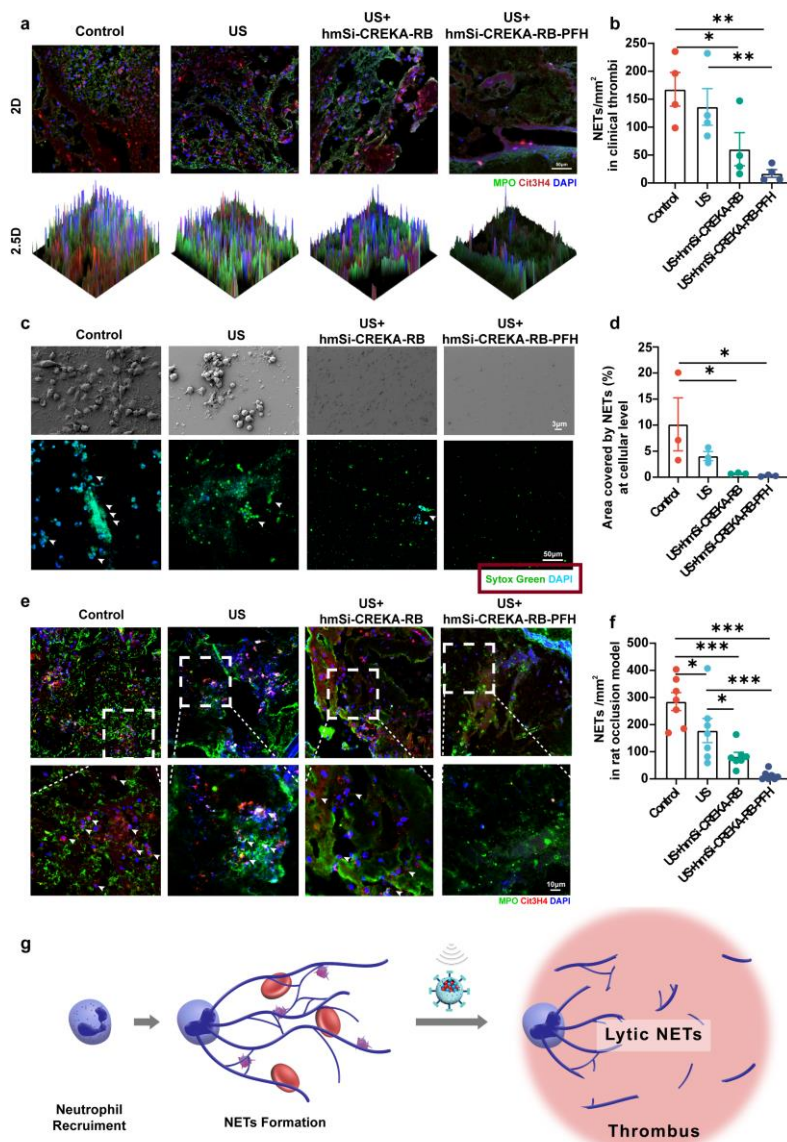


Figure 2. Theranostic platform caused lytic NETs. **a** Immunostaining of NETs in clinical thrombi after different treatments. **b** Quantification of NETs/mm² in clinical thrombi. n=4. Mean±SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001. At the cellular level, NETs were generated by treating neutrophils with PMA. **c** SEM images of NETs in different treatment groups and the corresponding Immunostaining with SYTOX GREEN at the cellular level. Arrows point to NETs (neutrophils and the filamentous structure around the nucleus). **d** The proportion of NET-like structures at the cellular level, n=3. Mean±SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001. **e** Immunostaining of NETs in rat carotid artery thrombus in different groups. Arrows point to NETs (DNA colocalized with Myeloperoxidase and Cit3H4). **f** Quantification of NETs/mm² in rat carotid artery

thrombus. n=7. Mean±SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001. **g** Scheme of the formation and lytic of NETs in thrombus.

Comment 6: Comment 17: One image is not enough to demonstrate anything. The authors need to show quantitative data with relevant replicate number N.

Response: Thanks for your comment. We quantified the fluorescence intensity and added it to the revised manuscript (Figure S18b).

Revision: Then, the target ability of the hmSi-CREKA-RB-PFH was demonstrated, obvious fluorescence signal of FITC-labelled CREKA at thrombus site could be seen after tail vein injection (Figure S18a). There was no significant difference in fluorescence area after 1 hour, so all the treatment started 1 hour after injection (Figure S18b).

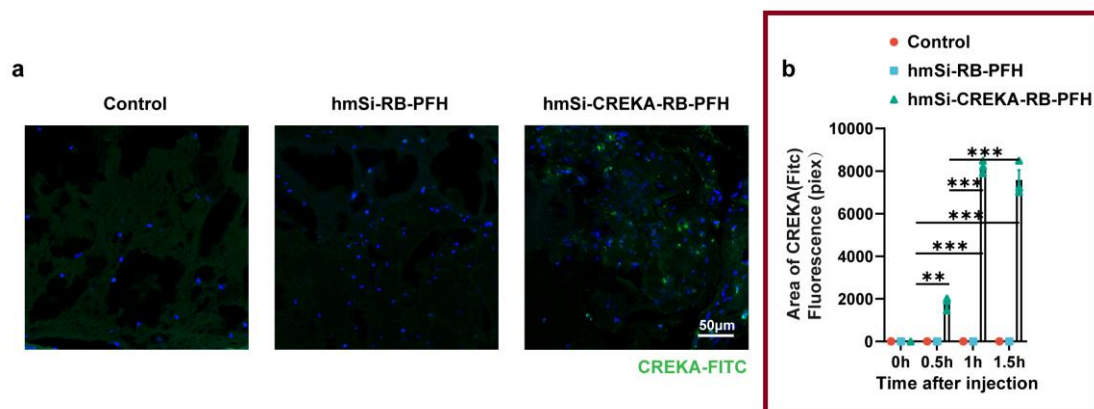


Figure S18. The thrombus-targeting ability of hmSi-CREKA(FITC)-RB-PFH. a Fluorescence images and **b** Statistics of fluorescence area after different injection time. Mean ± SEM. n=3, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001.

Minor points:

Comment 7: Additional comment to the RNA sequencing: can the authors give the baseline that was used to measure relative expressions changes?

Response: Thanks for your comment. In the RNA sequencing part, there are two groups: the control group was carotid vessels of normal rats, and the other group was treated with combined therapy after thrombolysis. The blood vessels were collected on the first and seventh day after patency. So the baseline was normal rat's carotid vessels

expression.

Comment 8: Comment 13: Nowhere are the dipeptides defined as N-epsilon-gamma-glutamyl-Lysine bond (which are only evoked once in the abstract). They should be defined in the “Detailed analysis of the composition...” paragraph of the intro.

Response: Thanks for your comment. We have supplemented the definition of dipeptides in the revised manuscript.

Revision: During the thrombosis process, the conversion of fibrinogen to fibrin immediately follows after platelet activation. And under the influence of factor XIII (FXIII), glutamate and lysine among fibrin-monomers will be crosslinked into a covalent bond, the ϵ -(γ -glutamyl)lysine isopeptide bond, consolidating the thrombus to withstand the blood shearing force¹⁰⁻¹², cross-linking of fibrin makes the clot more resistant to the fibrinolytic system.

Comment 9: Comment 14: The proposed modification by authors is satisfactory. However, this additional setup is not explained neither in the methods nor the manuscript. It should be added, especially since the resulting conclusion is important: thus the authors should comment in the text about how this setup allowed them to show that heat alone with US could destroy bonds, but that a more physiological setup with flow could show that the NPs were necessary in that case for thrombolysis.

Response: Thanks for your suggestions. We added the additional setup in the revised supplementary information and the influence of thermal effect was discussed.

Revision: At first, we found a huge decrease of NETs happened on US group (Figure S5a). Given that photothermal therapy has been reported as a new thrombolysis method, and considering the potential thermal effects generated by continuous US irradiation, we applied an extracorporeal circulation device (Figure S5b) to exclude the influence of heat.

In vitro experiments, we found that NETs and isopeptide bond levels were also significantly reduced based on ultrasound alone, which we suspected might be related to the thermal effect caused by ultrasound. This idea was verified by designing a

flowing water system. However, heat accumulation is less in vivo due to the skin barrier and the circulatory system, indicating the single effect of US is limited, and effective thrombolysis requires a combination of more powerful methods (such as we propose nanomaterials that are sensitive to ultrasound) .

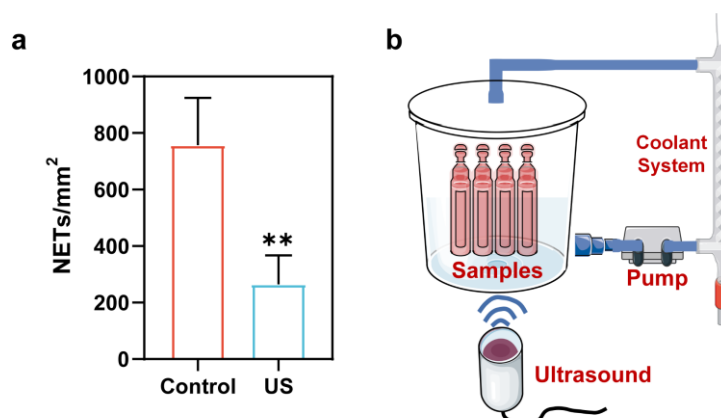


Figure S5. Removal of thermal effect. **a** Quantification of NETs/mm² in clinical thrombi. n=4. Mean±SEM, one-way ANOVA analysis, *P<0.05, **P<0.01, ***P<0.001. **b** Schematic diagram of extracorporeal circulation device.

Comment 10: Comment 18: the “D-dimer“is never introduced: is it the isopeptide bond degradation product? How is measured? And why did the authors remove the FDP data?

Response: Thank you for the comment. D-dimer consists of two covalently bound fibrin D domains, produced when plasmin cleaves fibrin, so it is a specific biomarker of fibrinolysis(*Thrombosis Research* 189 (2020): 5-12. <https://doi.org/10.1016/j.thromres.2020.02.009>).

According to the literature, stabilized fibrin degradation results in the generation of a heterogeneous mixture of so-called “fibrin degradation products” (FDPs), differing mostly in size and composition. Some enzymes may also breakdown fibrinogen to create fibrinogen degradation products. However, D-dimer generation only occurs through the breakdown of stabilized fibrin, thus making it a highly specific biomarker of fibrin degradation(*Blood* 113, no. 13 (2009): 2878-87. <https://doi.org/10.1182/blood-2008-06-165845>; *Clinical Chemistry and Laboratory Medicine (CCLM)* 61, no. 5

(2023): 841-50. <https://doi.org/10.1515/cclm-2022-0633>). So we removed the FDP data and used D-dimer level to characterized fibrinolysis. We sent the blood samples to the clinical laboratory of the hospital to determine the concentration of D-dimer by turbidimetric inhibition immuno assay.

Revision: D-dimer is a specific biomarker of fibrin degradation. Blood samples in normal, thrombosis, and thrombolysis periods were collected for D-dimer detection.

Comment 11: Finally, language quality is not good enough, with as pointed out by another reviewer instances of non-idiomatic expressions that could be generated by automatic translators or text generators. Thus, the authors must proofread their manuscript with the help of a native English speaker to use appropriate expressions. Examples in the introduction include: “lengthy thrombi”, “According to the progression of thrombosis”, in-depth research based on tPA-resistant”, “Along with time, the recanalized”, “liquid to gaseity”, “due to the strongly hydrophobic about PFH”, “loaded. Which indirectly “...

Response: Thank you for the comment. We have carefully reorganized the logic of the manuscript, added more experimental details, and strengthened analysis of the results. We also re-checked and revised the spelling, word using and grammar to make our manuscript more readability. Hoping that the current manuscript can better meet the standards for publication.

Comment 12: The introduction mentions “To date, as the most commonly used thrombolytic treatment, tissue plasminogen activator (tPA) can lead to fibrinolysis.” tPA always aims for fibrinolysis (due to its mechanism of action) and then acts as a thrombolytic. Rephrase.

Response: Thanks for your valuable comments and we have revised this sentence.

Revision: To date, tissue plasminogen activator (tPA) can lead to fibrinolysis by transforming plasminogen to plasmin, which made it the most commonly used thrombolytic treatment.

Comment 13: Fig. S4D: What is “thrombin time”? Please explain.

Response: Thanks for your comments. Thrombin time (TT) is the time it takes for blood to clot after standardized thrombin is added to the plasma([Chapter 133 - Thrombin Time and Fibrinogen Evaluation." In *Transfusion Medicine and Hemostasis \(Third Edition\)*, edited by Beth H. Shaz, Christopher D. Hillyer and Morayma Reyes Gil, 793-98: Elsevier, 2019; Determination of Fibrinogen and Thrombin Time \(Tt\)." In *Hemostasis and Thrombosis: Methods and Protocols*, edited by Emmanuel J. Favaloro and Giuseppe Lippi, 105-10. New York, NY: Springer New York, 2017\)](#)). We compared the thrombin time after three consecutive days of treatment with normal rat blood, and there was no significant difference, indicating that the treatment method proposed in this manuscript had no effect on the coagulation system.

Special thanks to you for your good comments. We have submitted the revised version (with marks in red) and pure version (without marks) in the PDF format of manuscript and Supplementary Information. We have tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers’ warm work earnestly, and hope that the corrections will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Reviewer #4 (Remarks to the Author):

In this manuscript authors adequately addressed important as well as minor concerns previously raised by editing the text and images as recommended and by performing additional experiments.

Of note, they now include the use of US + hmSi-CREKA-PFH treatment as in vivo control in the rat occlusion model, thereby increasing the rigor of the study. These results demonstrate that US + hmSi-CREKA-PFH treatment did not improve lumen

patency compared to challenged control animals and establishes more definitely the improved efficacy of US + hmSi-CREKA-RB-PFH.

Next, they deepen RNA sequencing analysis to investigate immune and endothelial cell responses to the US + hmSi-CREKA-RB-PFH at 1 and 7 days after treatment and additionally show that the vasculature recover at 28 days. Additional analysis of VE-Cadherin staining for EC junction stability (or dextran perfusion) and perivascular/smooth muscle cell counts would improve this data in establishing complete recovery of the vasculature.

Rigor is additionally improved by including n=3 animals in the pig occlusion animal model so that blood velocity, D-dimer and lumen patency could be analyzed statistically.

Response to reviewer 4:

We would like to thank you very much for your comments. Your suggestions have been very helpful to us. We have revised the manuscript very careful as follows and hope it is more suitable for publication in *Nature Communications*.

Response: Thanks for your comment. The endothelial connectivity and smooth muscle reconstitution of samples at different time (1, 7, and 28 days) were analyzed by immunofluorescence staining. The data was added in Figure 7 and S22.

Revision:

The results of Gene Set Enrichment Analysis (GSEA) also showed that developments related to vascular smooth muscle were upregulated (Figure S22d), immunofluorescence staining revealed that the reconstruction of smooth muscle in recanalized vessels approached that of normal blood vessels 28 days later (Figure 7c and 7d).

Given the importance of endothelial cells (ECs) in maintaining the long term patency of the vessels, the morphology of ECs and its biological functions were tested. ZO-1, a tight- junction protein between cells, also indirectly reflects the reconstruction of ECs. There was no reconstruction of α -SMA and ECs in the control group (Figure S23a-S23f). As for recanalized vessels, almost no ECs were observed at the completion of

treatment due to the damage brought by thrombosis (Figure 7c and 7e). ECs with poor continuity and large gaps were observed after 7 days of reperfusion. Over time, there was a distinct layer of ECs with tight connections at 28 days (Figure 7f and S23f). The number of endothelial cells per unit area increased with time (Figure 7e), indicating that the unobstructed blood flow is the premise of vascular reconstruction. As an important enzyme in cardiovascular system, endothelial nitric oxide synthase (eNOS) can catalyze the generation of nitric oxide (NO) to regulate vasomotion. The blood vessels after treatment were taken at 0, 7, and 28 days for eNOS immunofluorescence staining, and the display of eNOS showed a consistent reconstruction trend with the ECs (Figure 7g and S23g).

Supplementary Information: Functional verification of the recanalized vessels

The treated vessels and the contralateral normal blood vessel were harvested at Day 0, Day 7 and Day 28 after recanalization. A portion of the blood vessels were fixed with 4% paraformaldehyde and cut into frozen sections for immunofluorescence staining of CD31(1:100, abcam, ab281583), α -SMA(1:200, Boster, BM00020), ZO-1(1:200, Proteintech, 21773-1-AP) and eNOS (1:50, abcam, ab300071) to testify the morphology of endothelial cells and its ability to generate nitric oxide.

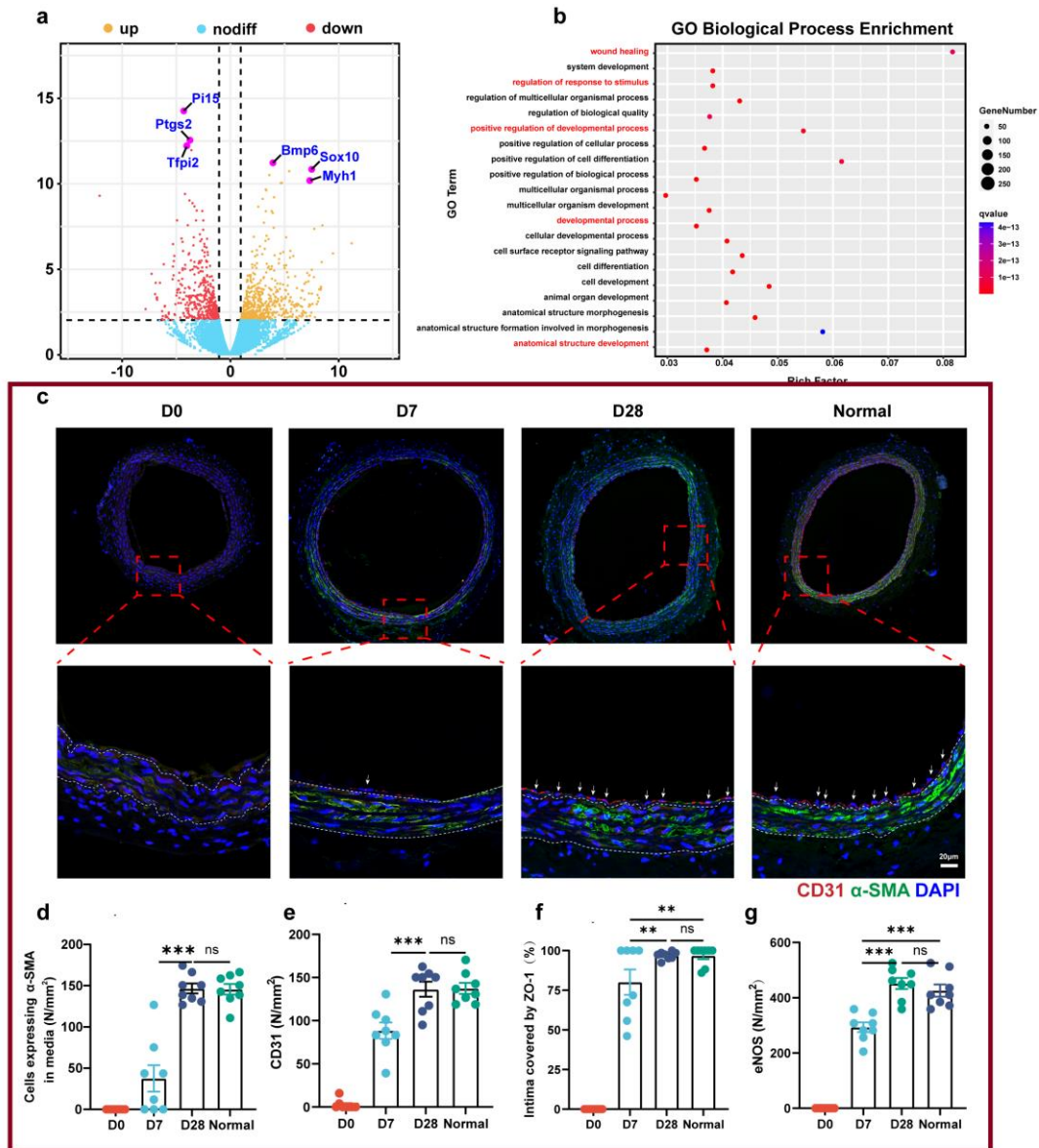


Figure 7. Reconstruction of the recanalized vessels treated with US responsive theranostic platform. **a** Volcano plots showing the identified upregulated and downregulated genes a day and a week after the combination therapy ($|\text{fold-change}| \geq 1.0$ with $q \text{ value} < 0.05$). $n=3$. **b** GO biological process analysis of genes significantly changed between the two groups ($|\text{fold-change}| \geq 1.0$ with $P \text{ value} < 0.05$). **c** Immunofluorescence staining of CD31 and α -SMA. The number of **d** α -SMA, **e** CD31, **f** ZO-1 and **g** eNOS. $n=8$. Mean $\pm$ SEM, one-way ANOVA analysis, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.00$; ns, $P > 0.05$.

Special thanks to you for your good comments. We have submitted the revised version (highlighted revisions) and pure version in the PDF format of manuscript and Supplementary Information. We have tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the corrections will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I appreciate the very thorough revisions. I have 2 more requests.

1. The methodology for MSB color thresholding does not account for the fact that collagen will be stained blue. Either collagen needs to be accounted for separately in the analysis or the data needs to be more accurately described as collagen and fibrin, rather than just analysis of the fibrin.

2. The data re: efficacy of technology at 10cm is disappointing. This limitation needs to be addressed in the discussion, as it is highly clinically relevant given that the deep veins of the pelvis are commonly greater than this depth.

Reviewer #2 (Remarks to the Author):

Thanks to the authors for their responsive to my critiques. I still have a few more issues that need to be improved. Although overall the manuscript is improved.

1)The toxicity testing of the particles is not correctly stated. The authors write "The biological distribution analysis was performed to detect the aggregation of the submicron particles in major organs, and no remarkable accumulation after the three-time administration under the detection of fluorescence signal (Figure S4e and S4f). I am not sure what the authors are trying to show here. I meant by toxicity is are the organs showing normal morphology or are these abnormal changes. I don't understand the authors response. Is there a positive control for S4E and F. Is it not clear to me what this is showing.

2) It is not enough to say thrombi were obtained from AIS patients. more description is needed. How were they obtained? What were the patients receiving? Ask a clinician to help with this characterization which is very poor. Dividing the samples into 4 does not rule out heterogeneity. Was histological analysis performed to examine each of 4 sections used? How can you say change occurred in NETs when you did examine the specimens before treatment? Either rewrite to be accurate with what you did or perform what I have asked.

3) I don't see a schematic diagram for S5b? There is no B? How does extracorporeal device exclude heat?

3)NET staining in S8 not too convincing. You say a great amount of Nets appeared relative to what?

4)S21B you say no embolism occurred?

5)Procine occlusion model is poorly explained. I don't even understand what you did.

Reviewer #3 (Remarks to the Author):

The authors answered all my questions, with the exception of the one concerning Zeta potential measurements.

This has significantly improved the quality of this manuscript, including supplementary experiments and controls that clearly enhance the scientific demonstration in the proposed article.

However, when measuring surface charge, it is essential not to use osmosed or MilliQ water, as they do not contain sufficient salts in solution (no ionic strength) and can therefore generate differences in results.

In order to obtain comparable results and to be able to conclude on the surface modifications of the proposed nanoobjects, an aqueous solution with a controlled ionic strength should be used, such as a 1 mM KCl aqueous solution.

I recommend that authors carry out their potential zeta experiments not in water but in 1 mM KCl.

Reviewer #4 (Remarks to the Author):

This manuscript presents an ultrasound-based system which allows for the monitoring and effective trombolysis of tPA resistant thrombi. The authors have been highly responsive to prior reviewers' comments and now present data that are strong and convincing.

Response to the Review Comments

Reviewer #1 (Remarks to the Author):

I appreciate the very thorough revisions. I have 2 more requests.

Response: Thank you very much for your recognition. Your suggestions were very helpful to us. We added some staining experiment and discussion about the depth of ultrasound tissue penetration. We hope our manuscript is more suitable for publication in *Nature Communications*.

Comment 1. The methodology for MSB color thresholding does not account for the fact that collagen will be stained blue. Either collagen needs to be accounted for separately in the analysis or the data needs to be more accurately described as collagen and fibrin, rather than just analysis of the fibrin.

Response: Thank you for your question.

Firstly, according to the literatures (*Stroke*. 2021 Nov; 52 (11): 3718-3727); *Stroke* 2021 Mar; 52 (3): 1131-1142), collagen is not the main component in clinical thrombi, it rather serves as the starting point of thrombosis, that is, after endothelial cell injury, the exposed subendothelial collagen fibers activate platelets and coagulation factor XII, activating the endogenous coagulation system. However, some literatures suggest through H&E staining and Masson staining that collagen in specimens retrieved by thrombectomy were part of the blood vessels in contact with the thrombi or thrombectomy device. It may be pulled down during the thrombectomy and could be observed at the edge of thrombus (as shown below) (*J Neurointerv Surg*. 2019 Mar; 11 (3): 232-236.).

Secondly, H&E and MSB staining (Figure 4a and Figure S13a) in our manuscript, we did not observe these collagen regions reported in the literature. We further performed Masson staining and immunofluorescence staining of

type I collagen (the main components of blood vessel walls) on the obtained clinical thrombus to exclude the influence of collagen. There was no similar areas with the literature from Masson staining, and immunofluorescence staining did not show a positive collagen signal (n=8). In summary, we believe that collagen does not exist in the clinical thrombi used in our manuscript.

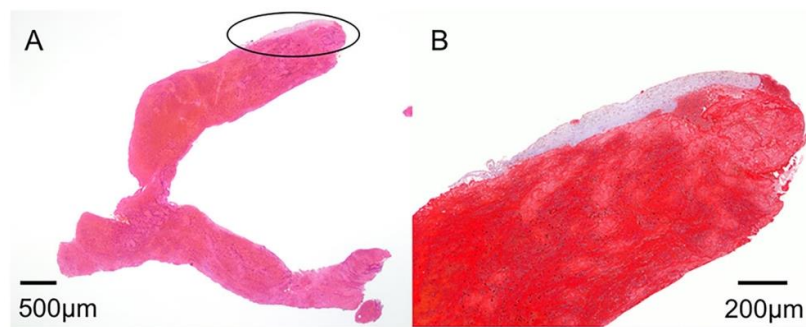


Figure: A) H&E staining B) Masson's trichrome. A piece of collagen fiber attached at the edge of the thrombus (ellipse) (*J Neurointerv Surg.* 2019 Mar;11(3):232-236.)

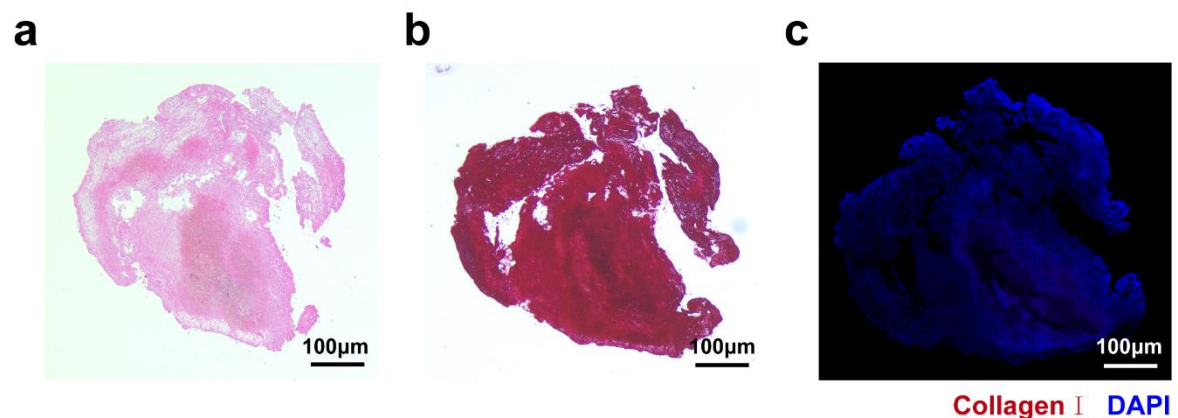


Figure: a) H&E staining, b) Masson staining and c) the immunofluorescence staining of type I collagen of clinical thrombus.

Comment 2. The data re: efficacy of technology at 10cm is disappointing. This limitation needs to be addressed in the discusison, as it is highly clinically relevant given that the deep veins of the pelvis are commonly greater than this

depth.

Response: Thanks for your suggestion, we believe that this limitation is indeed necessary to be raised in the discussion, as it is related to clinical practice. We have added a description of the limitations of ultrasound used in the article in the revised discussion.

Revision (Discussion Part): Additionally, in rat and pig trials, our designed theranostic platform has demonstrated good thrombolytic capability, but it may still be challenging in clinical application. For example, deep vein thrombus usually shielded by solid tissue or bone, which makes noninvasive ultrasound access difficult. However, the depth of tissue penetration of ultrasound depends on experimental parameters such as intensity, amplitude, focus, beam uniformity and effective radiating of transducer head. For instance, high intensity focused ultrasound has been used to treat tumors that are deeper than 10 cm. We believe that ultrasound-based therapies offer a great potential for clinical deep tissue thrombosis.

Special thanks to you for your good comments. We have submitted the revised version (with marks in red) and pure version (without marks) in the PDF format of manuscript and Supplementary Information. We have tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for your warm work earnestly, and hope that the corrections will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Reviewer #2 (Remarks to the Author):

Thanks to the authors for their responsive to my critiques. I still have a few more issues that need to be improved. Although overall the manuscript is improved.

Response: Thanks for your questions. Your suggestions help us to improve our manuscript. We have conducted supplementary experiments based on the following issues and sent our manuscript for scientific editing via Springer Nature Author Services to make it more readable (Order number: YDLPJR24). We hope our revised manuscript is more suitable for publication in Nature Communications.

Comment 1: The toxicity testing of the particles is not correctly stated. The authors write "The biological distribution analysis was performed to detect the aggregation of the submicron particles in major organs, and no remarkable accumulation after the three-time administration under the detection of fluorescence signal (Figure S4e and S4f). I am not sure what the authors are trying to show here. I meant by toxicity is are the organs showing normal morphology or are these abnormal changes. I don't understand the authors response. Is there a positive control for S4E and F. Is is not clear to me what this is showing.

Response: Thanks for your question. Organ fluorescence imaging usually used to verify in vivo distribution and biosafety of biomaterials (*Angew. Chem. Int. Ed.* 2020, 59, 13557 –13561; *Adv.Mater.*2023, 35, 2209789). In our manuscript, the organ fluorescence intensity represents the accumulation of submicron particles in major organs after injection. Inspired by your comments, we redesigned the experiment. The rats with carotid thrombosis were divided into three groups: without injection (Control), zero day after continuous injection (0d post injections), and seven days after continuous injection (7d post injections). The heart, liver, spleen, lung, kidney and blood vessels were

extracted for ex vivo fluorescence imaging (Figure S4d and S4e). H&E staining of major organs was also added in Figure S4f. There are two conclusions to be drawn from this experiment: ①The blood vessels with thrombus had obvious fluorescence, indicating that the submicron particles can target thrombus in vivo. ②Although there was a certain fluorescence signal in liver and kidney after continuous injections, it returned to the level of control group after 7 days, indicating that submicron particles could be metabolized and excreted, and would not accumulate in the organs for a long time, indicating its good biosafety.

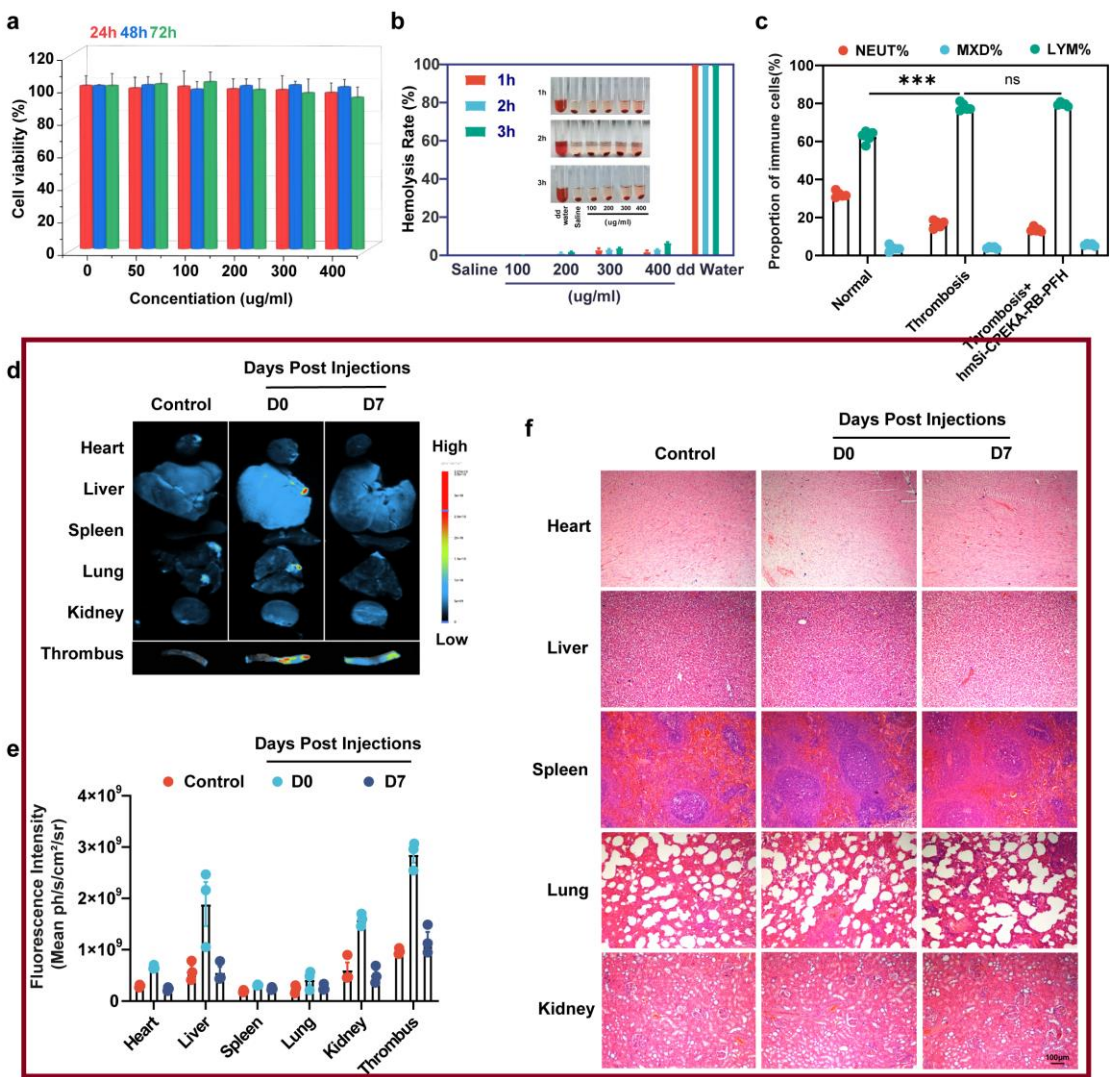


Figure S4. Biosafety of hmSi-CREKA-RB-PFH. **a** Cytotoxicity of hmSi-CREKA-RB-PFH with different concentrations through CCK8 detection kit. **b** Hemolysis rate of RBC after incubated with distilled water (positive control),

saline (negative control), and hmSi-CREKA-RB-PFH of different concentrations for 1, 2, and 3 h, respectively (inset the Photographs of RBCs in different groups). **c** Changes of immune cells after hmSi-CREKA-RB-PFH injection. n=5. Mean±SEM, one-way ANOVA analysis, ***P<0.001, ns, P>0.05. **d** Ex vivo fluorescence images and **e** fluorescence intensity of major organs and blocked vessels after hmSi-CREKA(FITC)-RB-PFH injection for three successive days. **f** H&E staining of major organs with hmSi-CREKA(FITC)-RB-PFH injection for three successive days.

Revision:

(Revised Manuscript): Organ fluorescence imaging is used to show the biological distribution of submicron particles in major organs (Figure S4d and S4e). The fluorescence of the injection submicron particles at the blocked vessels demonstrated its ability to specifically target thrombus. Additionally, after three days of injection, the fluorescence signals in the liver and kidney suggested that the particles might be excreted by these two organs. On day seven following delivery, these signals recovered to the same level as the control group. H&E staining demonstrated that it will not cause morphological changes in major organs, indicating it is biologically safe (Figure S4f).

(Supplementary Information): In Vivo Toxicity Assay

0.2 mL hmSi-CREKA(FITC)-RB-PFH (5 mg/mL) was injected into rats with carotid thrombus through tail vein for three days. The major organs of rats were harvested on day 0 and day 7 after injections for ex vivo fluorescence imaging (Vilber, Newton 7.0 Bio) and H&E staining, including heart, liver, spleen, lung and kidney. The vessels with thrombus were taken as positive control. Fluorescence intensity were analyzed in Kuant analysis software.

Comment 2: It is not enough to say thrombi were obtained from AIS patients. more description is needed. How were they obtained? What were the patients

receiving? Ask a clinician to help with this characterization which is very poor. Dividing the samples into 4 does not rule out heterogeneity. Was histological analysis performed to examine each of 4 sections used? How can you say change occurred in NETs when you did examine the specimens before treatment? Either rewrite to be accurate with what you did or perform what I have asked.

Response: Thank you for your comments. The clinical thrombi were obtained from acute ischemia stroke (AIS) patients who have failed intravenous thrombolysis within the therapeutic time window (4.5h), and underwent endovascular thrombectomy within 24 hours from symptom onset. The description of this section is located in the "Clinical Thrombi treatment and Immunofluorescence" section of the supplementary materials, and we have further described the source of thrombus in the revised manuscript.

We divided every clinical thrombus into four parts as evenly as possible and randomly placed them in four groups for ex vitro treatment. This method of ex vitro treatment is derived from references (*Stroke*. 2018;49:754–757; *Stroke*. 2019;50:3228–3237; *Circulation Research*. 2015;116:1182–1192.). To further clarify whether heterogeneity was excluded, we performed the full scan of NETs on the entire clinical thrombus (slice along the center point of the maximum diameter). The experimental results showed that NETs were uniformly distributed throughout the thrombus, and there was no significant difference in NETs content among four groups. In summary, we believe that the distribution of NETs on our obtained thrombi were relatively even, the exclusion of heterogeneity permits comparisons following different treatments.

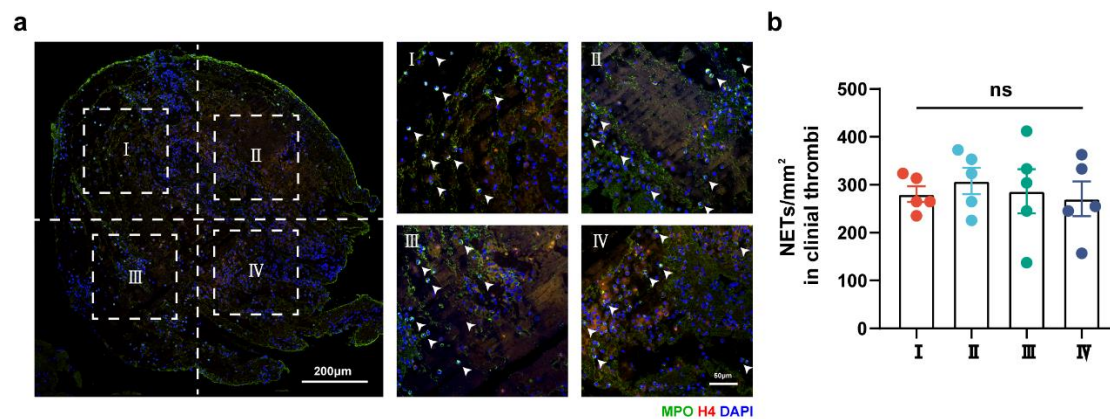


Figure: Immunofluorescence staining of NETs in clinical thrombi. **a** Full scan and **b** the number of NETs on clinical thrombi. $n=5$. Mean $\pm$ SEM, one-way ANOVA analysis, ns, $P>0.05$.

Revision (Revised Manuscript): We obtained clinical thrombi from AIS patients who have failed intravenous thrombolysis within the therapeutic time window (4.5h), and underwent endovascular thrombectomy within 24 hours from symptom onset.

Comment 3: I don't see a schematic diagram for 5b? There is no B? How does extracorporeal device exclude heat?

Response: Thank you for your question. Figure s5b was presented in our uploaded Supplementary Information (as shown below). The extracorporeal device generates the flow of water in the equipment through a peristaltic pump. When the water flows through the sample pool above the ultrasound, it will absorb US-induced heat and remove this part of heat through water circulation.

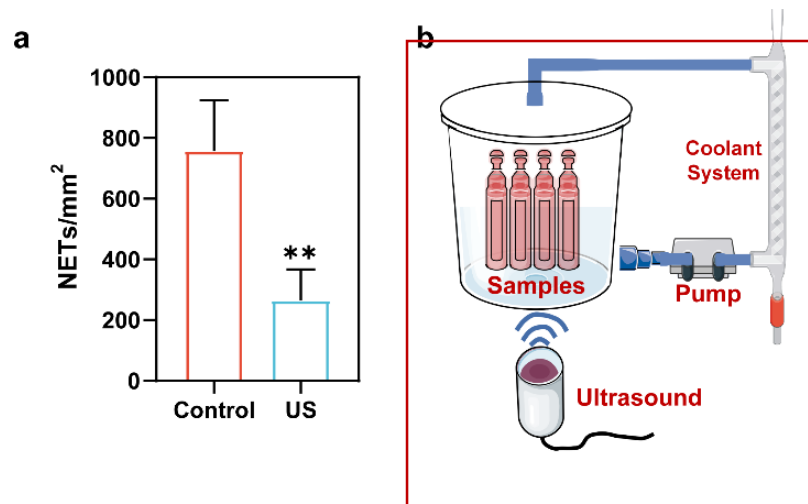


Figure S5. Removal of thermal effect. **a** Quantification of NETs/mm² in clinical thrombi. n=4. Mean±SEM, Independent-sample T test, *P<0.05, **P<0.01. **b** Schematic diagram of extracorporeal circulation device.

Comment 4: NET staining in S8 not too convincing. You say a great amount of Nets appeared relative to what?

Response: Thanks for your question. We chose the fourth day as the starting time of treatment because it was reported in the literature that NETs peak 3-5 days after stroke onset (*Nature Communications* 11, no. 1 (2020)). In order to further enhance the scientific validity of the data, we have conducted statistical analysis on the fluorescence of NETs from 0 to 5 days after thrombosis, the content of NETs in the thrombus reached its peak on the forth day. And the corresponding data has been supplemented in the revised manuscript as Figure S9.

Revision:

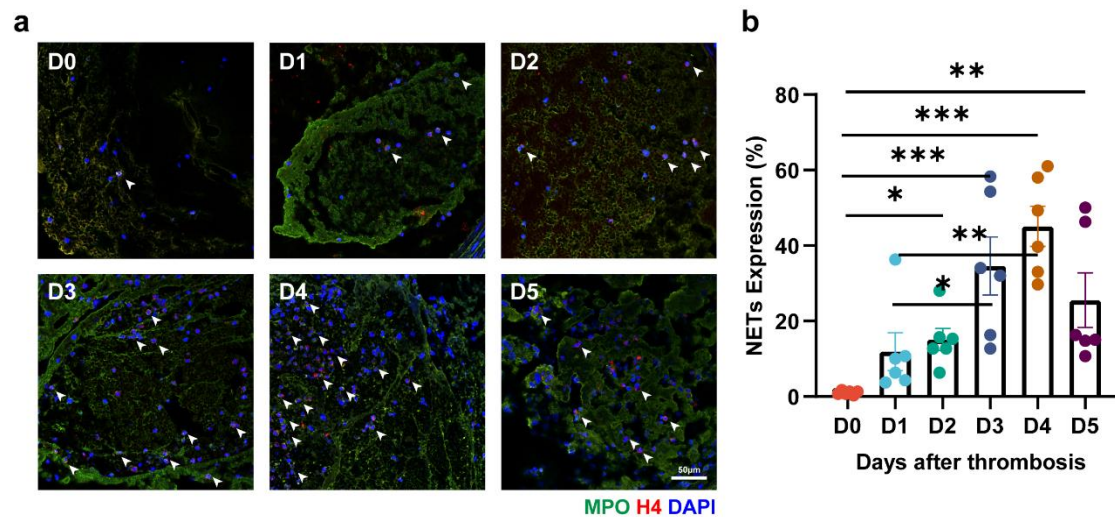


Figure S9. NETs formation in vivo. **a** The immunofluorescence staining of NETs in rat common carotid arteries from D0 to D5 after thrombosis. **b** Percentages of NETs expression at different time points. n=6. Mean±SEM, Kruskal-Wallis Test, *P<0.05, **P<0.01, ***P<0.001.

Comment 5: S21B you say no embolism occurred?

Response: Thank you for your question. Figure S21 shows the safety assessment after treatment. The platelets showed slight activation (2.11%) on the first day after treatment, this phenomenon subsided at seventh day after recanalization (Figure S21b). The mild activation rate has no significant effect on platelets, and we preliminarily believe that it will not cause re-blockage (*Adv. Funct. Mater.* 2023, 2312866). According to your question, we further added the Doppler ultrasonography on the recanalized vessels to rule out the risk of secondary embolism and the relative patency rate on first and seventh day were closed to 100%. The corresponding ultrasound data has been supplemented in Figure S21.

Other data in our manuscript (Figure 7 and Figure S23) also supported that no embolism occurred in recanalized vessels. At 28 days, endothelial cells and smooth muscle cells were well reconstructed, the endothelial cells are tightly

connected, and the amount of endothelial nitric oxide synthase is close to normal blood vessels. Combined with the above results, we believed that the blood vessels will not be blocked again after recanalization.

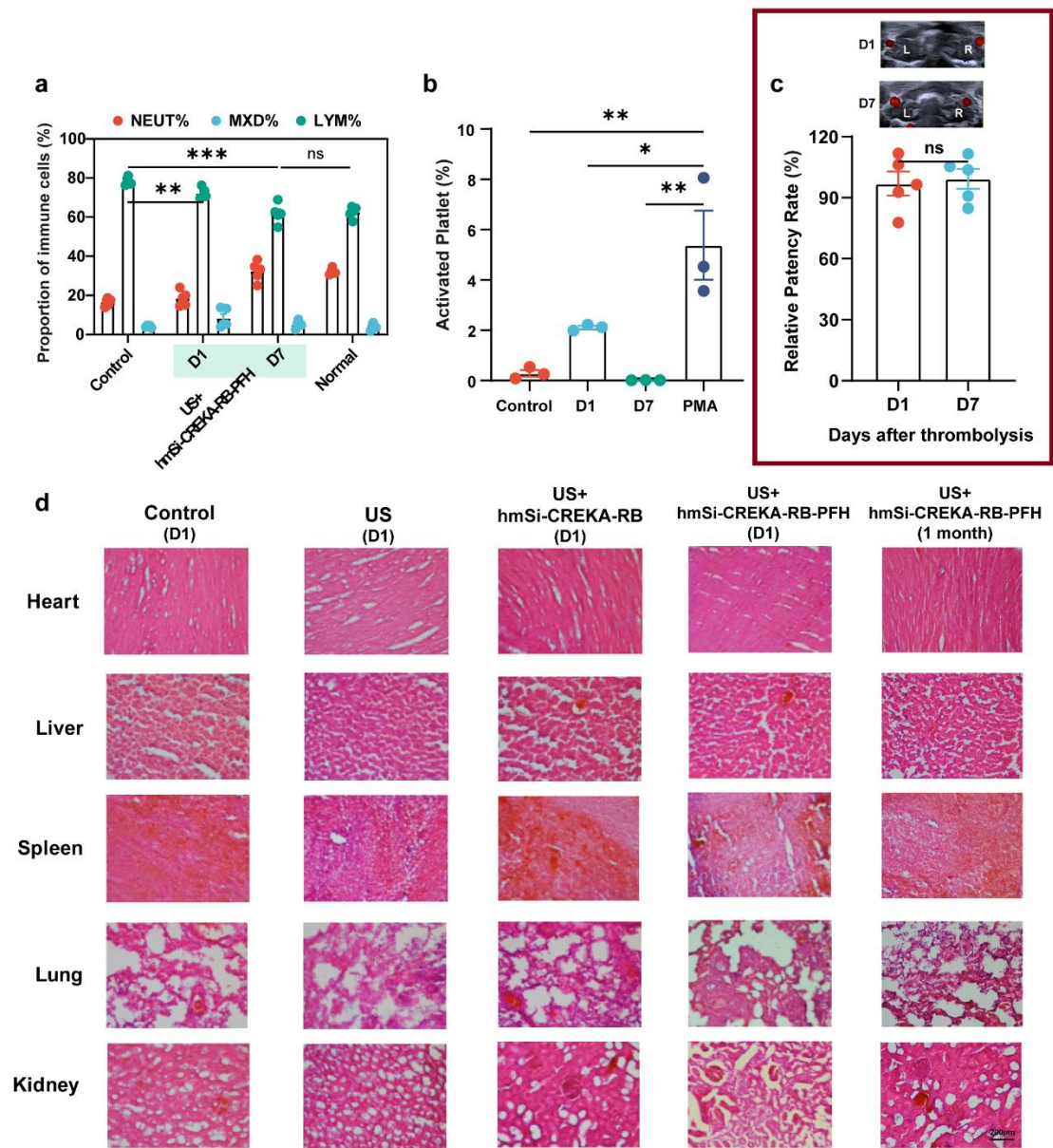


Figure S21. Safety assessment after treatment. **a** Proportion of immune cells in the blood of rats with different treatment (D1 and D7 means the first and the seventh day after completing three consecutive days of treatment, respectively). n=5. NEUT%, neutrophil%. MXD%, monocyte%. LYM%, lymphocyte%. **b** Platelet activation rate in different time after treated with US+hmSi-CREKA-RB-PFH. n=3, Mean ± SEM, one-way ANOVA analysis, **P<0.01, ***P<0.001, ns,

P>0.05. **c** Doppler ultrasound and the relative patency rate of recanalized vessels. n = 5, Paired-samples T tests. ns, P>0.05. **d** H&E staining of major organs after different treatments.

Comment 6: Procine occlusion model is poorly explained. I don't even understand what you did.

Response: Thanks for your question. The pig occlusion model is based on the following considerations:

Firstly, after observing the thrombolytic potential of our constructed platform in various thrombus models in rats, we further tested the thrombolytic effect in pig's thrombus to examine its clinical translational potential.

Secondly, according to literatures, thrombi with a length exceeding 8 mm can rarely achieve recanalization, and the thrombus length is associated with poorer clinical outcomes (*International Journal of Stroke*, 2015; 10 (6): 950-955; *Neurology* 97.20_ Supplement_2 (2021): S68-S78; *Stroke*. 2017; 48:1895-1900). Therefore, we constructed a thrombus with a length greater than 8mm in the pig thrombus model (as shown in Figure 8c) to explore the impact of this platform on long thrombi.

Similarly, we injected our submicron particles into the femoral artery of pigs after thrombus formation and performed 30 min treatment. The ultrasound imaging suggested a satisfactory recanalization effect (video 4). The D-dimer detection and H&E staining also supported thrombolysis result.

We have improved this part with more extensive descriptions of experimental procedures. In addition, we have sent our manuscript for scientific editing to improve the coherence of our explanation.

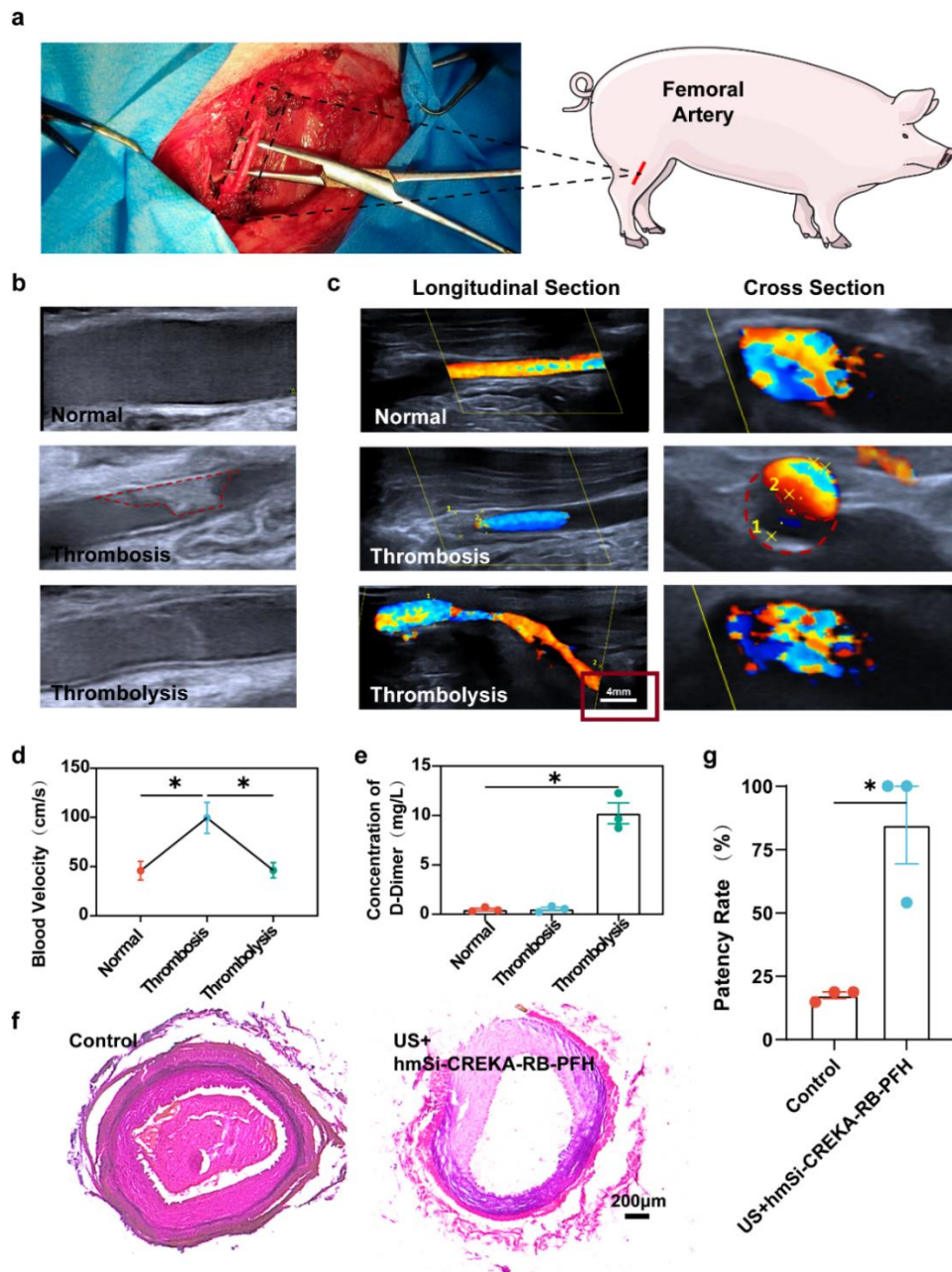


Figure 8. The US-responsive theranostic platform has a therapeutic effect in pigs. **a** Digital photos of an exposed femoral artery. **b** Real-time US (B-mode) and **c** Doppler ultrasound images showing thrombosis and thrombolysis in a femoral artery. **d** Real-time assessment of blood flow velocity. $n=3$. **e** Changes in D-dimer before and after treatment. $n=3$. Mean $\pm$ SEM, paired T test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. **f** Histopathological staining and **g** the corresponding patency rate. Mean $\pm$ SEM. $n=3$. Independent samples T-test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Revision:

(Revised Manuscript): We next constructed occlusion models in the pig femoral artery (Figure 8a), producing obvious thrombosis (Figure 8b). Then, hmSi-CREKA-RB-PFH was injected through the ear vein, after which US examination was conducted. Doppler ultrasonography revealed that the blood flow signal of healthy blood vessels vanished after thrombus formation (Figure 8c), the blood flow velocity increased from 45.85 cm/s to 99.53 cm/s as the blood vessel became narrower. After three, 10-min US sessions, the blood flow signal recovered, and the flow velocity decreased to 46.22 cm/s (Figure 8d), approaching the velocity in normal blood vessels. Blood samples from the normal, thrombosis, and thrombolysis periods were also collected to measure the level of D-dimer, a specific marker of fibrin degradation. There was no significant difference in the D-dimer level between the normal and thrombosis periods. However, afterwards, the D-dimer level increased 20-fold (Figure 8e), indicating that the thrombus was effectively dissolved. The results of H&E staining also showed the degree of vascular occlusion (Figure 8f), with a corresponding increase in the relative patency rate from 17.47% to 84.69% (Figure 8g). Immunofluorescence staining presented a large number of isopeptide bonds in the control group. However, the fluorescence of this crosslinks in the treatment group decreased, and fibrin was observed to be enriched in the position where the isopeptide bonds had not yet been destroyed (Figure S24). In addition to the excellent recanalization effect, we witnessed contrast improvement induced by the transition of PFH in carotid artery clot. The morphology of the thrombus became clearer and the resolution of US imaging was enhanced over time (Figure S25), indicating the feasibility of hmSi-CREKA-RB-PFH submicron particles as a contrast-enhancing agent for real-time monitoring of thrombosis treatment.

(Supplementary Information): Therapeutic Efficacy in Pig Thrombus Model

Domestic pigs weighing 60-80 kg were on a diet one day before surgery. Before operation, ketamine 6 mg/kg and diazepam 0.2 mg/kg were injected intramuscularly, after fixation, endotracheal intubation, oxygen, and sevoflurane were then supplied. Venous cannula (18 ~ 20g) was placed by ear vein puncture, and normal saline and propofol were intravenously drip. Under sterile conditions, the femoral artery was separated and rinsed with normal saline; Then the blood vessels were covered with filter paper strips (7 mm×3 mm) that were soaked in 50% ferric chloride solution for 15min, and the blood vessels were rinsed with normal saline after being left for 5 min. Then, hmSi-CREKA-RB-PFH (15mL, 20mg/mL) was slowly injected through the ear vein, US (1.0 MHz, 1.5 W/cm²) was performed for 30 min 1h after injection. The size and blood flow of the thrombus were observed with Doppler ultrasound (VINNO 6VET/6LAB) every 10 min. Blood samples from the normal, thrombosis, and thrombolysis periods were collected for D-dimer detection, which was performed in the Laboratory Department of Second Affiliated Hospital of Army Medical University Hospital. Finally, histological and immunofluorescence staining were conducted on the 20 μm frozen carotid artery sections.

Special thanks to you for your good comments. We have submitted the revised version (with marks in red) and pure version (without marks) in the PDF format of manuscript and Supplementary Information. We have tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for your warm work earnestly, and hope that the corrections will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Reviewer #3 (Remarks to the Author):

The authors answered all my questions, with the exception of the one concerning Zeta potential measurements. This has significantly improved the quality of this manuscript, including supplementary experiments and controls that clearly enhance the scientific demonstration in the proposed article.

However, when measuring surface charge, it is essential not to use osmosed or MilliQ water, as they do not contain sufficient salts in solution (no ionic strength) and can therefore generate differences in results.

In order to obtain comparable results and to be able to conclude on the surface modifications of the proposed nanoobjects, an aqueous solution with a controlled ionic strength should be used, such as a 1 mM KCl aqueous solution. I recommend that authors carry out their potential zeta experiments not in water but in 1mM KCl.

Response: Thanks for your suggestion. We have carried out the potential zeta experiment in 1mM KCl to conclude the surface modifications. The related results and description have been revised in the manuscript.

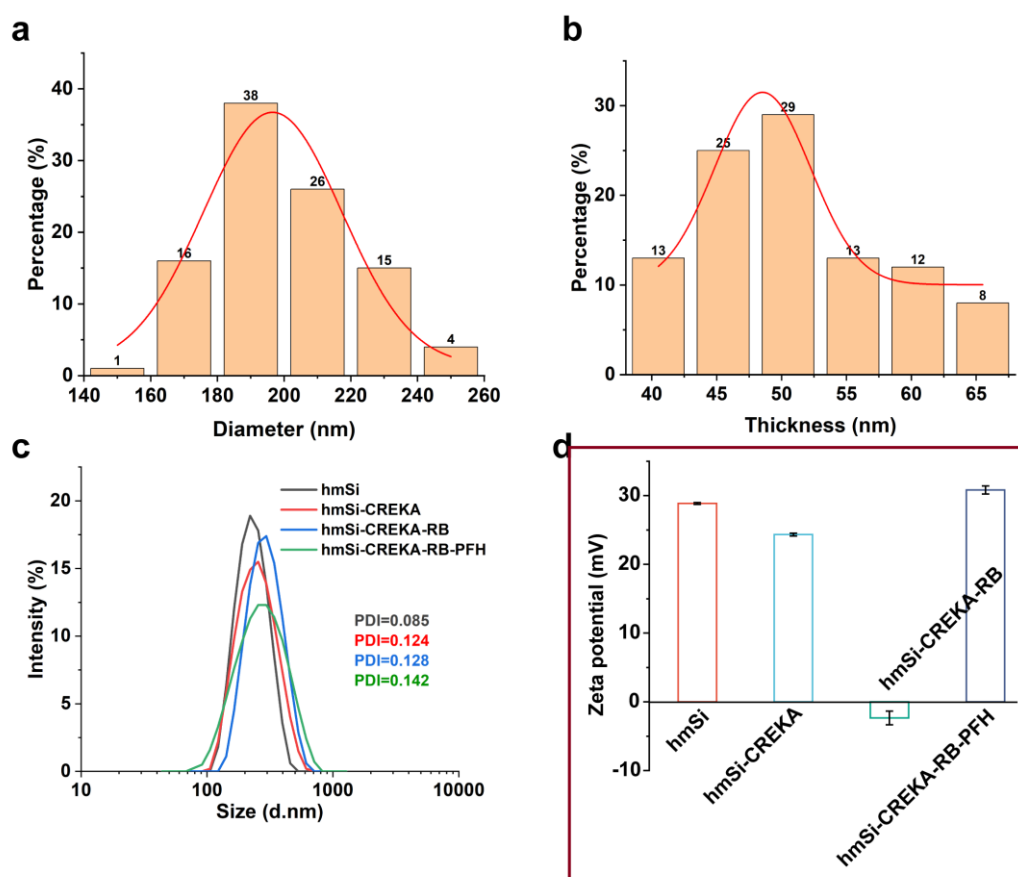


Figure S1. Characterization of hmSi-CREKA-RB-PFH. **a** Diameter and **b** Thickness of hmSi. **c** Intensity distribution of DLS and **d** Zeta potentials of hmSi, hmSi-CREKA, hmSi-CREKA-RB and hmSi-CREKA-RB-PFH.

Revision :

(Revised Manuscript): As for zeta potential, it changed from 28.9 to 24.3, -2.3 and 30.8 mV, the electronegativity of CREKA and RB led to a charge transition to negative terminal (Figure S1d).

(Supplementary Information): Dynamic light scattering (DLS) was used to measure the size distribution and ζ potential of submicron particles with a concentration of 1 mg/mL; The size distribution of submicron particles (hmSi, hmSi-CREKA, hmSi-CREKA-RB, hmSi-CREKA-RB-PFH), were measured in aqueous solution. The measurement of ζ potentials was performed in 1 mM KCl.

Special thanks to you for your good comments. We have submitted the revised version (with marks in red) and pure version (without marks) in the PDF format of manuscript and Supplementary Information. We have tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for your warm work earnestly, and hope that the corrections will meet with approval. Once again, thank you very much for your comments and suggestions.

Best regards,

Wen Zeng

Reviewer #4 (Remarks to the Author):

This manuscript presents an ultrasound-based system which allows for the monitoring and effective trombolysis of tPA resistant thrombi. The authors have been highly responsive to prior reviewers' comments and now present data that are strong and convincing.

Response: Thank you very much for your comments and recognition of our manuscript.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

Comments 1-3: The authors addressed the reviewer's questions.

Comment 4:

Regarding evaluating NETs in immunofluorescence (IF), the authors indicated in the Methods section that the nuclear counts of MPO and H4-positive in the trace area were determined in 0.102 mm² regions. Please confirm that the Y-axis of Figure S9.

Comment 5:

In Figure S21c, the relative patency rate exceeds 100% for several vessels. The luminal patency rate [(lumen-thrombus)/lumen x 100%] would be more appropriate.

The colors of the legends and dots in the figure appear in a different order in several Figures. This inconsistency is observed in Figures S21a, S4d, and 1g.

Comment 6:

Did you evaluate the histology of distal tissue emboli in the porcine experiment? This data could support the claim that there is no risk of secondary embolization, as mentioned in the conclusion. If such data are not available, the tone of the conclusion should be adjusted to reflect this uncertainty.

Reviewer #3 (Remarks to the Author):

I'd like to thank the authors for fully responding to my comments.
I have no further comments on this manuscript.

Response to the Review Comments

Reviewer #2 (Remarks to the Author):

Comments 1-3: The authors addressed the reviewer's questions.

Response: Thank you very much for your recognition. Your suggestions were very helpful to us. We hope our revised manuscript is more suitable for publication in *Nature Communications*.

Comment 4:

Regarding evaluating NETs in immunofluorescence (IF), the authors indicated in the Methods section that the nuclear counts of MPO and H4-positive in the trace area were determined in 0.102 mm² regions. Please confirm that the Y-axis of Figure S9.

Response: Thank you for your question. We chose to use percentage representation of NET expression by comparing the number of NETs in d1-d5 with the number of NETs on day 0 from thrombus onset according to a previous study (*Nature Communications* 11, no. 1 (2020)). In order to unify the evaluation of NETs throughout the text, we have changed this percentage to the absolute value of NETs.

Revision:

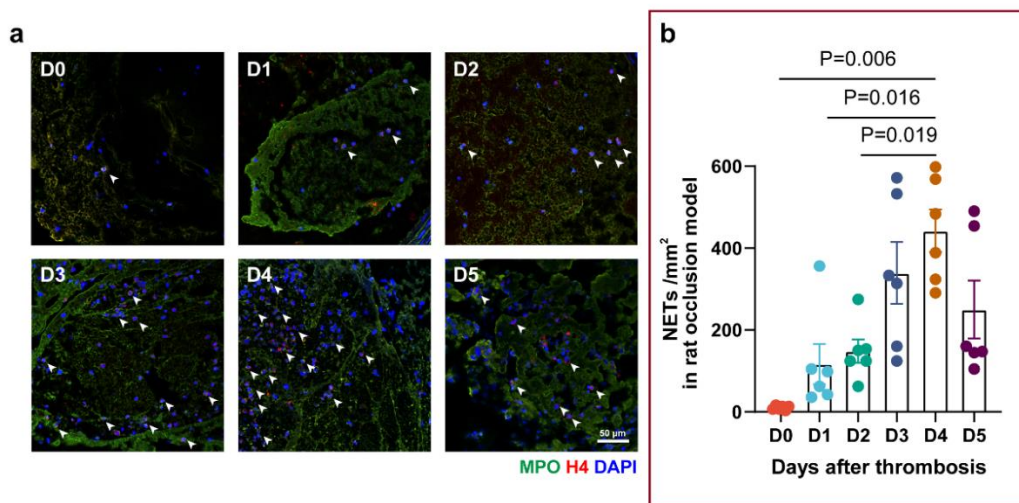


Figure S9. NETs formation in vivo. a The immunofluorescence staining of

NETs in rat common carotid artery from D0 to D5 after thrombosis and **b** The number of NETs at different time points. n=6 samples. Welch's ANOVA with Tamhane's T2 post hoc analysis were used to compare differences among six groups. Data are presented as mean±SEM. Source data underlying graph **b** is provided as a Source Data file.

Comment 5:

In Figure S21c, the relative patency rate exceeds 100% for several vessels. The luminal patency rate $[(\text{lumen-thrombus})/\text{lumen} \times 100\%]$ would be more appropriate.

Response: Thank you for your question. We used two methods to calculate the patency rate of the lumen, one is the relative patency rate calculated by the ratio of blood vessel flow velocity, and the other is the absolute patency rate obtained through H&E staining of the final sample based on the formula you suggested (Figure 6i) .

According to literatures, blood flow velocity is correlated with the size of the vascular lumen (*J Clin Ultrasound. 2014 Jan;42(1):16-23*) , and by comparing the blood flow velocity on the treatment side with the normal control side, one can approximately reflect the degree of vascular patency. Although individual differences have been ruled out, the blood flow velocity of the two blood vessels is not completely equal. In order to name it more formally, we changed the y-axis to “Blood Velocity (% of contralateral artery)” in the corresponding figures.

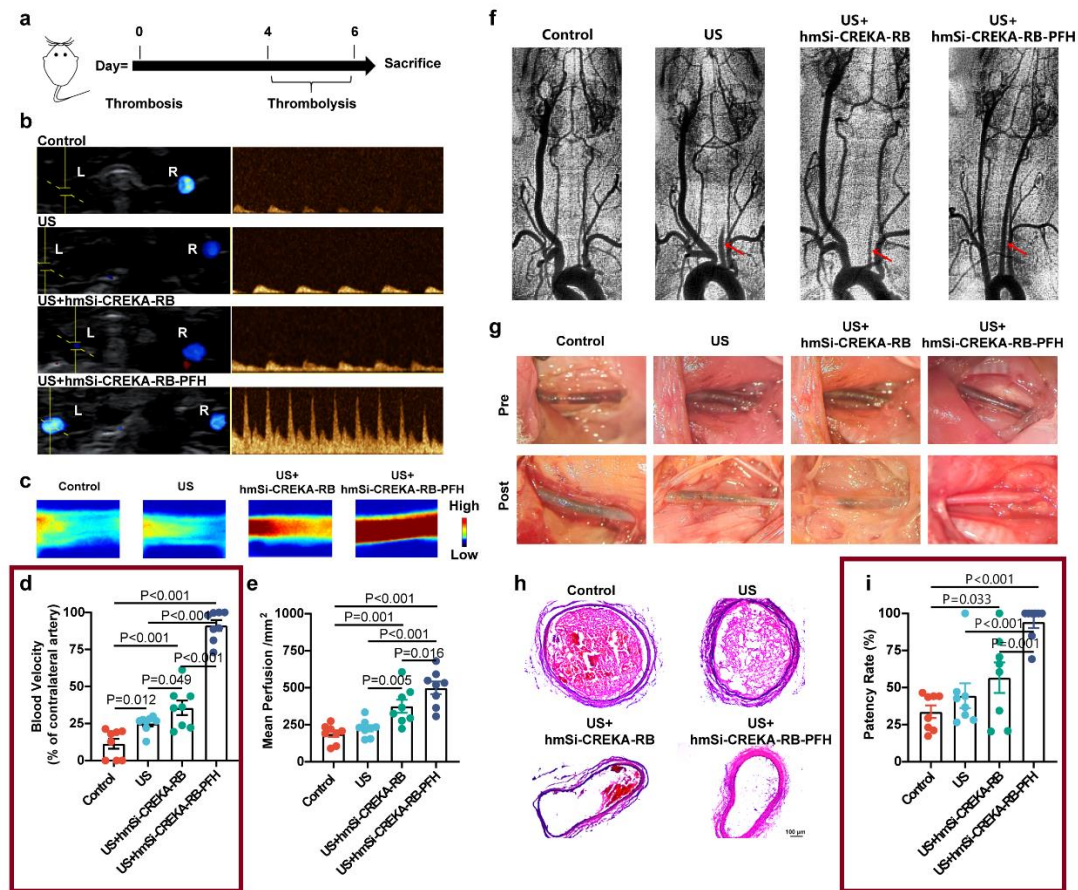
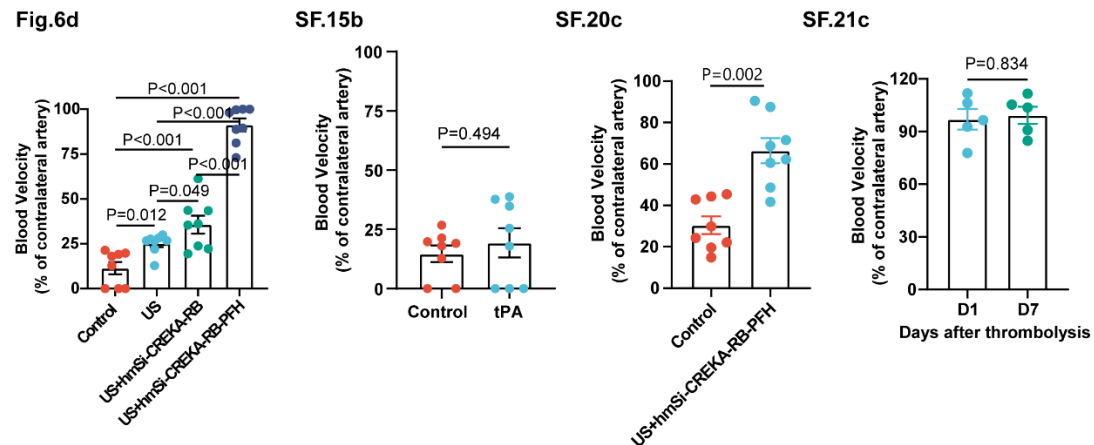


Figure 6. The therapeutic effect of the US-responsive theranostic platform in rats. **a** Schematic illustration of the experimental timeline. **b** Real-time Doppler ultrasound images in different treatment groups. **c** Laser speckle imaging of carotid arteries. **d** Percentage of blood flow velocity between blood vessels in different treatment groups and normal contralateral blood vessels. $n = 8$ samples. **e** Mean blood perfusion of carotid artery in different treatment groups. $n = 8$ samples. **f** DSA of the carotid artery of rats after treatments. The red arrows indicate the site of recanalization. **g** Digital photos of the carotid artery after each treatment. **h** Histopathology of treated vessels and **i** the patency rate of the vessels. $n = 8$ samples. Data are presented as mean $\pm$ SEM. one-way ANOVA with the LSD post hoc test was employed to compare the differences between groups. Source data underlying graph **d**, **e** and **i** are provided as a Source Data file. Each experiment was repeated three times or more independently with similar results.

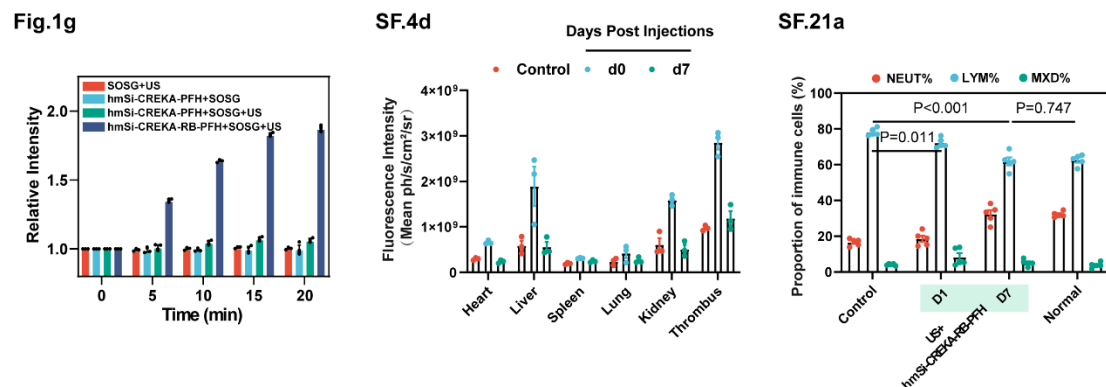
Revision:



Comment 6: The colors of the legends and dots in the figure appear in a different order in several Figures. This inconsistency is observed in Figures S21a, S4d, and 1g.

Response: Thank you for your question. We have standardized the color order in Figures S21a, S4d, 1g and all the other legends and dots in the revised manuscript.

Revision:



Comment 7:

Did you evaluate the histology of distal tissue emboli in the porcine experiment? This data could support the claim that there is no risk of secondary embolization, as mentioned in the conclusion. If such data are not available, the tone of the conclusion should be adjusted to reflect this uncertainty.

Response: Thank you for your comments. Due to the randomness of the site

of occlusion after thrombus detachment, we did not achieve histological evaluation of secondary occlusion of distal blood vessels. In vitro induced blood clots were treated in combination therapy (Fig 5e), and residual thrombus in rats after thrombolysis was washed by simulated shear stress in vitro (Fig S11c), in both cases, the hydrated particle size of fragments was less than 10um, which theoretically would not block small distal vessels (*Adv Sci. 2019 Jul 4;6(17):1901378*). We have supplemented this uncertainty in the revised discussion section.

Revision (Discussion): The risk of secondary distal vessel occlusion following endovascular thrombectomy for major vessel occlusions has been reported. Although our in vitro experiments suggested a low risk of distal small vessel occlusion, due to the unpredictability of the site of occlusion following thrombus detachment, we didn't thoroughly examine this risk through histological evaluation.

Reviewer #3 (Remarks to the Author):

I'd like to thank the authors for fully responding to my comments.

I have no further comments on this manuscript.

Response: Thank you very much for your recognition. Your suggestions were very helpful to us. We hope our revised manuscript is more suitable for publication in *Nature Communications*.