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Research Article

Stealth hemostatic anchors with vWF-driven navigation and plasmin-triggered tranexamic release for hematoma containment in cerebral hemorrhage

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ABSTRACT

The high mortality and disability rates associated with spontaneous intracerebral hemorrhage (sICH) are primarily attributed to secondary injuries caused by hematoma expansion from continuous bleeding or rehemorrhage. Rapid hemostasis to prevent hematoma progression is critical in clinical emergencies for improving surgical outcomes and patient prognosis. For internal hemorrhages inaccessible to external interventions, especially for sICH, intravenous hemostatic strategies are essential regardless of ultimate surgical eligibility. This study reported a stealth hemostatic anchor system based on peptide-drug conjugates. Tranexamic acid (TXA), a clinically approved antifibrinolytic agent, served as the hemostatic component, while a von Willebrand factor (vWF)-binding peptide (VBP) enabled targeted delivery by specifically binding to (vWF) exposed at vascular injury sites. A plasmin-cleavable linker was incorporated to control TXA release, ensuring site-specific drug activation. The plasmin-responsive peptide-drug conjugate (RPDC) was synthesized by covalently linking TXA to VBP via the plasmin-cleavable linker. *In vitro* and *in vivo* experiments verified the targeted hemostatic efficacy of RPDC, especially demonstrating 42% reduction in hematoma volume ($P < 0.001$ vs. saline; $P < 0.05$ vs. free TXA) with mitigated peri-hematoma pathology in the collagenase-induced ICR mouse ICH model. These results highlight the potential of the stealth hemostatic anchor as a precision

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therapeutic strategy for managing sICH, particularly in cases of internal hemorrhages inaccessible to surgical intervention or visual inspection. The plasmin-dependent targeting mechanism enables precise drug localization at cryptic hemorrhage sites, but further studies in larger animal models are needed to confirm its efficacy. This design offers a theoretical framework for advancing emergency interventions in cerebral hemorrhage and addressing challenges related to inaccessible bleeding sites.

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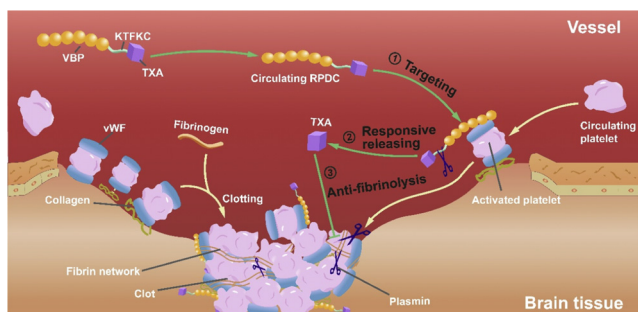
1. Introduction

Spontaneous intracerebral hemorrhage (sICH) represents one of the most catastrophic neurological emergencies, characterized by the rupture of intracerebral blood vessels, often as a consequence of chronic cerebrovascular disease. With an estimated global incidence of 2 million cases annually—constituting 10%–15% of all stroke events—sICH stands as the predominant contributor to mortality and long-term disability in hemorrhagic stroke patients, posing a formidable challenge to modern medicine [1,2]. The pathophysiological cascade of sICH is significantly exacerbated by hematoma expansion (HE), a critical phenomenon defined as the progressive increase in intracranial blood volume due to persistent or recurrent bleeding following the initial hemorrhage [3–5]. This expansion not only amplifies the mechanical mass effect but also triggers a series of secondary injuries, leading to devastating neurological sequelae including cognitive impairment, visual deficits, and motor dysfunction. In severe cases, HE can precipitate life-threatening complications such as intracranial hypertension and brain herniation, often progressing too rapidly to permit surgical intervention [3,6]. The clinical imperative to address HE is underscored by its profound impact on patient outcomes. Early intervention to either eliminate the hematoma or prevent its expansion has emerged as a cornerstone of emergency management, offering the potential to significantly enhance survival rates and improve long-term prognosis [7,8]. Current therapeutic strategies primarily focus on two fronts: acute blood pressure (BP) reduction and hemostatic intervention [9,10]. However, the optimal parameters for BP management remain contentious, with limited evidence demonstrating substantial reductions in mortality or disability, as illustrated by INTERACT3: its bundled care strategy incorporating rapid BP lowering demonstrated overall functional benefit, but efficacy varied substantially by hemorrhage location and treatment timing. In contrast, hemostatic therapies have shown more promising results in curtailing HE, though their application is complicated by the unique anatomical and physiological constraints of cerebral tissue [11,12]. The challenges inherent in managing sICH are further compounded by the limitations of conventional hemostatic methods. Traditional approaches such as external compression or mechanical compression are rendered ineffective by the intracranial nature of the

hemorrhage [12]. Consequently, the direct administration of coagulants has emerged as a crucial therapeutic modality in the acute management of sICH, representing a critical area of ongoing research and clinical innovation.

Hemostasis represents an exquisitely orchestrated biological process, meticulously regulated through precise temporal and spatial control mechanisms that maintain the delicate equilibrium between thrombosis and thrombolysis [13,14]. However, this physiological balance is vulnerable to disruption under pathological conditions, particularly in cases of trauma-induced coagulopathy or systemic inflammatory responses. Such disruptions can precipitate a cascade of hemostatic dysregulation characterized by structurally compromised fibrin clots, hyperfibrinolysis, and disseminated bleeding, often culminating in life-threatening complications such as disseminated intravascular coagulation [15–17]. Consequently, there is a need for additional strategies, including pharmacological agents, biomaterials, and advanced nanotechnologies, to either reduce fibrinolysis or enhance fibrin strength and stability, thereby restoring hemostatic balance and improving clinical outcomes. Central to this therapeutic paradigm is the fibrinolytic system—A sophisticated enzymatic network responsible for blood clot dissolution and vascular patency maintenance [18]. At the core of this system lies plasminogen and its active enzyme, plasmin [19,20]. In sICH, the fibrinolytic system assumes particular significance. The local tissue injury and inflammatory responses characteristic of sICH trigger plasminogen activation, leading to elevated plasmin levels. While this physiological response facilitates hematoma resolution, excessive plasmin activity can paradoxically exacerbate the pathological process, contributing to HE, cerebral edema, and secondary neurological injury [21]. Therefore, modulating the activity of the fibrinolytic system may represent a novel therapeutic strategy for the management of sICH.

The antifibrinolytic drugs commonly utilized in clinical practice encompass aminocaproic acid, aminomethylbenzoic acid and tranexamic acid (TXA). Among these, TXA exhibits the most potent antifibrinolytic efficacy [22–24]. TXA is a lysine analogue, which exerts its pharmacological action through a sophisticated molecular mechanism, competitively binding to the lysine-binding sites of plasminogen and tissue plasminogen activator (tPA). This molecular interaction effectively disrupts the critical binding of these fibrinolytic components to lysine residues on the fibrin surface,



Scheme 1 – Construction of plasmin-responsive peptide-drug conjugate (RPDC) and its intelligent targeted hemostasis mechanism.

thereby attenuating plasmin generation and subsequent clot dissolution [25]. Recent and ongoing clinical trials have demonstrated the beneficial effects of TXA in reducing active bleeding both prehospital and during hospitalization [23,24,26]. Particularly noteworthy is its demonstrated efficacy in intracerebral hemorrhage, where timely administration has shown the potential to alter the disease trajectory [27]. However, direct administration of TXA is fundamentally limited by its non-targeted systemic delivery, which necessitates high doses (typically 1–2 g bolus + infusion) that increase risks of thrombotic events by approximately 2% in high-dose regimens [28] and may lead to thrombotic events (e.g., myocardial infarction, ischemic stroke) [28,29]. Furthermore, TXA's short plasma half-life (~2 h) requires repeated dosing, exacerbating off-target effects without guaranteeing sufficient drug accumulation at the hemorrhage site, including off-target thrombosis, thromboembolism, and neuropathy. Therefore, it is urgently necessary to precisely deliver TXA to the bleeding site and control its release to enhance its safety and efficacy.

To address these issues, we engineered a stealth hemostatic anchor system, the plasmin-responsive peptide-drug conjugate (RPDC), designed to dynamically regulate fibrinolytic system activation through a sophisticated feedback mechanism (Scheme 1). This innovative approach leverages a von Willebrand factor (vWF)-binding peptide (VBP), a 15-amino acid sequence TRYLRHPQSWVHQI, to navigate the RPDC to bleeding sites by binding to vWF on activated platelets. A plasmin-cleavable peptide (KTFKC) is incorporated to detect plasmin activity and trigger the release of TXA, which in turn inhibits plasmin activation and stabilizes blood clots. This dual-action mechanism enables rapid hemostasis and limits HE. Our study validated the targeted and intelligent hemostatic efficacy of RPDC through rigorous *in vitro* and *in vivo* experiments, and assessed neurological outcomes in a mouse model of ICH. The RPDC platform represents a transformative approach to hemostatic therapy, offering unprecedented spatial and temporal control over antifibrinolytic activity. This work establishes a new technological paradigm for the management of sICH and other invisible hemorrhagic disorders, with potential implications for revolutionizing clinical approaches to critical bleeding scenarios.

2. Materials and methods

2.1. Materials

TXA (98%) was purchased from Shanghai Yien Chemical Technology Co., Ltd. (Shanghai, China). Urokinase-type plasminogen activator (uPA) was obtained from Wuhan Renfu Pharmaceutical Co, Ltd. (Wuhan, China). Acetonitrile ($\geq 99.9\%$, gradient grade) and collagenase IV were bought from Sigma-Aldrich. Calcein-AM/PI Live/Dead Assay Kit, Methylthiazolyldiphenyl-tetrazolium bromide (MTT), and DAPI Staining Solution were obtained from Beyotime biotechnology (Shanghai, China). DMEM medium, RPMI 1640 medium, fetal bovine serum (FBS) and 1% double antibiotics were purchased from Gibco of ThermoFisher Scientific. HRP-anti-CD31 and FITC-anti-Fibrinogen antibodies were purchased from Abbot Trading Co., Ltd. (Shanghai, China). The human umbilical vein endothelial cells (HUVECs) and rat cortical neuron cells (RN-cs) were obtained from Shanghai Qingqi Biotechnology Development Co., Ltd. (Shanghai, China). HUVECs were used between passages 3–8 and were cultured in RPMI 1640 medium containing 10% FBS and 1% double antibiotics. RN-cs were used between passages 1–2 and were cultured in DMEM medium containing 10% FBS and 1% double antibiotics.

SPF-grade male ICR mice (25–35 g) were supplied by the Animal Center of Guizhou Medical University (license number: SCXK(Gui)2023–0002). The animals were maintained in a well-ventilated facility with controlled environmental conditions: temperature 18–25 °C, relative humidity 40%–70%, and a 12 h light-dark cycle. Mice were housed in groups of five per cage with *ad libitum* access to food and water. Prior to the experiments, the mice underwent a one-week acclimatization period. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Medical University (Approval number: 2403421).

2.2. Synthesis and molecular weight determination of TXA-peptide drug conjugate

The plasmin-responsive peptide-drug conjugate (RPDC, sequence: Tranexamic acid-KTFKC-TRYLRHPQSWVHQI) and non-responsive conjugate (NPDC, sequence: Tranexamic acid-TRYLRHPQSWVHQI) were synthesized by Nanjing Peptide Biotechnology Co., Ltd. via Fmoc-based solid-phase synthesis on Fmoc-Ile-Wang resin (1.5 g) using iterative deprotection (20% piperidine/DMF), washing (DMF/DCM), and coupling of Fmoc-amino acids (HBTU/HOBt/DIPEA activation), followed by terminal tranexamic acid conjugation and TFA/TIS/H₂O (95:2.5:2.5, v/v/v) cleavage. Crude products were purified by preparative HPLC (Beijing Innovation Tongheng LC system, CXTH-3000 workstation) with C₁₈ column and ACN/H₂O gradient elution (optimized via analytical scouting), collecting fractions guided by target mass confirmation. Purified conjugates were analyzed by analytical HPLC (Condition details are provided in the supplementary materials). Molecular weights were confirmed using LC-MS (LCMS-2020, SHIMADZU) controlled by LabSolutions software (v5.99).

for system operation and data analysis. Measurements were conducted in ESI positive mode under standardized conditions: capillary voltage 4.5 kV, source temperature 250 °C, desolvation line temperature 400 °C, and cone gas flow 1.5 l/h.

2.3. *In vitro plasmin-responsive drug release testing*

The drug-responsive release characteristic was determined. Briefly, 1.0 ml RPDC and NPDC solution (10 mg/ml) were each mixed with 100 μ l uPA solution (50,000 U/ml) and transferred into dialysis bags (MWCO = 3.5 KDa). The dialysis bags were subsequently immersed in phosphate-buffered saline (PBS; 35 ml) at pH 7.4. The release study was conducted in a thermostatic tank under gentle shaking at 37 °C. Next, at certain times (15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h), 1.0 ml dialysis fluid outside the bag was sampled and an equal amount of fresh release medium was supplied immediately to keep the same volume. The release amount of TXA was determined using an LCMS (Condition details are provided in the supplementary materials).

2.4. *In vitro coagulation effect evaluation of RPDC*

The coagulation effects of RPDC and NPDC were preliminarily assessed using an *in vitro* blood inversion assay. First, the reagents treated with uPA were prepared. Briefly, four 1.5 ml EP tubes were prepared, each containing 270 μ l of one of the following solutions: saline, RPDC solution (equivalent to 0.3 mg/ml TXA), NPDC solution (equivalent to 0.3 mg/ml TXA) or TXA solution (0.3 mg/ml). Subsequently, 30 μ l of uPA solution (1 U/ μ l) was added to each tube to cleave the -KTFKC- linker and release TXA. The mixtures were thoroughly mixed and incubated at 37 °C for 5 min. In parallel, four additional 1.5 ml EP tubes were prepared, each containing 200 μ l mouse anticoagulated blood. Then, 20 μ l pre-incubated mixture was added to the anticoagulated blood tubes accordingly. The coagulation status of the blood samples was observed and documented by inverting the EP tubes at 5, 10, 15, 30, 45 and 60 min post-addition.

2.5. *TEG analysis of the anti-fibrinolytic effect of RPDC*

Thromboelastography (TEG) was used to further evaluate the anti-fibrinolytic effect of RPDC. Firstly, the TEG instrument (Guizhou Jinjiu Biotechnology Co., Ltd.) was debugged and set the conditions at 37 °C. The blood cups containing 300 μ l sodium citrate-anticoagulated blood were divided into four groups: uPA, uPA+TXA, uPA+RPDC, and uPA+NPDC. Then, the reagents (RPDC, NPDC, TXA, uPA and saline) were added to the blood groups accordingly and incubated at room temperature for 5 min. Subsequently, 30 μ l CaCl₂ (0.2 mol/l) was added and incubated for 1 min, immediately loaded the cup onto the TEG instrument and clicked "start". The critical parameters such as R (clot formation time), K (the time from the R time endpoint to the point where the tracing amplitude reaches 20 mm), MA (maximum amplitude), α -angle and LY30 (the rate of reduction in the amplitude of the blood

clot within 30 min after the MA value is determined) were obtained.

2.6. *Hemostatic efficacy of RPDC in the ICH model*

The collagenase-induced intracerebral hemorrhage (ICH) mouse model [30] was established to evaluate the hemostatic efficacy of RPDC. Briefly, after weighing and anesthetizing the mice, the animals were secured in a prone position on a stereotaxic frame (model 68025, RWD Life Science Co., Ltd., Shenzhen, China). The position of the syringe was adjusted to ensure accurate targeting relative to the bregma point (2.2 mm lateral and 0.2 mm anterior). A burr hole was drilled at this location, and the syringe needle was advanced to a depth of 3.5 mm. Collagenase IV (0.075 U in 2 μ l) was then infused at a rate of 0.1 μ l/min. Following the injection, the needle was left in place for 5 min before being retracted at a speed of 1 mm/min. The aseptic bone wax was used to seal the needle hole, followed by skin suturing. Sham-operated mice underwent the same procedure but were injected with sterile saline instead of collagenase IV. The ICH mice were randomly divided into four groups (10 per group): model, TXA, RPDC and NPDC group. Drugs were administered via tail vein injection (TXA at a dose of 14 mg/kg). 24 h post-intervention, the mice were euthanized and subjected to cardiac perfusion. Subsequently, brain tissues were harvested and fixed with 4% paraformaldehyde for 48 h.

For the measurement of hemorrhage volume. The fixed brain tissues were sectioned into 8 equally spaced coronal slices, each with a thickness of 1 mm. Hemorrhagic areas were quantified using ImageJ software (version 1.44) [31]. For each slice, the rostral surface (side A) and the caudal surface (side B) were imaged. The mean hemorrhage area for each slice was derived by averaging the summed areas of sides A and B. The total hemorrhage volume for each mouse brain was calculated by multiplying the mean hemorrhage area of each slice by the slice thickness (1 mm). Furthermore, the brain tissue slices with hemorrhage were embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E) to evaluate pathological alterations in the peri-hematoma brain tissue.

In addition, the permeability of the blood-brain barrier (BBB) in ICH mice was evaluated. In detail, the ICH mice were randomly divided into five groups (six mice per group): sham group, model group, TXA group, RPDC group, and NPDC group. Each group of mice was administered the corresponding drug via tail vein injection (TXA at a dose of 14 mg/kg). 18 h later, mice were injected with 0.12 ml of 2% Evans blue (EB) solution per 30 g body weight via intraperitoneal injection. 6 h after EB injection, the mice were euthanized, and brain tissues were collected following cardiac perfusion. The brain tissues were weighed, homogenized, and treated with formamide. After centrifugation, the absorbance of the supernatant was measured at 620 nm, and an EB standard curve was plotted to calculate the EB content in the brain tissues. The extent of the BBB disruption was determined based on the EB content in the brain tissue. EB content was calculated using the formula: EB content = B $\times$ formamide volume/wet weight, where B

represents the measured EB content ($\mu\text{g/ml}$) in the brain tissue.

2.7. Targeting hemorrhage localization of RPDC

To further elucidate the hemostatic efficacy of RPDC, the *in vivo* distribution, and hemorrhagic site targeting were investigated. In detail, ICH mice were divided into two groups (nine per group): RPDC and control. The mice in the RPDC group were intravenously injected with Cy5-labeled RPDC (Cy5-RPDC) at a dose of 5 mg/kg (calculated by Cy5), while the control group was administrated with the same dose of free Cy5 to simulate free drug's distribution. Cy5-RPDC was synthesized by conjugating Cy5 NHS ester (Lumiprobe) to primary amines on RPDC in 0.1 M NaHCO_3 buffer (pH 8.5) for 2 h at 25 °C, followed by PD-10 column purification (Cytiva) and UV-Vis validation ($\epsilon_{649}=250,000 \text{ M}^{-1}\text{cm}^{-1}$, labeling efficiency >95%). The *in vivo* biodistribution of Cy5 was tracked using the small animal *in vivo* imaging system (PerkinElmer Inc., American) at 1, 12 and 24 h post-injection. Then, the mice were anesthetized and euthanized to harvest brain tissues and monitored by the small animal *in vivo* imaging system.

The brain tissues at 24 h were collected and fixed for immunofluorescence analysis. Brain sections were stained with DAPI (blue, staining neuron nuclei), HRP-anti-CD31 (red, labeling blood vessel), and FITC-anti-Fibrinogen (green, labeling thrombus) respectively. The Cy5-RPDC was purple. The fluorescence images were captured by a fluorescence microscope (NIKON ECLIPSE E100, Japan) using a 20 × objective lens (scale bar: 50 μm).

2.8. Statistical analysis

Statistical analyses were conducted using SPSS version 26.0 (IBM, America). To determine statistical significance, one-way ANOVA was employed, followed by Dunnett's post hoc test for comparisons among three or more groups concerning a single variable. A *P*-value threshold of <0.05 was set to establish statistical significance.

3. Results and discussion

3.1. Synthesis and molecular weight determination of TXA-peptide drug conjugates

We engineered RPDC by conjugating a VBP and TXA via KTFKC, as depicted in Fig. 1A. For comparison, a NPDC was synthesized by directly linking VBP to TXA without the cleavable sequence (Fig. 1B inset). Both conjugates featured chemically modified TXA carboxyl termini (the active site) to maintain inactive states until targeted release. Both RPDC (yield: 30.2%) and NPDC (yield: 30.5%) were synthesized by Nanjing Peptide Biotechnology Co., Ltd. using solid-phase synthesis. HPLC analysis (Fig. S1) demonstrated high purity for both conjugates (RPDC: 98.24% at 11.87 min; NPDC: 98.48% at 11.74 min). The results of LC-MS spectrum (Fig. 1A and B) confirmed molecular weights of RPDC (2681.18 Da) and NPDC (2073.41 Da). The dominant $[\text{M} + 2\text{H}]^{2+}$ peak at m/z 1341.40 (calc. 2681.18 Da) in RPDC MS spectrum

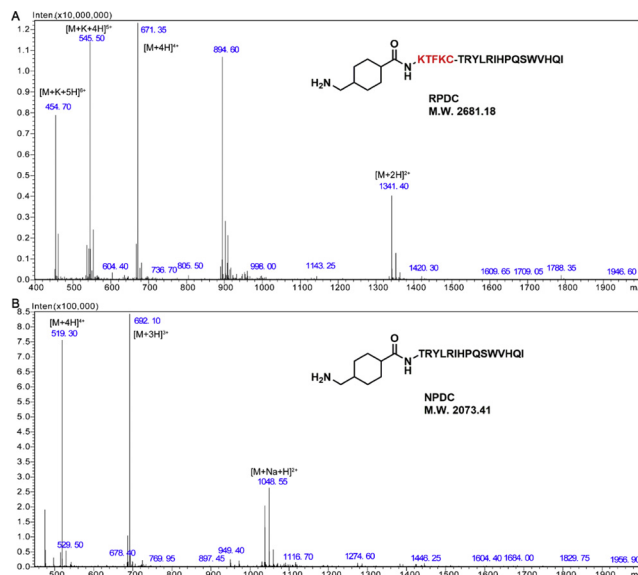


Fig. 1 – Mass spectrum of RPDC (A) and NPDC (B) with chemical structure of inset figure of RPDC and NPDC.

matches the theoretical mass of Tranexamic acid-KTFKC-TRYLRIHPQSWVHQI (calc. 2681.20 Da, error <0.05%). For NPDC (Fig. 1B), the $[\text{M} + \text{Na} + \text{H}]^{2+}$ adduct at m/z 1049.55 (calc. 2073.10 Da for Tranexamic acid-TRYLRIHPQSWVHQI, error <0.05%) and corroborate the expected mass. This precise molecular engineering establishes a foundation for targeted, plasmin-responsive drug delivery.

3.2. In vitro plasmin-responsive drug release testing

To validate the site-specific drug release capability of RPDC at vascular injury sites, we conducted *in vitro* plasmin-responsive drug release experiments using dialysis method. This assay was designed to simulate pathological fibrinolysis at bleeding sites, where uPA converts plasminogen to active plasmin, which specifically cleaves the Lys-Thr bond within the KTFKC linker of RPDC to release TXA. Specifically, RPDC solutions containing uPA (50,000 U/ml) were encapsulated in the cellulose membrane dialysis bags (MWCO 500 Da), where the membrane retained large peptide conjugates (>5 kDa) while allowing liberated TXA (MW 157 Da) to diffuse. The released TXA was quantified over incubation time. The cumulative drug release profile over time was then plotted, as illustrated in Fig. S2. RPDC exhibited rapid and sustained release (>50% TXA within 24 h) due to plasmin-specific cleavage, whereas negligible release (<10%) occurred from NPDC (lacking the plasmin-sensitive sequence) ($P < 0.001$). These results confirm RPDC's plasmin-triggered release mechanism and its potential for targeted hemostasis.

3.3. In vitro safety assessment of the TXA-peptide drug conjugates

To evaluate the safety profiles of RPDC and NPDC, we conducted comprehensive *in vitro* cytotoxicity and hemocompatibility assessments. Using the MTT assay

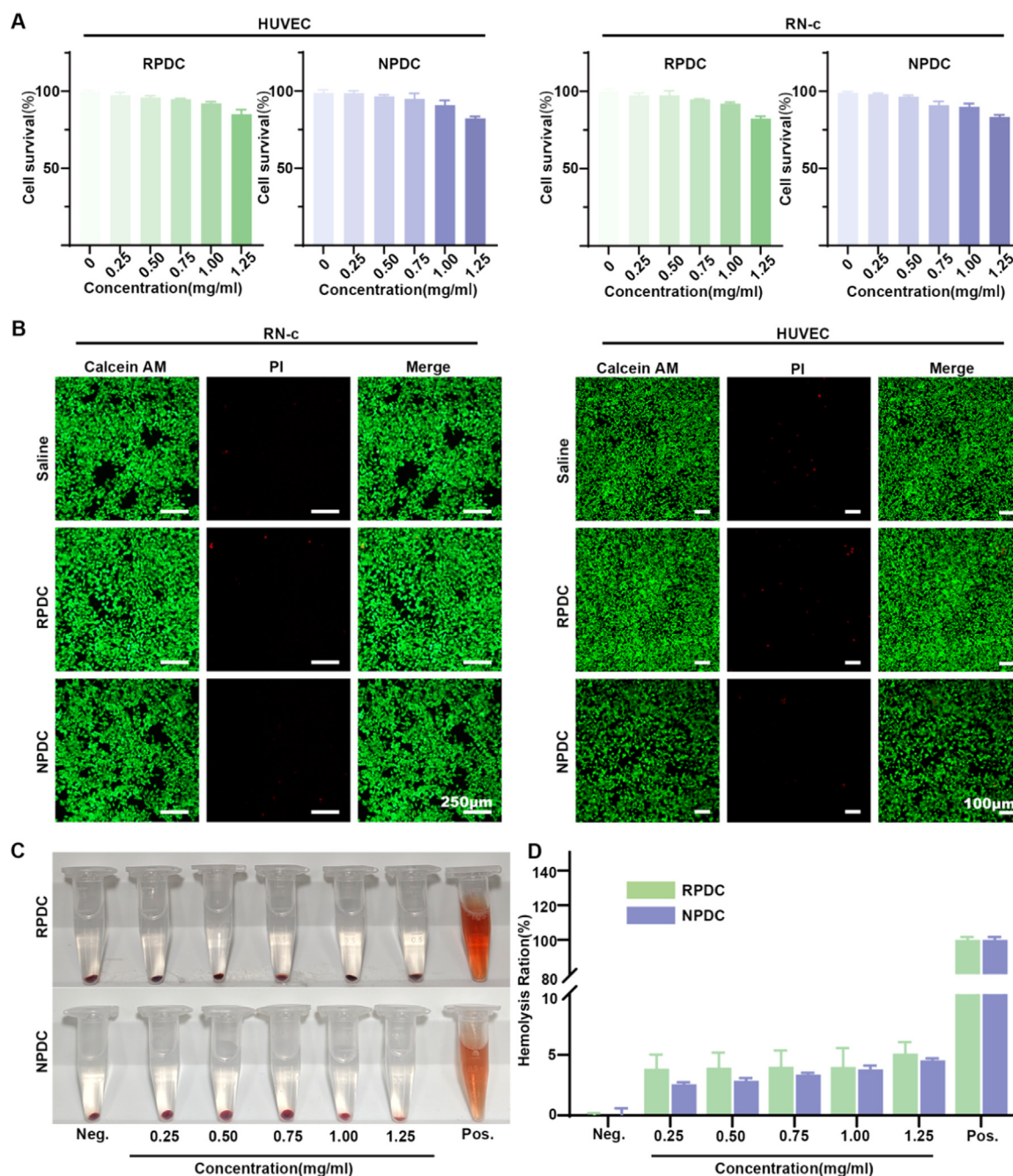


Fig. 2 – In vitro safety assessment of TXA-peptide drug conjugates. (A) Cell viability of HUVECs and RN-cs following treatment with series concentrations of RPDC and NPDC for 24 h; (B) Live/dead staining of HUVECs and RN-cs following treatment with series concentrations of RPDC and NPDC for 24 h (Scale bar: 100 μm); (C) Representative photos of blood samples following treatment with series concentrations of RPDC and NPDC for 2 h; (D) Hemolysis rates of samples in (C).

following a 24 h incubation period at 37 °C, we examined the effects of these conjugates on HUVECs and RN-cs. As shown in Fig. 2A, both RPDC and NPDC demonstrated minimal cytotoxicity at concentrations ranging from 0.25 mg/ml to 1.00 mg/ml, with cell viability consistently exceeding 95%. However, at concentrations above 1.0 mg/ml, a 5%–10% reduction in cell viability was observed, indicating potential cytotoxic effects on both cell types. To further validate these findings, live/dead cell staining was performed at 1.00 mg/ml.

The results, depicted in Fig. 2B, revealed that the majority of cells in all treatment groups remained viable, underscoring the favorable biocompatibility of both conjugates at this concentration. Collectively, these findings indicate that RPDC and NPDC exhibit favorable cell compatibility at 1.00 mg/ml.

Furthermore, hemolysis experiments were conducted using PBS as the negative control and Triton X-100 (1%) as the positive control to evaluate the blood compatibility of RPDC and NPDC. Hemolysis results demonstrate that

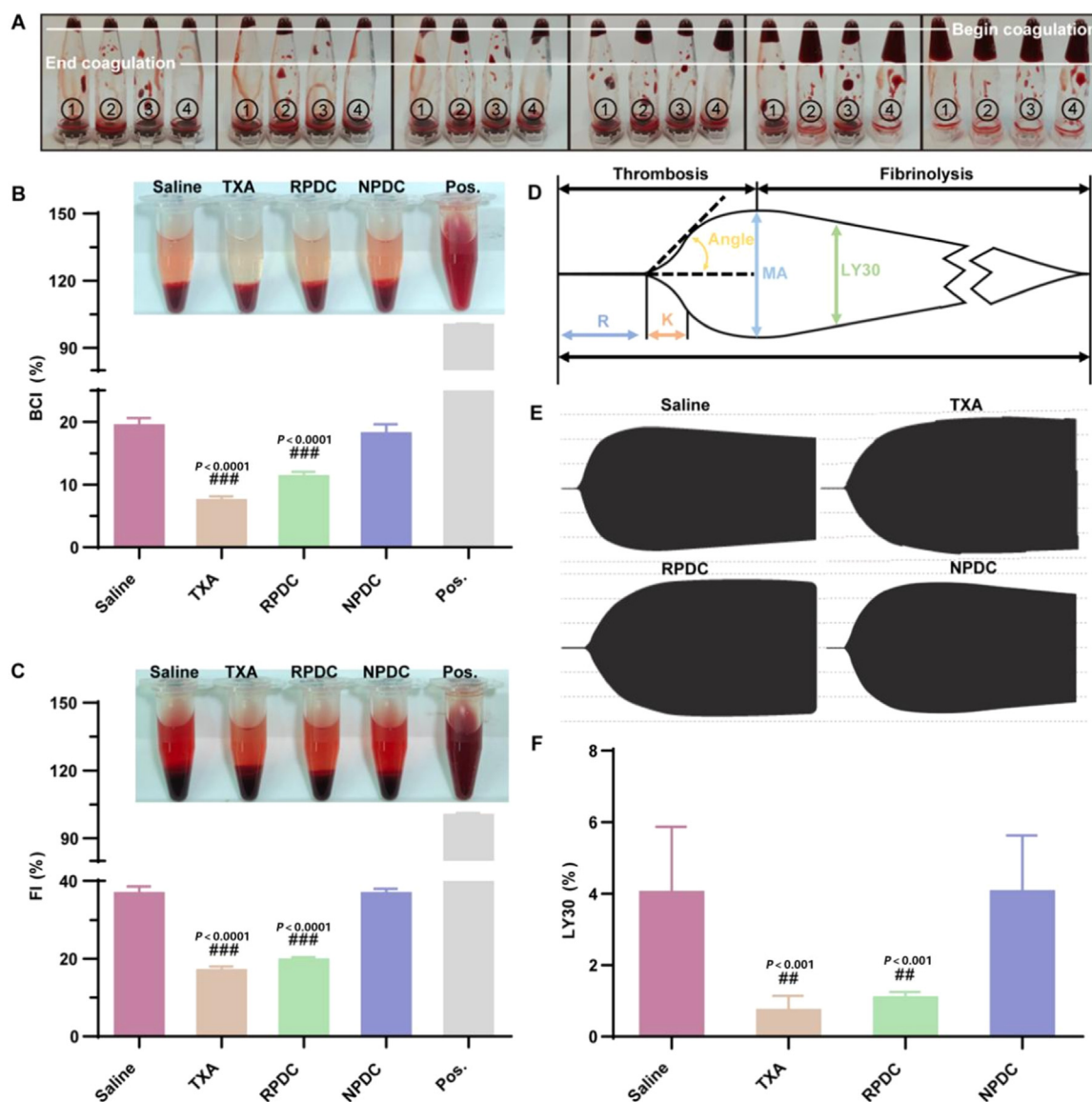


Fig. 3 – In vitro coagulation effect evaluation of RPDC (A) Representative inverted blood images following different treatments at 5, 10, 15, 30, 45, 60 min, ① Saline; ② RPDC; ③ NPDC; ④ TXA; (B) Representative photos of blood samples and BCI values in different groups. $^{##}P < 0.01$, $^{###}P < 0.001$, compared to saline group; (C) Representative photos of blood samples and FI values of different groups. $^{##}P < 0.01$, $^{###}P < 0.001$, compared to saline group; (D) Explanation diagram of TEG. (E) Representative TEG in different groups; (F) Statistical chart of LY30 in different groups. $^{##}P < 0.01$, $^{###}P < 0.001$, compared to saline group.

even at a concentration of 1.25 mg/ml, RPDC and NPDC exhibited low hemolytic activity (Fig. 2C), with hemolysis rates remaining below 5% (Fig. 2D), confirming their excellent hemocompatibility. Based on these findings, a concentration of 1.00 mg/ml was selected for subsequent experiments.

3.4. In vitro coagulation effect of RPDC

To assess the coagulation efficacy of RPDC, we first employed an in vitro blood inversion assay using fresh venous blood from ICR mice. As illustrated in Fig. 3A, within the first 10 min, only the clot size in the RPDC (②) group increased. By 15 min, a

significant increase in clot size was observed in both the RPDC (②) and TXA (④) groups, while no changes were noted in the NPDC (③) and saline (①) groups. This indicates that RPDC initiates coagulation within 15 min, matching the efficacy of TXA. Complete coagulation was achieved in the RPDC and TXA groups by 45 min (defined as no liquid blood observed visually), with no residual blood at the EP tube cap. In contrast, the NPDC and saline groups required 60 min for complete coagulation, indicating that RPDC significantly accelerates the blood coagulation process, reducing the coagulation time by 15 min compared to natural coagulation. Overall, RPDC demonstrated a hemostatic effect comparable to that of TXA, indicating that RPDC responds to uPA to release TXA and

exert its coagulation function. Conversely, NPDC, which lacks plasmin responsiveness, exhibited no significant difference in hemostatic efficacy compared to saline, as its conjugated TXA remains inactive. These findings preliminarily indicate that the designed RPDC enables controlled TXA release at bleeding sites, enhancing both efficacy and safety.

The blood coagulation index (BCI) and fibrinolysis index (FI) are critical metrics for evaluating hemostatic and antifibrinolytic performance, respectively. A lower BCI indicates superior coagulation, while a lower FI signifies enhanced antifibrinolytic activity [32]. As depicted in Fig. 3B, the RPDC and TXA groups exhibited significantly lower BCI values compared to the saline and NPDC groups, indicating the superior coagulation performance of RPDC. Similarly, lower FI values were observed in the RPDC and TXA groups compared to the saline and NPDC groups further demonstrating the better antifibrinolytic performance of RPDC (Fig. 3C). These findings confirm the fact that RPDC responds to uPA to release TXA, which in turn exerts antifibrinolytic and pro-coagulant effects.

TEG is a laboratory assay that measures the viscoelastic properties of whole blood clotting [33]. To further evaluate clot stability, we conducted TEG on whole rat blood under uPA-induced conditions. The key parameters, including R (clot formation time, in min), K (the time from the R time endpoint to the point where the tracing amplitude reaches 20 mm, in min), MA (maximum amplitude, in millimeters, mm), α -angle (in degrees, °), and LY30 (the rate of reduction in the amplitude of the blood clot within 30 min after the MA value is determined, in percent, %) were recorded and analyzed (Fig. 3D). As depicted in Fig. 3E and 3F, both RPDC and TXA significantly reduced LY30 compared to the saline and NPDC groups, indicating enhanced thrombus stability due to their antifibrinolytic properties. However, RPDC did not significantly affect the parameters (Fig. S3) including R, K, MA or α -angle of clot formation, similar to TXA, suggesting that RPDC does not interfere with basic coagulation function. These findings collectively demonstrate that RPDC effectively stabilizes clots without compromising intrinsic coagulation mechanisms, making it a promising candidate for targeted hemostasis.

3.5. *In vivo* hemostatic effect of RPDC

The hemostatic properties *in vivo* of the RPDC were preliminarily evaluated by the amount of bleeding and hemostatic time both in the mouse liver injury model and mouse-tail amputation model (Fig. 4). For the mouse liver injury model (Fig. 4A–4D), hemorrhage was induced by creating a standardized 2 cm deep puncture in the exposed liver lobe using an 18-gauge needle. From the photographs of the samples in Fig. 4B, it was visually apparent that both RPDC and TXA significantly reduced the amount of bleeding in mice with liver injury. Quantitative analysis further revealed that RPDC and TXA exhibited significantly lower blood loss compared to the control and NPDC groups ($n = 6$, $P < 0.001$, $\alpha = 0.05$) (Fig. 4C). Additionally, the hemostatic times for RPDC and TXA were 233 s and 220 s, respectively, which were markedly shorter than those observed in the control (335 s) and NPDC (347 s) groups ($P < 0.001$) (Fig. 4D).

In the mouse-tail amputation model (Fig. 4E and 4I), the tail was transected at 1.5 cm from the tip to generate controlled hemorrhage. Both RPDC and TXA demonstrated a significant reduction in hemorrhage severity, as evidenced by the sample photographs in Fig. 4F. The blood loss in the control (saline) group was 293 mg. In contrast, treatment with RPDC reduced blood loss by 50%, achieving a comparable effect to that of TXA. However, the NPDC group showed no significant difference in blood loss compared to the control group (Fig. 4G). As illustrated in Fig. 4H, the hemostatic times of the RPDC and TXA groups were significantly shorter than those of the control group ($n = 3$, $P < 0.001$, $\alpha = 0.05$). Notably, the hemostatic time of the NPDC group did not differ significantly from that of the control group. These findings suggest that RPDC is capable of responding to plasmin and releasing TXA at the bleeding site *in vivo*, where TXA subsequently exerts antifibrinolytic and pro-coagulant effects. In contrast, NPDC inhibits the activity of TXA due to its non-plasmin-cleavable property. It can be inferred that TXA within RPDC is released only upon activation of the fibrinolytic system during bleeding, thereby enhancing the safety profile of TXA.

3.6. Hemostatic efficacy of RPDC in the ICH model

Encouraged by the *in vivo* hemostatic efficacy, we further investigated the restricting HE effect of RPDC in a mouse ICH model. Hematoma size is a critical determinant and biomarker for predicting the prognosis of patients with sICH [34]. Therefore, a collagenase-induced ICH mouse model was established using collagenase IV to evaluate the hemostatic efficacy of the RPDC. An illustration in Fig. 5A explained the entire animal study and photographic images of the surgical procedure. Animals were secured in a stereotactic frame in the prone position, and the stereotactic injection site was determined relative to the bregma (2.2 mm lateral and 0.2 mm anterior). For the ICH mouse groups, we designated the following: sham group (mice underwent a similar stereotactic injection but received saline), model group (injection saline), TXA group (14 mg/kg), RPDC group (equivalent TXA, 14 mg/kg), and NPDC group (equivalent TXA, 14 mg/kg). Compared to the sham and model groups, both RPDC and TXA groups demonstrated significant therapeutic effects, reducing hematoma volume by $42.3\% \pm 3.1\%$ and $27.3\% \pm 4.85\%$ ($P < 0.001$ vs. saline control, Fig. 5B and 5C). Moreover, the RPDC group exhibited a statistically significant further reduction in hematoma volume compared to the TXA group ($P < 0.05$, Fig. 5C). Furthermore, in the collagenase-induced ICH mouse model, the RPDC treatment group showed a significant reduction in brain lesion area and fewer pathological signs of peri-hematoma injury assessed by H&E staining (5 μ m sections stained for 5 min with H&E Y, Fig. 5D). These findings further substantiate that RPDC is capable of responding to plasmin *in vivo*, thereby releasing TXA, which subsequently inhibits the activation of the fibrinolytic system and promotes coagulation, thereby restricting HE. In contrast, NPDC showed no therapeutic benefit, as peptide conjugation blocked TXA's active site.

Furthermore, the permeability of the BBB in ICH mice was assessed using EB extravasation to evaluate the integrity of cerebral micro-vessels following RPDC treatment. As

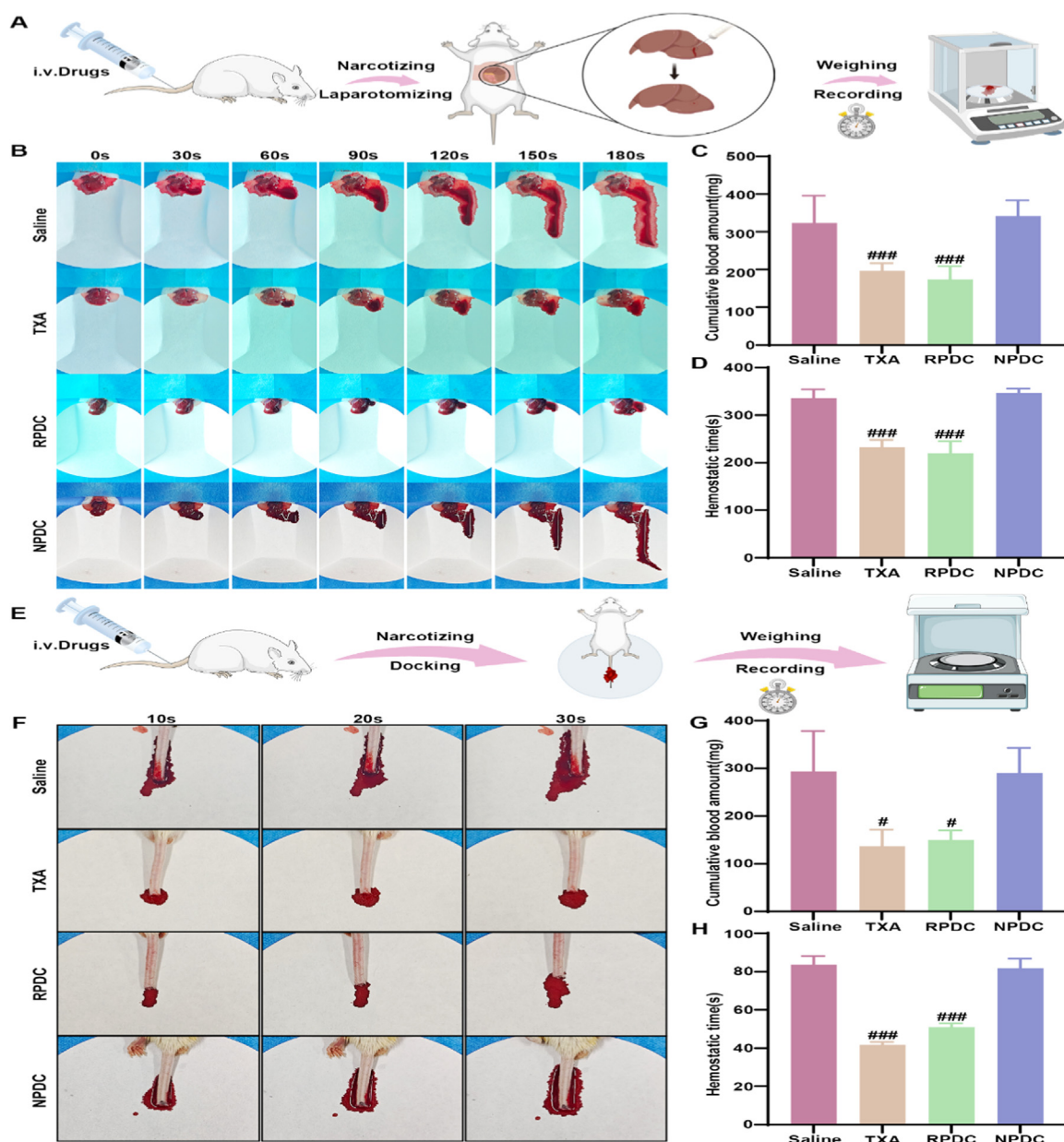


Fig. 4 – Evaluation of the in vivo hemostatic effect of RPDC. (A) Scheme representation of the mouse liver injury model during hemostasis; (B) Representative photographs of the bleeding samples in different groups of the mouse liver injury model; (C) Blood loss and (D) hemostatic time in the mouse liver injury model; (E) Schematic diagram of mouse-tail amputation model; (F) Representative photographs of the bleeding samples in different groups of the mouse-tail amputation model. (G) Blood loss and (H) hemostatic time in the mouse-tail amputation model. #P < 0.05, ###P < 0.001, compared to saline group.

shown in Fig. 5E, in the sham group, EB was confined to the vasculature, whereas in the model group, EB was extensively distributed throughout the brain tissue, indicating severe BBB disruption in the ICH pathology. In contrast, the RPDC group exhibited limited EB distribution in brain tissue, similar to the TXA group. However, the NPDC group showed extensive EB distribution, comparable to the model group. Additionally, quantitative analysis of EB content in brain tissue (Fig. 5F) further confirmed that RPDC treatment significantly reduced EB extravasation compared to the model group, with efficacy comparable to TXA, while NPDC showed minimal effect. These findings demonstrate

RPDC's dual mechanism: plasmin-responsive TXA release for targeted hemostasis and subsequent vascular stabilization to prevent HE. This targeted approach represents a significant advancement over conventional therapies, offering precise control of fibrinolytic activity while minimizing systemic effects.

3.7. Prognostic improvement in ICH mice by RPDC

Behavioral assessments provide critical insights into neurological recovery following ICH [35]. To evaluate RPDC's therapeutic potential, we employed a comprehensive

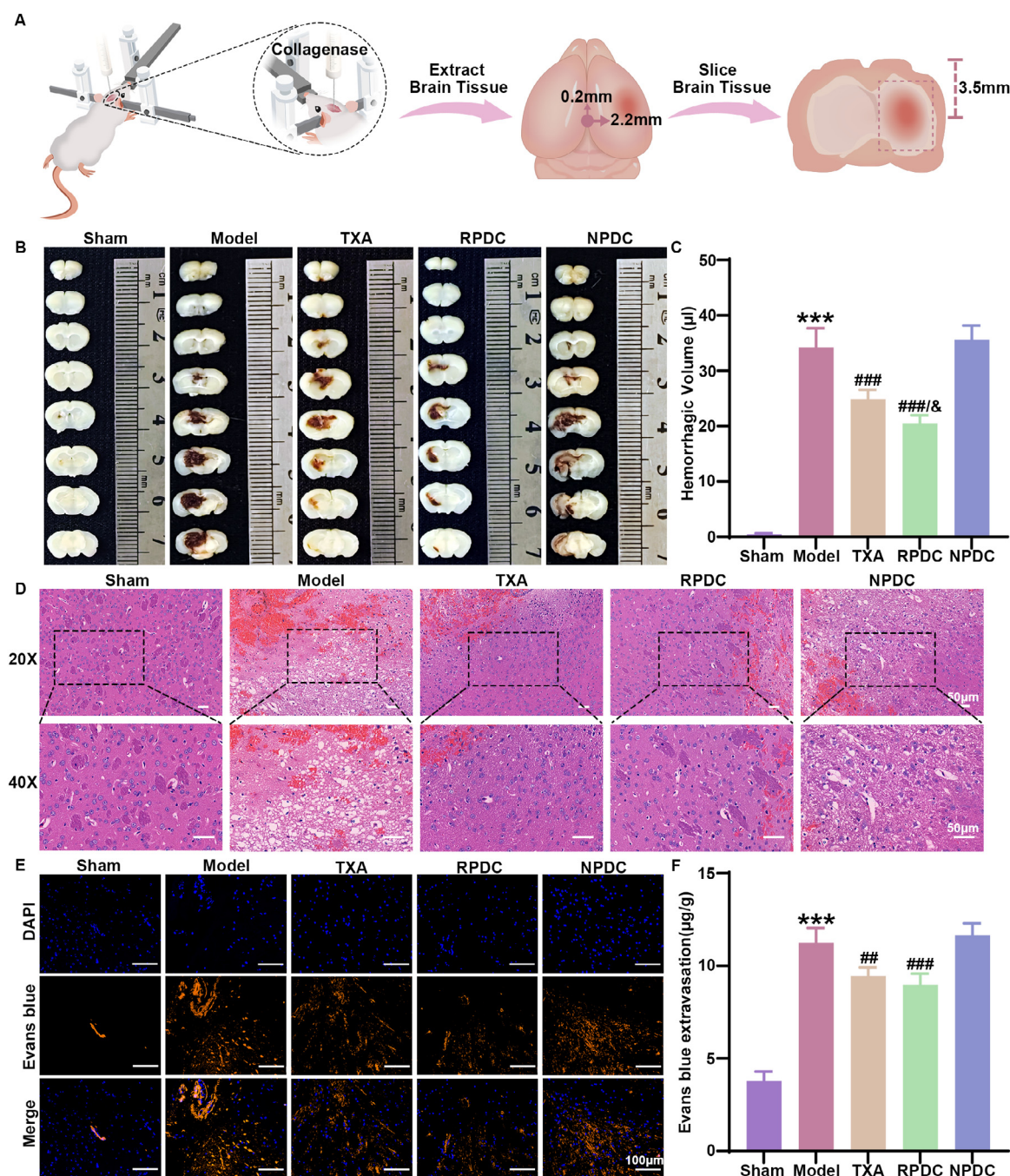


Fig. 5 – In vivo evaluation of the hemostatic efficacy of RPDC in the ICH model. (A) Schematic representation of the construction of an ICH mouse model and the brain tissue processing protocol; **(B)** Representative photographs of the brain tissue slices in different groups; **(C)** Statistics of brain hemorrhage volume in different groups; **(D)** H&E staining of the brain tissue slides in different groups; **(E)** Fluorescence imaging of EB distribution in brain tissue; **(F)** Quantitative analysis of EB content in brain tissue across different experimental groups. *** $P < 0.001$, compared to sham group; ## $P < 0.01$, ### $P < 0.001$, compared to model group; & $P < 0.05$, compared to TXA group.

behavioral experiment, including the modified Longa score (criteria: 0 = No deficit, normal; 1 = Mild neurological deficit, failure to fully extend the right forepaw; 2 = Moderate neurological deficit, circling to the right; 3 = Severe neurological deficit, falling to the right; 4 = No spontaneous movement or loss of consciousness), balance beam test

(performed on a solid cylindrical wooden beam: length 100 cm × diameter 1 cm, elevated 20 cm above the floor), and corner test (10 trials per mouse with ≥30 s intervals; only upright turns with full dorsal contact against the wall were recorded), complemented by survival and weight monitoring (Fig. 6). All behavioral assessments (Longa scoring, balance

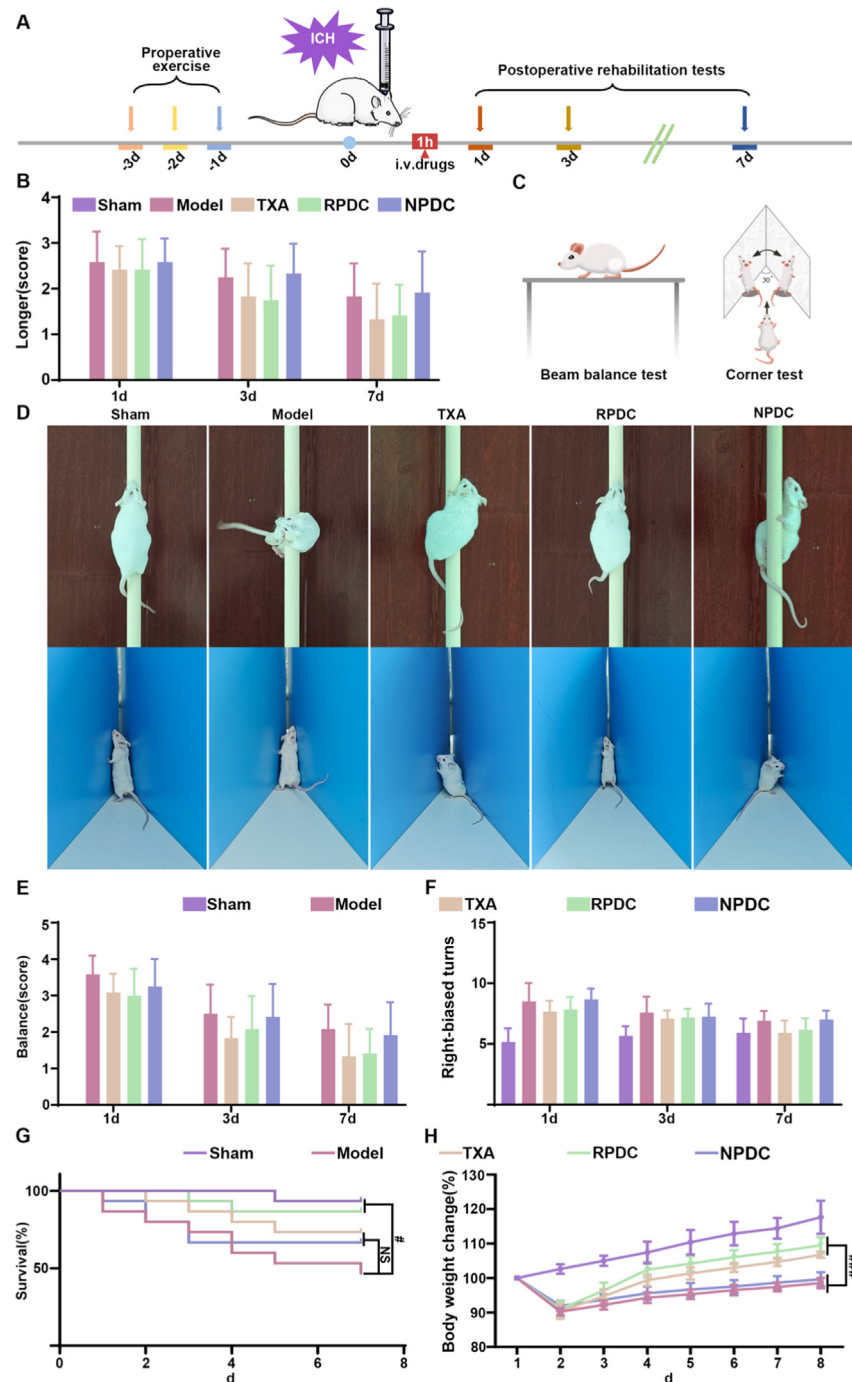


Fig. 6 – Prognostic evaluation in ICH mice. (A) Experimental procedure diagram. The longer score of different groups on Day 1, 3 and 7 post-surgery; (C) Schematic illustrations of the balance beam test and the corner test; (D) Representative photographs of the balance beam test and the corner test in different groups; (E) Score of balance beam test, and (F) percentage of right turns in corner test at 1, 3 and 7 d post-surgery; (G) Survival rate and (H) body weight of the mice in different groups during the period of the post-operative rehabilitation tests. #P < 0.05, compared to model group.

beam test, corner test) were performed by observers who were blinded to the treatment group assignments of the mice. As shown in Fig. 6A, a detailed postoperative rehabilitation training protocol was designed. Prior to the experiment, all animals underwent at least 3 d of preoperative motor training. Subsequently, an ICH model was established by injecting

collagenase into the brain, followed by drug intervention. Neurological function was assessed in each group of mice on Day 1, 3 and 7 using the balance beam test and corner test.

The Longa score for the sham group remained at 0. After ICH surgery, the Longa score significantly increased, while the

Longa scores of mice treated with NPDC and TXA showed a decreasing trend compared to the model group and NPDC group (Fig. 6B). Mice in the sham group were able to walk in a straight line, whereas mice in the model and NPDC groups exhibited a significant increase in hindlimb slips on the balance beam. In contrast, mice treated with RPDC and TXA showed fewer slips on the balance beam and had lower neurological severity scores compared to the other groups (higher scores indicate more severe injury) (Fig. 6C and 6D). In the corner test, the proportion of right turns (calculated from 10 valid trials per mouse) in the NPDC and TXA groups gradually decreased over time compared to the model group, indicating significantly better neurological function (Fig. 6C and 6D). These results suggest that RPDC treatment enhances cognitive and motor functions in ICH mice, primarily attributed to the release of TXA by RPDC, which limits HE. Additionally, body weight and survival rates were monitored for each group of mice (Fig. 6E and 6F). Post-ICH, mice in the model group experienced rapid weight loss and a significant decline in survival rate. In contrast, NPDC and TXA-treated mice initially lost weight but gradually regained it in subsequent days, accompanied by a notable increase in survival rate. These findings further support the efficacy of RPDC treatment in improving the prognosis of ICH mice.

Collectively, these findings underscore RPDC's therapeutic advantage: its plasmin-responsive TXA release not only limits HE but also promotes neurological recovery, offering a promising approach to improve ICH outcomes. The consistent superiority of RPDC over free TXA highlights the importance of targeted drug delivery in maximizing therapeutic efficacy while minimizing systemic complications.

3.8. Targeting hemorrhage localization of RPDC

vWF serves as a critical mediator in platelet recruitment to sites of vascular injury and represents a therapeutic target for antithrombotic interventions [36–38]. In this study, we harnessed this biological mechanism by engineering a VBP that enables precise targeting of RPDC to hemorrhagic sites. To unravel the therapeutic potential of RPDC, we conducted comprehensive investigations into its *in vivo* biodistribution and hemorrhagic site targeting efficiency. ICH model mice were administered Cy5-labeled RPDC (Cy5-RPDC, prepared via NHS-ester conjugation) or free Cy5 via tail vein injection, with subsequent fluorescence distribution tracked using small animal *in vivo* imaging systems (IVIS Lumina III; excitation: 649 nm, emission: 670 nm, exposure time: 500 ms, binning: 4). As demonstrated in Fig. 7A, the cerebral fluorescence intensity in ICH mice receiving Cy5-RPDC significantly exceeded that of the free Cy5 group. *Ex vivo* brain fluorescence analysis revealed that Cy5-RPDC demonstrated rapid and specific accumulation at cerebral hemorrhage sites, with fluorescence intensity peaking at 12 h and maintaining therapeutic levels through 24 h (Fig. 7B). This targeted accumulation far surpassed the non-specific distribution of free Cy5, highlighting RPDC's precision targeting capabilities.

Immunofluorescence studies provided further compelling evidence of RPDC's targeting specificity. While sham-operated mice showed intact cerebral vasculature, ICH mice exhibited characteristic hemorrhage and thrombus formation, and

mice treated with TXA showed extensive perivascular thrombi (Fig. 7C). Remarkably, Cy5-RPDC treatment resulted in extensive colocalization (Pearson's coefficient > 0.85, Fig. S7 & Table S1) of the conjugate with fibrin-labeled thrombi, demonstrating its ability to precisely localize at hemorrhagic sites. In contrast, free Cy5 fluorescence showed minimal overlap with thrombus locations. This targeted delivery enables the controlled release of TXA, which subsequently reinforces the fibrin network to form stable, occlusive thrombi. These findings conclusively demonstrate RPDC's ability to target hemorrhagic sites and thrombi, subsequently releasing TXA to reinforce the fibrin network and establish more stable occlusive thrombi.

3.9. *In vivo* safety assessment of the TXA-peptide drug conjugates

Hemostasis represents a sophisticated biological equilibrium, meticulously balancing coagulation activation for effective bleeding control against the risk of pathological thrombosis [39]. This delicate balance presents a fundamental challenge in hemostatic therapy, where therapeutic interventions must achieve effective clotting without precipitating thrombotic complications. To evaluate the safety profile of our innovative TXA-polypeptide drug conjugates, we conducted comprehensive assessments of their impact on coagulation parameters and systemic biocompatibility. Our investigation focused on key coagulation parameters, prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB) levels, which provide critical insights into the coagulation cascade's integrity. The coagulation parameters (PT/APTT/TT/FIB) were detected using a Rayto RT-6200 analyzer (Shenzhen Rayto life sciences) with matched reagents (PT; APTT). Remarkably, intravenous administration of RPDC at therapeutic doses (14 mg/kg TXA equivalent) showed no significant perturbation of these parameters, with all values remaining within normal physiological ranges (Fig. S4). This finding was further supported by consistent profiles for RBC, WBC, platelet counts, hemoglobin, and hematocrit consistent hematological profiles across treatment groups (Fig. S5), suggesting minimal systemic impact on blood components. Histopathological evaluation of 5 μ m-thick sections from major organs (heart, liver, spleen, lung, kidney) revealed excellent biocompatibility, with no evidence of pathological changes in cellular architecture or tissue organization (Fig. S6). These findings underscore the therapeutic advantage of our innovative RPDC, maintaining the delicate hemostatic balance while minimizing systemic effects.

3.10. *In vivo* pharmacokinetics evaluation of RPDC

Pharmacokinetic profiling of drug candidates is of critical importance. To effectively manage re-bleeding and persistent bleeding in patients with sICH, appropriate strategies to extend the half-life of therapeutic agents are essential [1,2]. In this study, we evaluated the *in vivo* pharmacokinetics of RPDC using Cy5-RPDC and free Cy5 (simulating unbound drug) in ICR mice. Blood samples were collected over 48 h post-tail vein injection, and Cy5 concentrations were quantified to generate

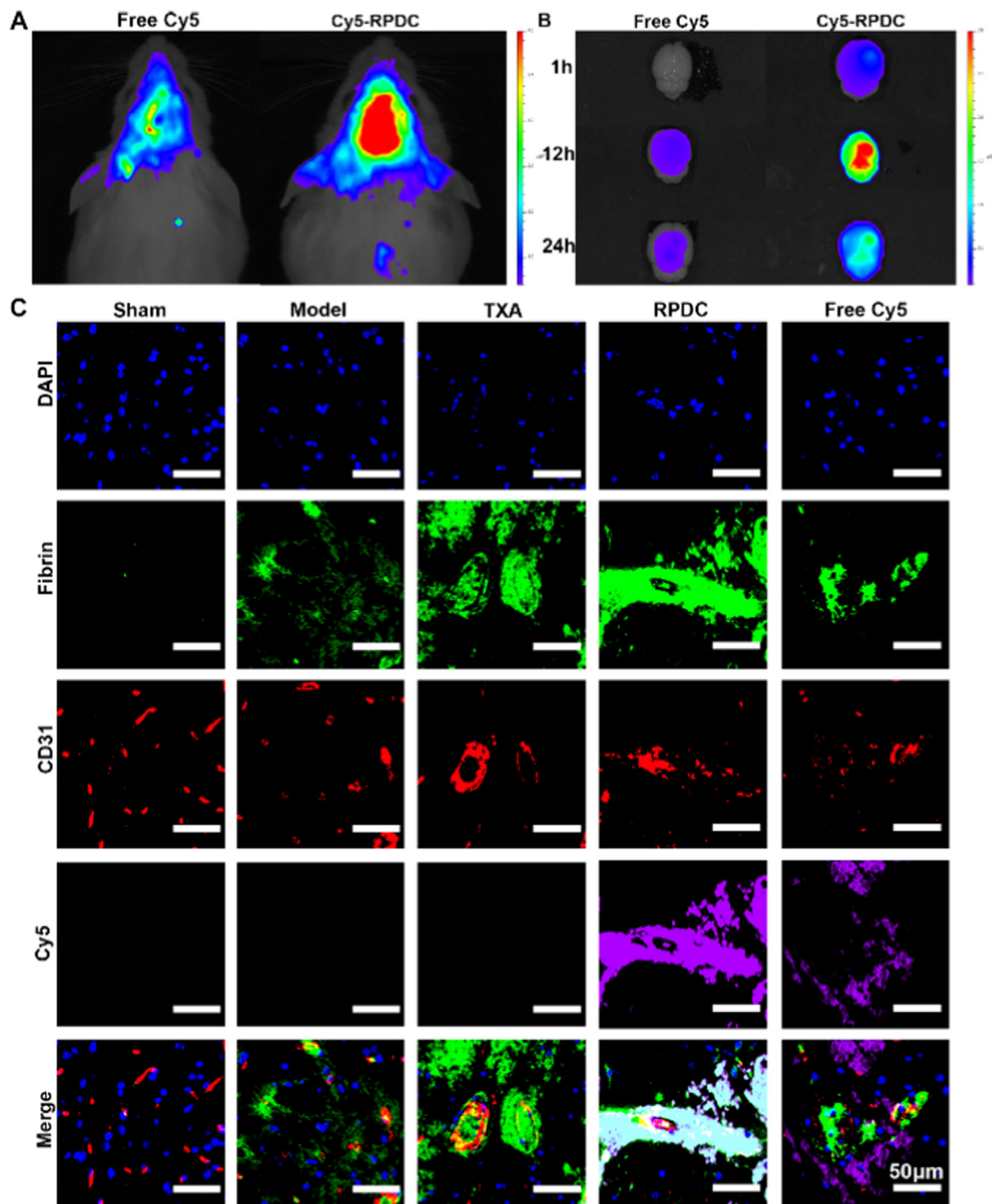


Fig. 7 – Targeting hemorrhage localization of RPDC. (A) In vivo fluorescence imaging of the brain in ICH mice following different interventions for 24 h; (B) Ex vivo fluorescence imaging of the brain tissues in ICH mice following different interventions for 1, 2 and 24 h; (C) Immunofluorescence staining of brain tissue in ICH mice following different interventions for 12 h, with DAPI staining the cell nuclei, FITC-fibrin antibody labeling fibrin fibers in thrombi, red-CD31 antibody labeling vascular endothelial cells, and Cy5 labeling RPDC.

time-concentration curves (Fig. S8). Non-compartmental analysis (Table S2) revealed that Cy5-RPDC exhibited a more extended elimination half-life ($T_{1/2}$) compared to free Cy5 (18.127 vs. 12.239), alongside a > 3 -fold increase in $AUC_{0-\infty}$, indicating prolonged systemic retention and slower clearance. These results demonstrate that RPDC's structural design could delay metabolic degradation, enabling sustained drug activity within the 6-h critical window for monitoring and intervening in sICH-associated HE. The enhanced pharmacokinetic profile positions RPDC as a promising candidate for real-time management of dynamic bleeding complications in neurological emergencies.

4. Conclusion

We engineered a plasmin-responsive hemostatic anchor (RPDC) enabling targeted TXA delivery to bleeding sites via vWF-binding peptide accumulation and enzymatic activation. In collagenase-induced ICH models, RPDC achieved a 42% reduction in hematoma volume with significant neurological improvement, demonstrating precise hemostatic control while minimizing systemic effects. This targeted strategy overcomes limitations of conventional antifibrinolytics through integrated molecular recognition and stimulus-responsive drug release. Future translation requires validation in large animal models, GMP-compliant manufacturing optimization, and immunogenicity assessment. RPDC represents a paradigm-shifting approach for managing HE in hemorrhagic disorders.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ajps.2025.101091](https://doi.org/10.1016/j.ajps.2025.101091).

The figures and tables with “S” before the serial number are included in the Supplementary material.

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