

CRISPR-Cas9 cleaves DNA microspheres to release drug for efficient thrombosis treatment

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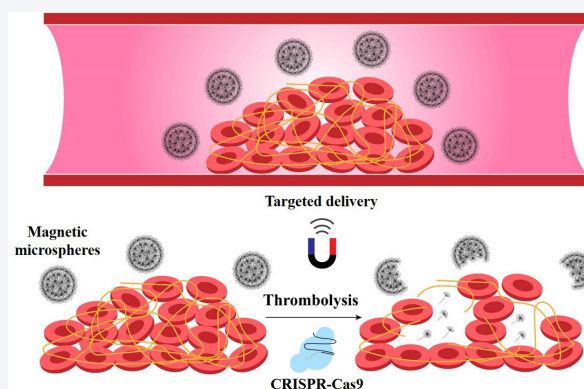
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ABSTRACT: The drug delivery nanocarriers are widely employed in thrombolytic therapy. However, the off targeting and ineffective drug release of delivery vehicles make it difficult to targeted accumulation, effective diffusion, and penetration into thrombus tissue. Herein, we reported a biocompatible magnetic microsphere constructed via a programmable DNA rolling circle amplification (RCA) reaction integrated with iron oxide magnetic nanoparticles (Fe_3O_4 NPs). By combining ultrasound and photoacoustic imaging for diagnosis with magnetic field-guided targeting for therapy, these magnetic DNA microspheres were further functionalized with CRISPR-Cas9 to enable precise regulation of thrombolytic drug release. This system promotes deep penetration into thrombi and enhances mass transfer within clots, thereby accelerating thrombolysis. As a result, the thrombolytic drug, tissue plasminogen activator (tPA), can be delivered selectively to thrombus sites, where it is rapidly released and penetrates the clot, significantly improving the thrombolytic rate and therapeutic efficacy.



KEYWORDS: rolling circle amplification (RCA), magnetic DNA microsphere, CRISPR-Cas9, targeted delivery, thrombosis

1 Introduction

Thrombosis, the formation of blood clots within arteries or veins, represents a primary pathological mechanism underlying acute ischemic cardiovascular diseases [1–3]. Vascular occlusion partially or completely obstructs the blood flow in the blood vessel, which can significantly exacerbate cardiovascular conditions, leading to serious clinical outcomes such as unstable angina, coronary artery infarction, myocardial infarction, atherosclerosis, ischemic stroke, and pulmonary embolism [4–8]. In the USA alone, thrombosis-

related myocardial infarction and stroke account for over 2000 deaths daily due to clots formed in coronary or cerebral arteries [9]. Current diagnostic techniques for thrombosis include ultrasound (US), venography, magnetic resonance imaging (MRI), photoacoustic imaging (PAI), and computed tomography (CT), among others. These modalities assess vascular morphology and composition, as well as the structure and function of related organs, to detect thrombotic events [10–14]. Nevertheless, there remains a clinical need for integrated strategies that combine accurate, timely diagnosis with effective therapeutic intervention.

In recent years, various drug delivery nanocarriers, such as magnetic nanomotors, liposomes, and synthetic polymers, have been developed for thrombolytic therapy [15–18]. Incorporating thrombolytic agents like tissue plasminogen activator (tPA) into these systems has been shown to improve patient survival rates and quality of life [19, 20]. However, limitations related to biocompatibility, targeting precision, and controlled drug release

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often hinder effective accumulation, penetration, and diffusion of these carriers within thrombus tissue. Furthermore, tPA itself faces challenges including relatively low biocompatibility, inefficient site-specific delivery, and suboptimal thrombolytic kinetics [21]. Thus, there is an urgent need to develop a dose-controllable, targeted delivery platform capable of controlled drug release for thrombotic diseases. Such a system could enhance the bioavailability of tPA, facilitate its localized delivery to specific regions of the thrombus, and ultimately accelerate clot dissolution.

DNA hydrogel is swollen, three-dimensional cross-linked networks formed through the self-assembly of DNA in an aqueous environment, making them suitable candidates for drug delivery [22, 23]. These materials exhibit advantageous characteristics such as programmable design, stimulus responsiveness, biodegradability, high encapsulation capacity, and selective permeability. Consequently, they hold significant potential for various biomedical applications, including gene delivery, biosensing, and tissue engineering [24–26]. For instance, Luo et al. developed a method to construct pure DNA hydrogels that form three-dimensional gel structures via DNA self-assembly, demonstrating promising utility in controlled drug release and cell-free protein production [27, 28].

The clustered regularly interspaced short palindromic repeats (CRISPR)-associated enzyme Cas9 is an RNA-guided endonuclease that target specific DNA sequences via RNA–DNA base-pairing, serving as an indispensable tool in biological research [29]. The

CRISPR-Cas9 enables precise cleavage at designated sites [30]. This capability makes it feasible to integrate CRISPR-Cas9 with DNA hydrogels, thereby allowing responsive, programmable regulation within drug-delivery systems.

To address these challenges in thrombolysis, we constructed a functionalized hydrogel system based on a programmable DNA rolling circle amplification (RCA) reaction. This hydrogel was integrated with biotin-modified iron oxide (Fe_3O_4) magnetic nanoparticles to form a biocompatible magnetic microspheres. The functionalized hydrogel formed by magnetic DNA microspheres can be structurally programmed through template sequence design combined with rolling circle amplification, and by modifying Fe_3O_4 NPs, the structure can rapidly target specific sites under the influence of a magnetic field, making it a biocompatible drug delivery carrier. Employing a combined strategy of ultrasound/photoacoustic imaging for diagnosis and magnetic-field-guided targeting for therapy, the system was further coupled with the CRISPR-Cas9 to regulate the on-demand release of the thrombolytic drug tPA. This approach promotes deep penetration into thrombi and enhances mass transfer within the clot core (Fig. 1). The magnetic drive enables precise targeting to selective sites in the thrombus, while the CRISPR-Cas9 system acts as a responsive release mechanism, collectively ensuring rapid drug penetration, improving thrombolytic rates, and enhancing therapeutic efficacy. This constructed delivery system effectively

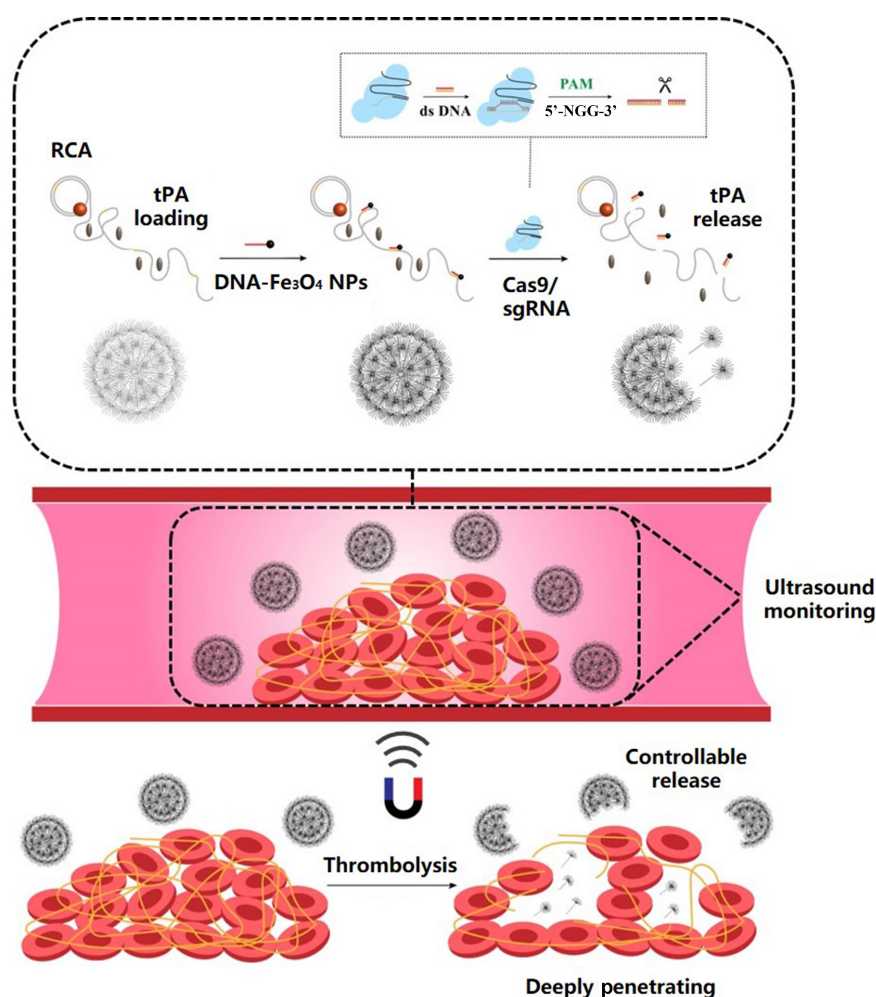


Figure 1 Schematic diagram of magnetic DNA microspheres drug releasing system regulated by CRISPR-Cas9 for the treatment of thrombus.

combines the DNA sequence programmability of the rolling circle amplification structure and the CRISPR-Cas9 technology, which can precisely identify and cut specific sequence sites to achieve the disassembly of the structure. It is also modified with magnetic nanoparticles and can be targeted to the thrombus site through a magnetic field, rapidly releasing thrombolytic drugs to achieve efficient thrombolytic therapy.

2 Experimental

2.1 Chemicals and materials

T4 DNA ligase and CRISPR-Cas9 protein were purchased from New England Biolabs (Beijing, China). All oligonucleotide sequences (Table S1 in the Electronic Supplementary Material (ESM)) were synthesized and modified by Sangon Biotech (Shanghai, China). Deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), dithiothreitol (DTT), and thrombin were purchased from Sigma-Aldrich (St. Louis, USA). phi29 DNA polymerase was purchased from ThermoFisher (Shanghai, China). Streptavidin-Fe₃O₄ NPs were purchased from Ruixi (Xi'an, China). tPA was purchased from Abcam (Shanghai, China). Single guide RNA (sgRNA) were purchased from Sangon Biotech (Shanghai, China). All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The chemicals mentioned were all used as received, and all solutions were prepared with Milli-Q water (18.2 MΩ/cm resistivity) from a Millipore system.

2.2 Preparation of magnetic DNA microspheres

5 μL of 10 μM Primer 1 was mixed with 5 μL of 10 μM single-stranded DNA (ssDNA) in T4 ligation buffer, then 2 μL of T4 ligase and adenosine triphosphate (ATP) were added into the solution, storing at 4 °C overnight. 2 μL of dATP, dCTP, dGTP, dTTP, and DTT coupling with phi29 DNA polymerase were dispersed in the phi29 buffer. All the solution was kept at 40 °C for 2 h, then adding 50 μL of mixtures of biotin labelled DNA and streptavidin-labelled Fe₃O₄ NPs, 1 μL of 100 μM primer2 and primer3 in sequence at 40 °C for another 2 h.

2.3 Synthesis of magnetic functionalization of DNA microspheres with Cas9/sgRNA

Circle DNA (30 μM) and dNTP (100 mM), according to the volume ratio of circle DNA to dNTP 2:1 were mixed in phi29 buffer, then DTT and phi29 polymerase were added and reacted at 40 °C for 1 h. Streptavidin-modified ferric oxide nanoparticles and biotin-modified deoxyribonucleic acid complex were added, following with adding primer2 (100 μM) and primer3 (100 μM). The magnetic DNA microgel was obtained after reaction at 40 °C for 1 h. The gel is dispersed and then centrifuged at high speed to remove the solution. In addition, 1 μM CRISPR-Cas9 and sgRNA complexes were added in magnetic gel (MG) formation, and the redundant Cas9/sgRNA ribonucleoprotein (RNP) complexes were removed by high-speed centrifugation to obtain MG/Cas9/sgRNA.

2.4 Scanning electron microscopy (SEM) imaging

The magnetic DNA microspheres were firstly tackled with critical point drying, the samples were left to dry on the silicon wafer under room temperature before imaging. Then, the samples were sprayed

with conductive platinum atoms and immobilized onto the substrate with electroconductive paste. After these procedures, the SEM images were obtained using scanning electron microscopy (Gemini, Zeiss, Germany for DNA microspheres; Hitachi, S4800, Japan for magnetic DNA microspheres) operated at an acceleration voltage of 2.0–5.0 kV.

2.5 Synthesis of magnetic DNA microsphere loading with tPA

The circle DNA, primer1 DNA, dNTP, DTT, and phi29 polymerase were mixed in the phi29 buffer and kept incubating in TGrade Dry Bath Incubator (TIANGEN, China) at 40 °C for 2 h. Then, 0.2 μM tPA was added into the solution, the primer2, primer3 and biotin labelled DNA with streptavidin-labelled Fe₃O₄ NPs were added into the mixture at the same temperature for another 2 h.

2.6 Quantitative determination of tPA in the MG/tPA by using bicinchoninic acid (BCA) kit

MG/tPA was diluted with deionized water, which was tested to detect the concentration of tPA by BCA kit (ThermoFisher Scientific, Shanghai, China). The absorbance at 562 nm was measured on a plate reader.

2.7 Magnetic targeting of DNA microspheres targeted delivery *in vitro*

10 μL of magnetic DNA microspheres were added into 1.5 mL centrifuge tube containing 500 μL saline and 100 μL prepared blood clots. The magnet was placed below the centrifuge tube for 1 min, the position of magnetic DNA microgels was monitored by naked eyes.

2.8 CRISPR-Cas9 regulated experiments of magnetic DNA microspheres *in vitro*

1 μM Cas9/sgRNA ribonucleoprotein complexes were added to the DNA microsphere/tPA and MG/tPA samples, respectively. Then the samples were characterized by SEM (Gemini, Zeiss, Germany for DNA microspheres; Hitachi, S4800, Japan for magnetic DNA microspheres).

2.9 Assessment of thrombolysis efficacy in *ex vivo* blood clots

The male Sprague–Dawley (SD) rats (8 to 10 weeks old) were anesthetized via isoflurane gas. 100 μL of fresh blood were obtained from the heart arteries and added in several 1 mL centrifuge tubes containing 50 U of thrombin solution. The tubes were placed at 37 °C for 3 h and then moved to 4 °C for 3 days. The prepared blood clots were put into 500 μL centrifuge tubes containing saline solution. Saline, MG, MG/tPA, MG/tPA/CRISPR, and only tPA were added into corresponding centrifuge tubes with slight vibration, which monitoring at a fixed point (0, 3, 6, 9 h) in time at 37 °C.

2.10 Construction of thrombus model

Thrombus formation: Rats were anesthetized with 10% chloral hydrate (100 μL) by intraperitoneal injection. The left femoral artery of the mouse was exposed with surgical instruments. After completing the exposure, a filter paper soaked in 5% ferric chloride

solution was placed on the surface of the femoral artery for 3 min. Then, the filter paper was removed, and the treated femoral artery was washed with sterile phosphate-buffered saline (PBS) buffer. Eventually, visible femoral artery thrombosis was formed. All the relevant animal experiments were approved by Ren Ji Hospital Ethics Committee (approval No: RJ2023–084A).

2.11 Magnetic targeting of DNA microspheres *in vivo*

The male SD rats (specific pathogen free (SPF) level) were anesthetized by 10% chloral hydrate solution. Then, the femoral artery of rats was treated with 5% ferric chloride solution. The treated femoral artery was imaged by ultrasonic and photoacoustic imaging system. After intravenously injected with 100 μ L of magnetic DNA microspheres for 10 min, the femoral artery was imaged again, utilizing magnetic targeting. The accumulations of magnetic DNA microspheres using magnet were monitored by US and PAI.

2.12 Assessment of thrombolysis efficacy in a rat model

100 μ L saline and MG/tPA intravenous injection volume were used, respectively ($n = 3$ for each group). For control and treatment groups, the magnet was placed close to the clot for 10 min. CRISPR-Cas9 performed shear regulation on MG/tPA, and the thrombolysis was monitored by blood flow variation.

2.13 Thrombolytic effect evaluation of MG/tPA *in vivo*

The effect of thrombolysis of the different groups in rats with lower extremity arterial thrombosis was treated by injecting saline and MG/tPA. A Doppler ultrasound imaging system was used to monitor and record the blood flow velocity and blood flow. The group was observed, and the blood flow velocity and blood flow were recorded, after which the rats were euthanized, and the blood vessels in the thrombus were removed and analyzed by pathological examination.

2.14 Histopathology examination

The vascular tissue was excised from the rats and collected for histological analysis ($n = 3$ per group). Thrombus tissues of the treated rats were harvested, fixed with 4% paraformaldehyde, and embedded in paraffin, which further stained with hematoxylin and eosin (H&E) before undergoing histopathological examination using an optical microscope.

2.15 Statistical analysis

All the experiments were conducted in triplicate unless elsewhere indicated. All the data were expressed as means $\pm$ standard deviation (s.d.). Statistical significance was performed by two-tailed unpaired *t* test analysis. Statistical significance was set at $*P < 0.05$, and extreme significance was set at $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

3 Results and discussion

3.1 Preparation and regulation of DNA hydrogels

The DNA hydrogel was synthesized via RCA, forming a network composed of flower-like DNA microspheres. SEM analysis showed that the microspheres exhibited a size distribution ranging from 2.4 to 3.6 μ m (Fig. 2(a)), with a predominant diameter of approximately 2.7 μ m (Fig. 2(b)). These DNA microspheres are entirely composed of DNA and have excellent biocompatibility (Fig. 2(c)). The results from dynamic light scattering (DLS) analysis displayed that zeta potential of DNA microspheres solution was -22.2 mV, which further proves that the microsphere structure is made up of DNA molecules (Fig. 2(d)). By incorporating a designed DNA primer containing specific Cas9 cleavage sites into the microspheres, the addition of the Cas9-sgRNA ribonucleoprotein complex enables a targeted response: the complex recognizes the engineered sequence and rapidly cleaves the

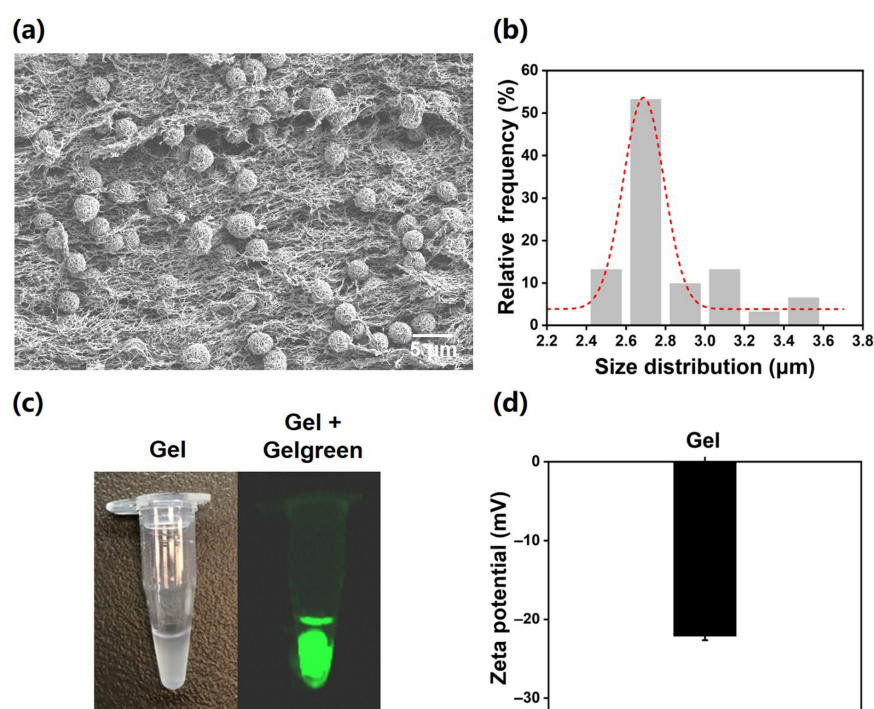


Figure 2 Synthesis and characterization of DNA microspheres. (a) SEM characterization of DNA microsphere structures. (b) Size distribution of synthesized DNA microspheres. (c) Hydrogel photo and imaging after adding Gelgreen dye. (d) Zeta potential of DNA microspheres by laser nanosize analyzer.

DNA framework, thereby achieving precise structural regulation. SEM characterization revealed that DNA microspheres with cleavage sites were synthesized with high structural consistency (Fig. 3(a)). After the addition of Cas9/sgRNA ribonucleoprotein complexes, the spherical structures undergo rapid structural cleavage, which can achieve release of the drugs loaded inside (Fig. 3(b)). This demonstrates the programmability of the DNA microsphere sequence and the rapid transformation of the structures, facilitating the fast controlled release of thrombolytic drugs. Furthermore, the individual DNA microspheres were characterized by SEM (Fig. 3(c)). After the CRISPR-Cas9 regulation, the structural changes in shape and dimension are quite obvious (Fig. 3(d)). Therefore, the regulation of DNA microspheres has been achieved successfully, further accelerating the release of thrombolytic drugs, which is conducive to the highly efficient treatment of thrombosis.

3.2 Preparation and characterization of magnetic DNA microspheres

Capitalizing on the ease of DNA modification, we first synthesized biotin-labeled DNA microspheres. These were then functionalized via the strong biotin–streptavidin interaction with DNA-conjugated iron oxide magnetic nanoparticles (Fe_3O_4 NPs) to obtain the final MG (Fig. S1 in the ESM). SEM was used to characterize the morphology and dimensions of the resulting structures. The images confirmed the successful and uniform incorporation of Fe_3O_4 NPs into the DNA microspheres (Fig. 4(a)). After modification with Fe_3O_4 NPs, the size of the magnetic DNA microspheres has increased to approximately 3.0 μm (Fig. 4(b)). The constituent elements of magnetic DNA microsphere were characterized by energy-dispersive X-ray spectroscopy (EDS) in Fig. 4(c), which contains C, P, O, Fe elements from DNA and Fe_3O_4 NPs. The proportion of weight percentage of each element was semi-quantificationally detected by EDS, indicating the specific

proportion of C, P, O, and Fe elements in magnetic DNA microspheres (Fig. 4(d)). Thanks to their magnetic component, these microspheres can be rapidly and precisely directed to specific locations under external magnetic field control (Fig. S2 in the ESM).

3.3 CRISPR-Cas9 regulated controlled release of tPA from magnetic DNA microspheres *in vitro*

In the magnetic DNA microsphere, the intact flower-like structure is displayed by SEM characterization (Fig. 4(e)). The variation of morphology and dimension from the magnetic DNA microsphere after regulation of CRISPR-Cas9 was also characterized via SEM (Fig. 4(f)), demonstrating that the structural cleavage responses can still be successfully achieved by adding the Cas9/sgRNA ribonucleoprotein complexes after modifying magnetic Fe_3O_4 NPs via DNA hybridization and recognizing the specific DNA sequence sites.

Following the CRISPR-Cas9-mediated release of tPA, the concentration of the released tPA in the solution was quantified by the BCA protein assay kit. A standard curve was first established for protein concentration, linear equation is $Y = 1.09249X + 0.03849$, the R^2 value is 0.9910, which demonstrates that there is a good linear relationship between the detection signal and the concentration (Fig. 4(g)). Subsequent measurement of the tPA loaded within the MG yielded a concentration of 0.05085 $\mu\text{g}/\mu\text{L}$ (Fig. 4(h)). These results confirm the successful and quantifiable loading of tPA into the MG delivery system.

3.4 Magnetically guided targeting and thrombolysis efficacy of tPA releasing from magnetic DNA microspheres *in ex vivo* blood clots

To evaluate thrombolytic efficacy *in vitro*, blood clot samples were treated with saline (control), MG (magnetic gel alone), MG loaded

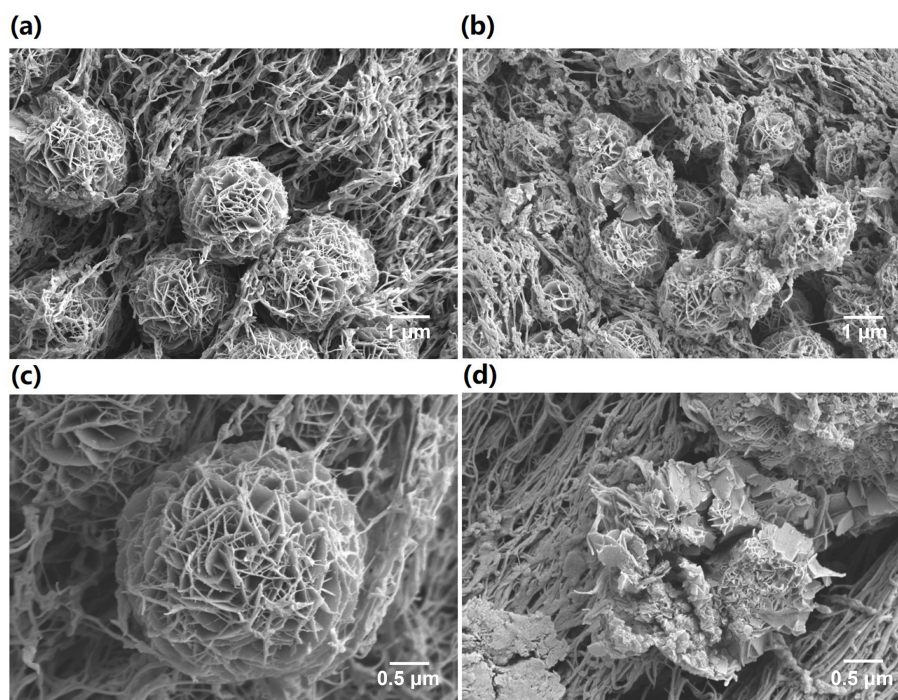


Figure 3 CRISPR-Cas9 regulated DNA microsphere structures. SEM characterization of DNA microspheres (a) before and (b) after CRISPR-Cas9 shearing. SEM images of individual DNA microsphere (c) before and (d) after CRISPR-Cas9 regulation.

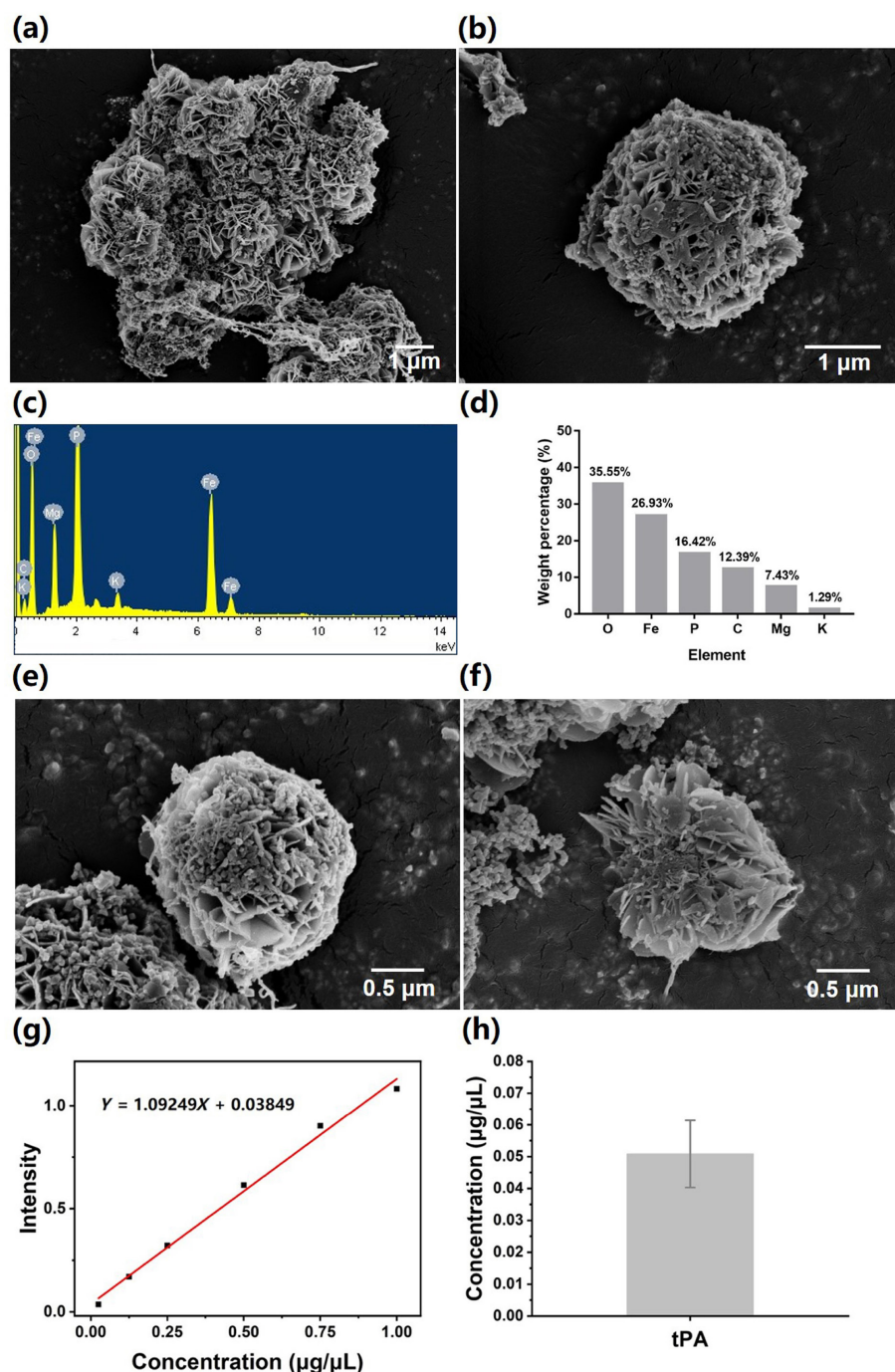


Figure 4 Synthesis and regulation of magnetic DNA microspheres. (a) SEM characterization of magnetic DNA microspheres, scale bar: 1 µm. (b) The image of individual magnetic DNA microsphere, scale bar: 1 µm. (c) Energy-dispersive X-ray spectroscopy (EDS) characterization of magnetic DNA microspheres. (d) The proportions of the elements of EDS detection. CRISPR-Cas9 regulated control release of tPA. SEM images of magnetic DNA microsphere (e) before and (f) after CRISPR-Cas9 regulation, scale bars: 0.5 µm. (g) Linear range of protein concentrations from BCA kit. (h) The concentration of released tPA quantified by the BCA protein assay kit.

with tPA (MG/tPA), MG/tPA with CRISPR-Cas9 regulation (MG/tPA/CRISPR), and free tPA. Under magnetic guidance, the DNA microspheres demonstrated rapid and targeted movement within the thrombus solution (Fig. S3 in the ESM). A clear dissolution of blood clots was observed in all groups except the saline (Fig. 5(a)) and MG (Fig. 5(b)) controls. After 6 h, significant clot dissolution occurred. The MG/tPA group had a slower release rate and thus had difficulty in rapidly dissolving the clots (Fig. 5(c)).

Notably, the MG/tPA/CRISPR group (Fig. 5(d)), having the same thrombolytic effect as free tPA (Fig. 5(e)), enabled rapid and controlled tPA release, which exhibited the most pronounced thrombolytic effect.

3.5 Construction and verification of femoral artery thrombosis model in rats

To evaluate thrombus formation *in vivo*, a ferric chloride (FeCl_3)-

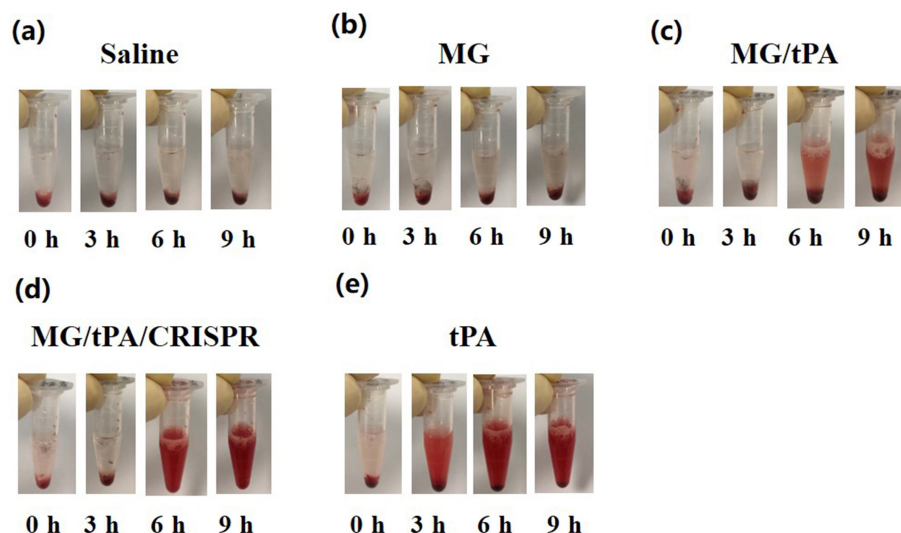


Figure 5 Assessment of dissolution efficiency in *ex vivo* blood clots. Representative photographs of the blood clot dissolution processes at 0, 3, 6, and 9 h after treating with (a) saline, (b) MG, (c) MG/tPA, (d) MG/tPA/CRISPR, and (e) tPA, respectively.

induced injury model was established in the femoral artery of SD rats, the thrombosis formation time was optimized. The results indicated that thrombosis could be formed within 3 min (Fig. 6(a)). Doppler ultrasound imaging was used to monitor blood flow before and after thrombus induction. Before thrombosis formation, the blood vessels had a significant blood flow (Fig. 6(b)). Following injury, a pronounced decrease in blood flow was recorded, confirming successful vessel occlusion through the variations of blood flow (Fig. 6(c)). Histological examination of hematoxylin and eosin (H&E)-stained sections further validated the model, revealing clear morphological differences between normal (Fig. 6(d)) and thrombosed (Fig. 6(e)) femoral arteries. These results collectively confirm the successful establishment of a lower-extremity arterial thrombosis model in rats.

3.6 The efficacy of thrombolysis in a rat model

The *in vivo* targeting performance of the system was evaluated using indocyanine green (ICG)-labeled magnetic DNA microspheres encapsulating tPA. Following intravenous injection via the tail vein, photoacoustic imaging was performed. Owing to the magnetic field-guided targeting capability of the MG/tPA complex, a strong photoacoustic signal was localized specifically at the site of femoral artery injury after administration of ICG-modified MG-tPA (Fig. 7(b)), showing a significant increase compared with the control group (Fig. 7(a)). This result confirms the high targeting accuracy of the designed construct. Both the photoacoustic average signal and the photoacoustic average threshold were notably enhanced after active targeting with ICG-modified MG-tPA (Figs. 7(c) and 7(d)). The value of PA Avr Thresh increased from 0.296 to 0.989 mm² ($n = 3$) due to the ICG-modified MG-tPA targeting to the site of thrombosis by magnetic field. Doppler ultrasound imaging was further used to monitor blood flow after thrombosis induction and subsequent drug delivery (Figs. 7(e) and 7(f)). A significant decrease in blood flow velocity was observed following thrombus formation (Fig. 7(g)). In contrast, administration of MG/tPA/CRISPR *in vivo* led to a marked recovery in blood flow velocity (Fig. 7(h) and Fig. S4 in the ESM). The blood flow velocity detected by ultrasound enhanced from 39.193 to 74.762 mm/s ($n = 3$), demonstrating that the thrombolytic drugs (tPA) are targeted delivered to the thrombus

site by magnetic microspheres and can be rapidly released, achieving an efficient thrombolytic effect. To evaluate the biosafety of tPA treatment with magnetic microspheres delivery, the result of routine blood test demonstrated that there was no significant change with control and treatment groups in SD rats (Fig. S5 in the ESM). The level of biochemical parameters in serum, including liver function related indexes and kidney metabolism related indicators, also exhibited no significant variation (Fig. S6 in the ESM). Meanwhile, H&E staining of the major organs isolated from the rats showed negligible changes in histomorphology with control and treatment groups, in which no obvious damage was found in the organs (Fig. S7 in the ESM). Therefore, no immune response and other side effects *in vivo* were induced in treatment of thrombolytic therapy. The therapeutic approach was also applied in a mouse model of inferior vena cava thrombosis (Fig. S8 in the ESM). Histological analysis of thrombosed tissues after MG/tPA/CRISPR treatment revealed substantially enhanced thrombolysis compared with controls (Fig. S9 in the ESM), demonstrating the broad efficacy and potential of this strategy for thrombus treatment.

4 Conclusions

In summary, we successfully developed a magnetically guided drug delivery system based on DNA microspheres self-assembled via programmable rolling circle amplification and functionalized with iron oxide nanoparticles. This system enables the targeted co-delivery of thrombolytic drugs to specific thrombus sites for integrated diagnosis and therapy. The tPA-loaded magnetic DNA microspheres allow precise thrombus targeting and visualization through US and PAI. Moreover, by incorporating CRISPR-Cas9 for regulatable drug release, the system promotes rapid penetration of tPA into the clot, enhancing intraluminal mass transfer and significantly improving thrombolytic rate and therapeutic efficacy. With its favorable biocompatibility and targeting capability, this magnetic DNA microsphere platform provides a promising strategy for precise diagnosis and effective treatment of thrombotic diseases.

5 Discussion

Thrombolytic therapy plays a crucial role in medicine, with its

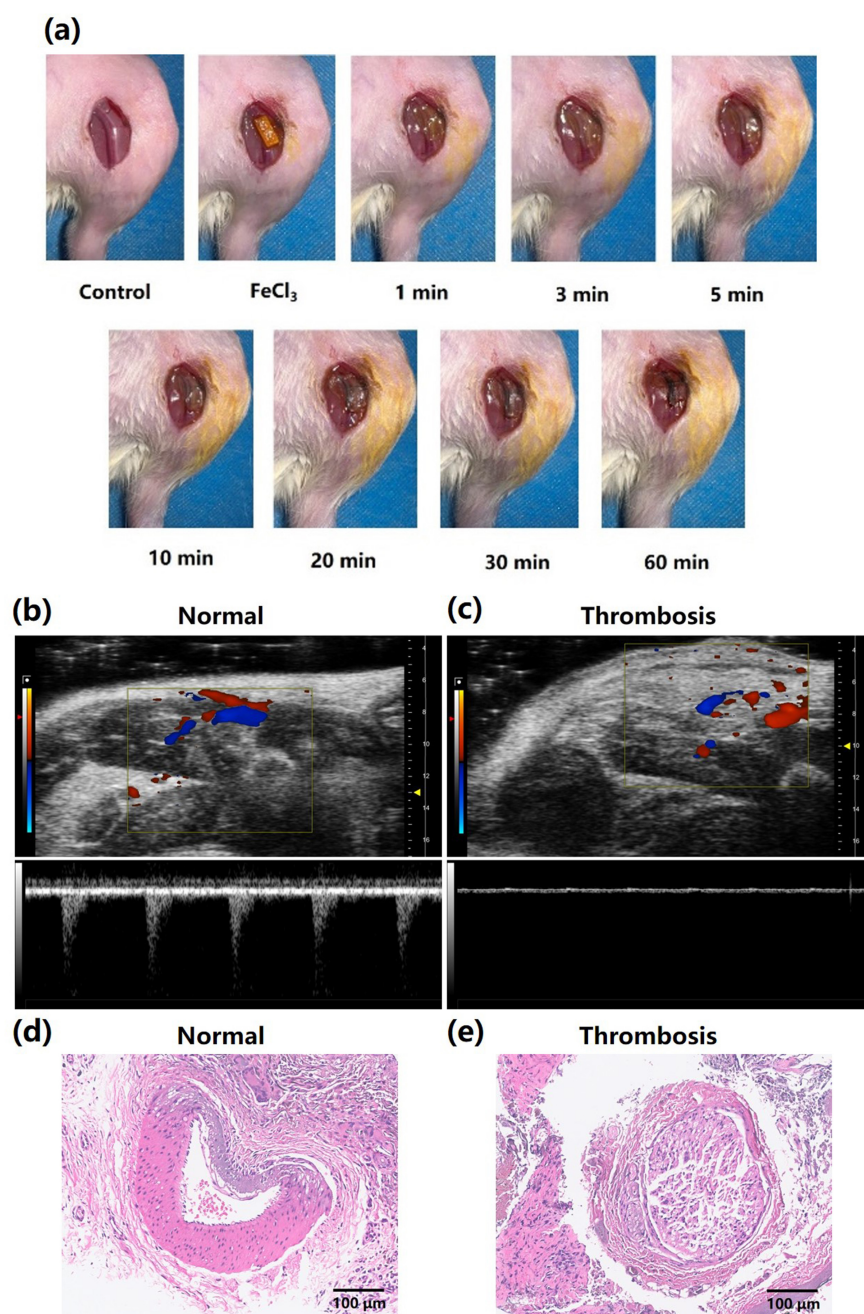


Figure 6 Construction of a lower extremity artery thrombosis rat model. (a) Establishment of the rat femoral artery thrombus model and condition optimization. The blood flow variations in the clotted femoral artery area (b) before and (c) after thrombosis monitoring with ultrasound. The histological images of the femoral artery (d) before and (e) after thrombus formation. Scale bars: 100 μm .

significance lying in saving lives, protecting organ functions, preventing recurrence, and improving the quality of life for patients. Under the action of thrombolytic drugs, the fibrinogen in the body can be activated and transformed into fibrinase, which can cleave the fibrin that constitutes the framework of the thrombus. When the fibrin network breaks down, the thrombus loses its supporting structure and disintegrates into fragments, which are then carried away by the blood, thus restoring the patency of the blocked blood vessels. However, traditional thrombolytic therapy is associated with bleeding complications, which is a major concern as it is a systemic rather than a localized effect. Thrombolytic drugs not only dissolve the thrombus in the blood vessels but also dissolve the

functional thrombi formed during the hemostasis process in other parts of the body or weaken the stability of the blood vessel walls. Moreover, there is a time sensitivity issue. The narrow time window makes traditional thrombolytic therapy extremely time-sensitive, causing many patients to miss the optimal treatment opportunity. The earlier the thrombolytic therapy is administered, the better effect can be achieved. Therefore, the precise delivery of thrombolytic drugs to the thrombus site and their rapid release are of great significance in thrombolysis.

DNA molecules and RCA amplification technology are widely used in disease diagnosis and treatment. In this study, DNA microspheres with sequence-programmable characteristics are used

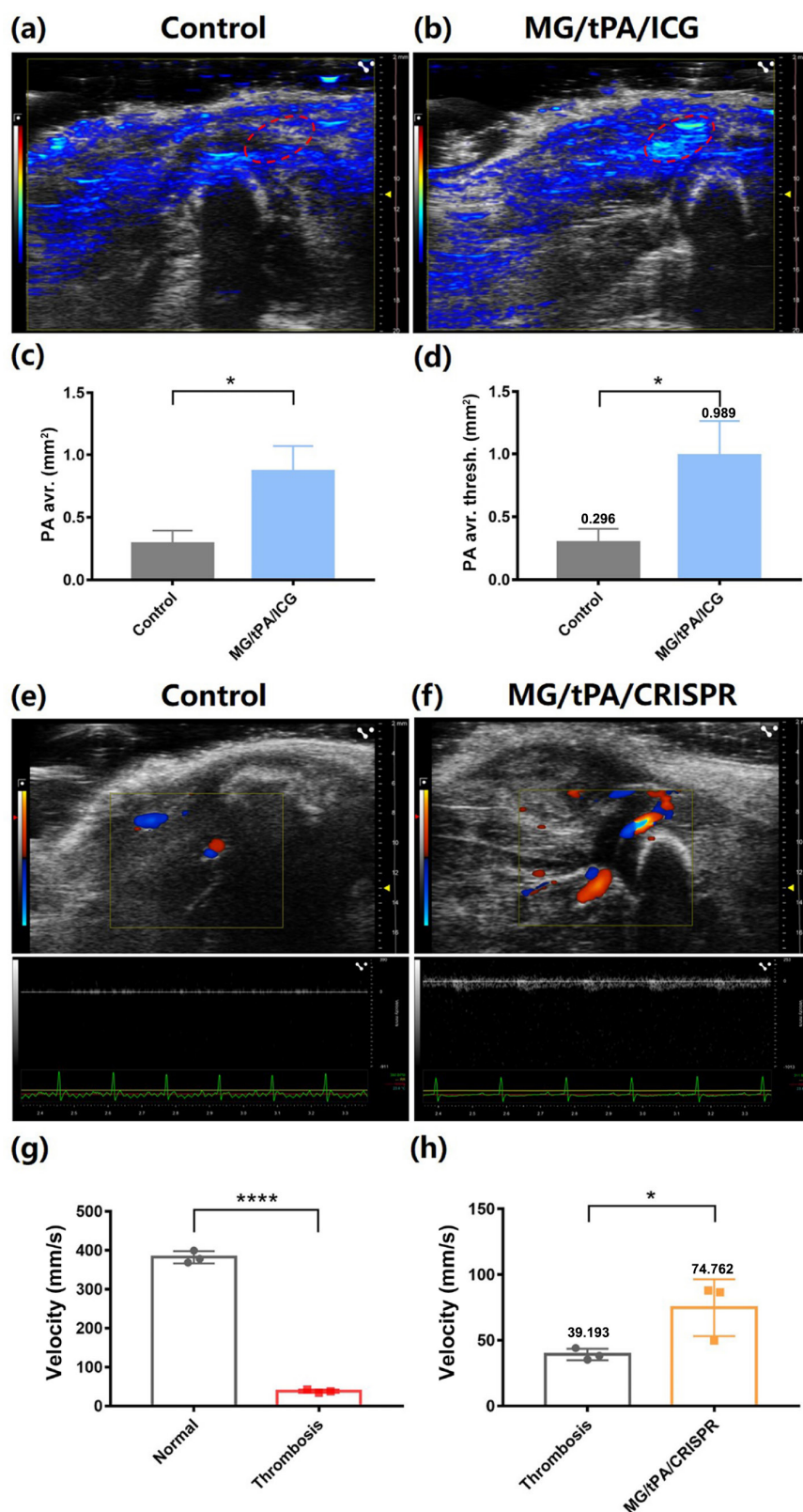


Figure 7 The efficacy of targeted delivery and lower extremity arterial thrombolysis in a rat model. The photoacoustic signal (a) before and (b) after magnetic DNA microspheres with ICG administration. (c) The PA average intensity and (d) PA average intensity threshold changes in the clotted femoral artery area before and after MG/tPA/ICG treatment. The blood flow variations (e) before and (f) after thrombolysis in the rat femoral artery thrombus model monitored by Doppler ultrasound flow imaging. (g) The blood flow variations in the femoral artery area were monitored by Doppler flow imaging before and after thrombolysis. (h) The blood flow variations in the femoral artery area were monitored by Doppler flow imaging before and after MG/tPA/CRISPR treatment. Data are presented as mean $\pm$ s.d., $n = 3$. Statistical significance was performed by two-tailed unpaired t test analysis. Statistical significance was set at $*P < 0.05$, and extreme significance was set at $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

as carriers, which have good biocompatibility and are easy to functionalize. In this research, iron oxide nanoparticles (Fe_3O_4 NPs) are used as functional moiety and bound to the DNA microspheres through DNA hybridization, providing magnetic targeting properties to facilitate the rapid enrichment of the microspheres at specific locations, which enabled efficient delivery of thrombolytic drugs to the thrombus. In addition, by combining the sequence-programmable characteristics of DNA with CRISPR-Cas9 technology, specific cleavage sequences are designed. In the presence of the Cas9/sgRNA complex, the microsphere structure can be precisely cleaved, rapidly releasing the thrombolytic drugs and enhancing the therapeutic effect on thrombosis.

The successful construction of the thrombolytic drug delivery system was verified through SEM, EDS, and ultrasound imaging. The MG/tPA/CRISPR can rapidly dissolve thrombi in an *in vitro* blood clot solution and was targeted to the thrombus site in an *in vivo* rat lower extremity artery thrombosis model, as demonstrated by photoacoustic imaging. After precise delivery and rapid release of the thrombolytic drugs, the blood flow velocity was monitored by Doppler ultrasound imaging. The increase in blood flow velocity confirmed that MG/tPA/CRISPR could efficiently achieve targeted delivery and rapid release of thrombolytic drugs.

Electronic Supplementary Material: Supplementary material (the images of synthesis and regulation, the routine blood test, liver and kidney function index detection, histological analysis, and further details of nucleic acid sequences) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94908971>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

S. Y. and N. N. W.: Data curation, validation, funding acquisition, writing manuscript, experimental design. Y. H. H., X. G. W., and Q. Y. T.: Data curation, validation. G. Q. T. and J. G. S.: Project administration, writing manuscript. P. F. L.: Project administration, funding acquisition, writing manuscript. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

All the animal experiments were carried out following the guidelines of the Ren Ji Hospital for the Care and Use of Laboratory Animals and were approved by Ren Ji Hospital Ethics Committee (Ethical No.: RJ2023-084A).

Use of AI statement

None.

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