

Synthesis, Biological Evaluation and Reversal of Sulfonated Di- and Triblock Copolymers as Novel Parenteral Anticoagulants

Justyna Swieton, Joanna Miklosz, Natalia Bielicka, Aleksandra Frackiewicz, Karol Depczynski, Marta Stolarek, Piotr Bonarek, Kamil Kaminski, Piotr Rozga, Shin-Ichi Yusa, Anna Gromotowicz-Poplawska, Krzysztof Szczubialka, Dariusz Pawlak, Andrzej Mogielnicki, and Bartlomiej Kalaska*

Despite targeting different coagulation cascade sites, all Food and Drug Administration-approved anticoagulants present an elevated risk of bleeding, including potentially life-threatening intracranial hemorrhage. Existing studies have not thoroughly investigated the efficacy and safety of sulfonate polymers in animal models and fully elucidate the precise mechanisms by which these polymers act. The activity and safety of sulfonated di- and triblock copolymers containing poly(sodium styrenesulfonate) (PSSS), poly(sodium 2-acrylamido-2-methylpropanesulfonate) (PAMPS), poly(ethylene glycol) (PEG), poly(sodium methacrylate) (PMAAS), poly(acrylic acid) (PAA), and poly(sodium 11-acrylamidoundecanoate) (PAAU) blocks are synthesized and assessed. PSSS-based copolymers exhibit greater anticoagulant activity than PAMPS-based ones. Their activity is mainly affected by the total concentration of sulfonate groups and molecular weight. PEG-containing copolymers demonstrate a better safety profile than PAA-containing ones. The selected copolymer PEG₄₇-PSSS₃₂ exhibits potent anticoagulant activity in rodents after subcutaneous and intravenous administration. Heparin Binding Copolymer (HBC) completely reverses the anticoagulant activity of polymer in rat and human plasma. No interaction with platelets is observed. Selected copolymer targets mainly factor XII and fibrinogen, and to a lesser extent factors X, IX, VIII, and II, suggesting potential application in blood-contacting biomaterials for anticoagulation purposes. Further studies are needed to explore its therapeutic applications fully.

1. Introduction

Anticoagulants are pivotal in the therapy of thrombotic diseases, for example, myocardial infarction, ischemic stroke, or venous thromboembolism, which still present significant challenges to global healthcare systems. Despite the availability of anticoagulants, medical needs associated with thromboembolic conditions are still not fully met. According to recent World Health Organization reports,^[1] ischemic heart disease and stroke remain the two top causes of mortality worldwide. Unfractionated heparin (UFH), a rapid-acting anticoagulant administered intravenously (*i.v.*), is primarily used to prevent coagulation during cardiovascular or percutaneous interventions.^[2] Despite its effectiveness, UFH possesses limitations, such as variable pharmacokinetics, high risk of bleeding, and the necessity for frequent monitoring and dose adjustments.^[3,4] Consequently, low-molecular-weight heparins (LMWHs) were introduced to address these shortcomings. While both UFH and LMWHs contain a pentasaccharide unit and sulfate groups, their interactions with antithrombin (AT)

J. Swieton, J. Miklosz, A. Frackiewicz, K. Depczynski, D. Pawlak, A. Mogielnicki, B. Kalaska
Department of Pharmacodynamics
Medical University of Bialystok
Mickiewicza 2C St., Bialystok 15-089, Poland
E-mail: bartlomiej.kalaska@umb.edu.pl
N. Bielicka, A. Gromotowicz-Poplawska
Department of Biopharmacy and Radiopharmacy
Medical University of Bialystok
Mickiewicza 2C St., Bialystok 15-089, Poland

M. Stolarek, P. Bonarek, K. Kaminski, K. Szczubialka
Faculty of Chemistry
Jagiellonian University
Gronostajowa 2 St., Krakow 30-387, Poland
M. Stolarek
Doctoral School of Exact and Natural Sciences
Jagiellonian University
prof. S. Lojasiewicza 11 St., Krakow 30-348, Poland
P. Rozga
Drug Discovery and Early Development Department
Adamed Pharma S.A.
Pienkow, Mariana Adamkiewiczza 6A St., Czosnow 05-152, Poland

The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/adhm.202402191>

DOI: 10.1002/adhm.202402191

and coagulation factors differ. UFH binds and accelerates AT's inhibition of several coagulation factors, mostly fIIa and fXa. In contrast, LMWHs, characterized by a shorter chain than UFH, primarily inhibit fXa activity by binding to AT, with limited ability to bind fIIa.^[5] Clinical data demonstrated comparable efficacy in treating deep vein thrombosis between home-treated LMWHs and hospital-administered UFH treatments.^[6,7] LMWHs enable subcutaneous (*s.c.*) administration and less frequent dosing regimens, offering convenience and improved patient compliance during chronic therapy. However, due to bleeding complications, there is a need for effective reversal agents that manage this adverse event and ensure patient safety.

Recent studies have highlighted the anticoagulant potential of heparin-mimicking polymers, as they possess the ability to interact with key coagulation factors, for example, fIIa and fXa, and anticoagulant proteins, such as AT and heparin cofactor II.^[8–11] Self-anticoagulant sponges have been developed for whole blood autotransfusion, providing anticoagulation by irreversibly depleting fXI and inhibiting fIIa.^[8] Similarly, hydrogel microspheres that bind fVIII, fIX, and fXI prevent clot formation in extracorporeal circuits.^[9] Universal Heparin Reversal Agent (UHRA) could be a promising candidate for clinical antithrombotic agents due to its unique polyP-targeting mechanism and low toxicity, with the potential to address the limitations of current antithrombotic therapies.^[12] Additionally, polyglycerol sulfate was found to significantly prolong clotting time, which indicates its effective anticoagulant properties.^[13]

Protamine is currently the only Food and Drug Administration-approved antidote for UFH, but its effectiveness against LMWHs is limited. Moreover, protamine's lack of specificity can lead to complications, including excessive bleeding and hypersensitivity reactions, necessitating careful titration and monitoring.^[14–17] Various compounds, such as Platelet Factor 4, Heparin Binding Copolymer (HBC), and UHRA are being investigated in preclinical settings to address these concerns.^[12,18–23] Despite promising advancements, challenges in achieving clinical translation of those antidotes remain. One promising candidate during clinical trials is andexanet alfa, a modified recombinant inactive fXa, which has shown efficacy in reversing the effects of direct oral anticoagulants,^[24–26] but it was less effective than protamine against UFH and LMWHs reversal.^[27]

In recent years, there has also been growing interest in the potential of synthetic polymers containing sulfate, sulfonate, and carboxyl groups as anticoagulant agents.^[28–33] Their well-controlled structure could offer customization of anticoagulant activity, precise and predictable pharmacokinetics, and mitigation of adverse effects observed with conventional therapies. Here, we have focused on developing novel synthetic block copolymers with sulfonate groups that mimic the anticoagulant activity of heparins. To obtain polymers with a well-defined and controllable molecular weight that enables

high reproducibility, the reversible addition-fragmentation chain transfer (RAFT) polymerization method was used. We have also demonstrated that the most promising candidate's activity was effectively reversed by a polymer-based cationic antidote – HBC, previously developed by us.^[19,20] We have also elucidated the potential mechanism of action of our selected anticoagulant.

2. Results

2.1. Characterization of the Studied Copolymers

We have prepared block copolymers via RAFT-controlled radical polymerization. A triblock copolymer (PEG₄₉-PAMPS₃₁₈-PMAAS₁₀₀) composed of poly(ethylene glycol) (PEG), poly(sodium 2-acrylamido-2-methylpropane sulfonate) (PAMPS), and poly(sodium methacrylate) (PMAAS) blocks was synthesized in accordance with a reference paper.^[34] The subscript numbers express degrees of polymerization estimated from proton nuclear magnetic resonance (¹H NMR) spectra. An ABA-type block copolymer (PSSS₈₉-PEG₂₂₉-PSSS₈₉) composed of poly(sodium styrene sulfonate) (PSSS) and PEG was synthesized using a bifunctional PEG macro chain transfer agent.^[35] A diblock copolymer (PAMPS₇₅-PAAU₂₈) composed of PAMPS and poly(sodium 11-acrylamidoundecanoate) (PAAU) was synthesized via RAFT.^[36] Diblock copolymers (PSSS₅₈-PAA_{*m*}; *m* = 33 and 63, and PEG₄₇-PSSS_{*n*}; *n* = 32 and 99) composed of PSSS, poly(acrylic acid) (PAA), and PEG were prepared via RAFT.^[37,38] **Figure 1** shows the chemical structures of the block copolymers used in this study. The number-average molecular weight (*M_n*), estimated from ¹H NMR, and molecular weight distribution (*M_w*/*M_n*), estimated from gel-permeation chromatography, were summarized in **Table 1**. The *M_w*/*M_n* values were relatively narrow, suggesting that the block copolymers have a well-controlled structure.

2.2. in vitro Anticoagulant Activity Measured by Activated Partial Thromboplastin Time (aPTT) Assay

The in vitro anticoagulant activity of the block copolymers or UFH was tested by measuring their influence on the aPTT prolongation in rat plasma (**Figure 2a**). The aPTT test measures the time required for blood to clot, evaluating the activity of the intrinsic coagulation pathway. All studied copolymers prolonged aPTT indicating anticoagulant properties. The most potent copolymers were PEG₄₇-PSSS₉₉ and PSSS₈₉-PEG₂₂₉-PSSS₈₉, which maximally prolonged aPTT at 50 μg mL⁻¹, similar to UFH.^[39] The influence of the overall chain charge on aPTT prolongation was confirmed as PSSS₅₈-PAA₆₃ demonstrated greater activity than PSSS₅₈-PAA₃₃ and PEG₄₇-PSSS₉₉ showed greater activity than PEG₄₇-PSSS₃₂. PSSS₈₉-PEG₂₂₉-PSSS₈₉ exhibited greater potency compared to PEG₄₇-PSSS₃₂. Because of PEG₄₉-PAMPS₃₁₈-PMAAS₁₀₀ weak anticoagulant activity, we discontinued studying this copolymer in further tests.

2.3. Viability of H9c2(2-1) Cells After Exposure to Copolymers

The in vitro cytotoxicity of the block copolymers was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

S.-I. Yusa
Department of Applied Chemistry
Graduate School of Engineering
University of Hyogo
167 Shosha, Himeji 671-2280, Japan

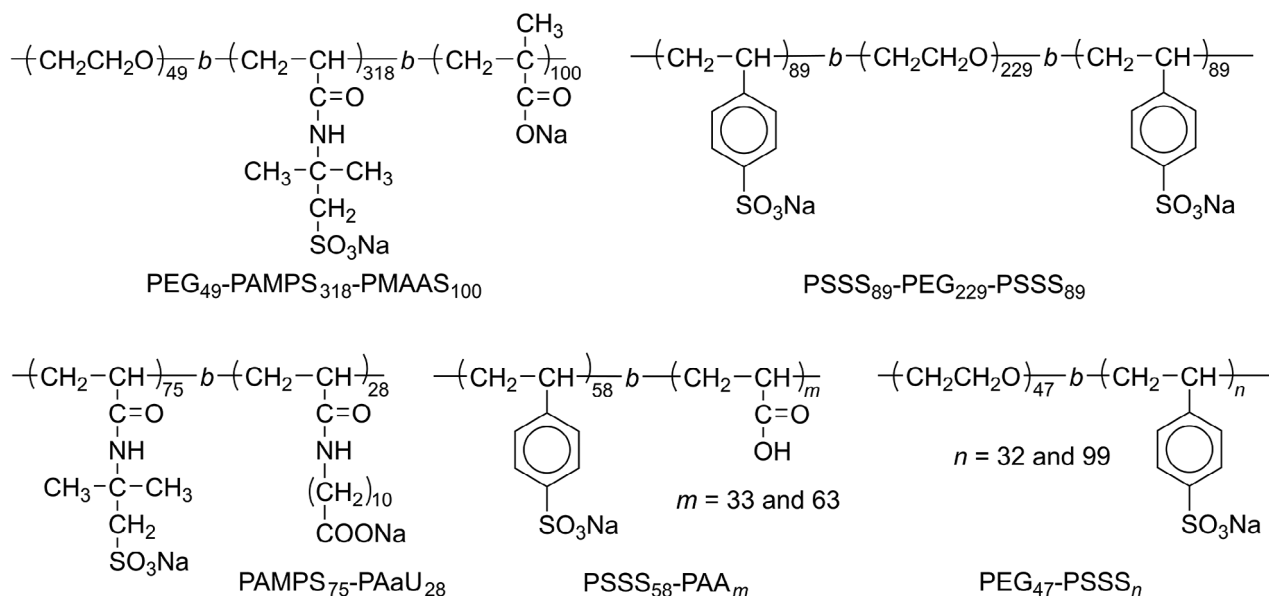


Figure 1. Chemical structures of the block copolymers used in this study.

bromide (MTT) assay in rat heart/myocardium cells. PAMPS₇₅-PAAU₂₈ reduced the viability of the cells in the whole range of tested concentrations, with half maximal inhibitory concentration of $\approx 250 \mu\text{g mL}^{-1}$ (Figure 2b). We observed similar toxicity of the polymer in the presence of fetal bovine serum (Figure S1). Although this polymer was nontoxic for Vero cells at concentrations up to $1000 \mu\text{g mL}^{-1}$,^[40] we discontinued studying this copolymer in further tests due to these safety concerns. An increase in cell viability above 100% after incubation with PSSS₈₉-PEG₂₂₉-PSSS₈₉, PSSS₅₈-PAA₆₃, PEG₄₇-PSSS₃₂, and PEG₄₇-PSSS₉₉ may be related to mild stimulation of cell proliferation, as documented in the literature for sulfated and sulfonated polymers, both natural and synthetic ones.^[41,42]

2.4. Blood Cell Count

Copolymers PSSS₈₉-PEG₂₂₉-PSSS₈₉ and PEG₄₇-PSSS₉₉ slightly, but statistically significantly, reduced platelet count at the concentration of $100 \mu\text{g mL}^{-1}$ (Figure 2c), whereas PSSS₈₉-PEG₂₂₉-PSSS₈₉ and PSSS₅₈-PAA₃₃ also slightly, but statistically significantly, increased white blood cell count in rat blood at the concentration of $100 \mu\text{g mL}^{-1}$ (Figure 2d). There was no influence

on red blood cell count or the rest of the blood count parameters within all three copolymer concentrations (Table S1).

2.5. Hemocompatibility

At the concentration of $1,000 \mu\text{g mL}^{-1}$, copolymers PSSS₅₈-PAA₃₃ and PSSS₅₈-PAA₆₃ induced hemolysis (Figure 2e). Interestingly, when the content of the PAA block decreased, the hemolysis rate increased. This indicated that the PAA block reduced the hemolytic effect of the PSSS block but was not as effective as the PEGylation.

2.6. Cardiorespiratory Safety in Rats

We investigated the influence of copolymers on various cardiorespiratory parameters, including mean blood pressure, heart rate, blood perfusion of paw vessels, peak CO₂, respiratory rate, and blood oxygen saturation 30 min post-administration at the dose of 10 mg kg^{-1} . After the *i.v.* administration of all copolymers, an immediate increase in blood pressure was visible, followed by subsequent pressure drop (Figure 3a). This effect was noticeable but less pronounced after the administration of PEG₄₇-PSSS₃₂. Moreover, PEG₄₇-PSSS₃₂ influenced other respiratory parameters (Figure S2) and blood count (Table S2) to a lesser extent than other copolymers. In our previous study,^[19] we observed that administration of UFH at the dose of 9 mg kg^{-1} also led to disturbances in blood pressure and heart rate in rats. Therefore, we have chosen PEG₄₇-PSSS₃₂ as our leading copolymer, having the most promising efficacy-to-safety profile, for further *in vivo* investigation.

2.7. Ex Vivo Total Thrombus Formation (T-TAS System)

The T-TAS System was used to evaluate thrombus formation using AR and PL chips, which simulate semi-physiological

Table 1. Characteristics of the studied polymers.

Polymers	M_n [NMR] [g/mol]	M_w/M_n
PEG ₄₉ -PAMPS ₃₁₈ -PMAAS ₁₀₀	85900	1.23
PSSS ₈₉ -PEG ₂₂₉ -PSSS ₈₉	47300	2.00
PAMPS ₇₅ -PAAU ₂₈	29600	1.33
PSSS ₅₈ -PAA ₃₃	14300	1.18
PSSS ₅₈ -PAA ₆₃	16500	1.17
PEG ₄₇ -PSSS ₃₂	8670	1.13
PEG ₄₇ -PSSS ₉₉	22500	1.20

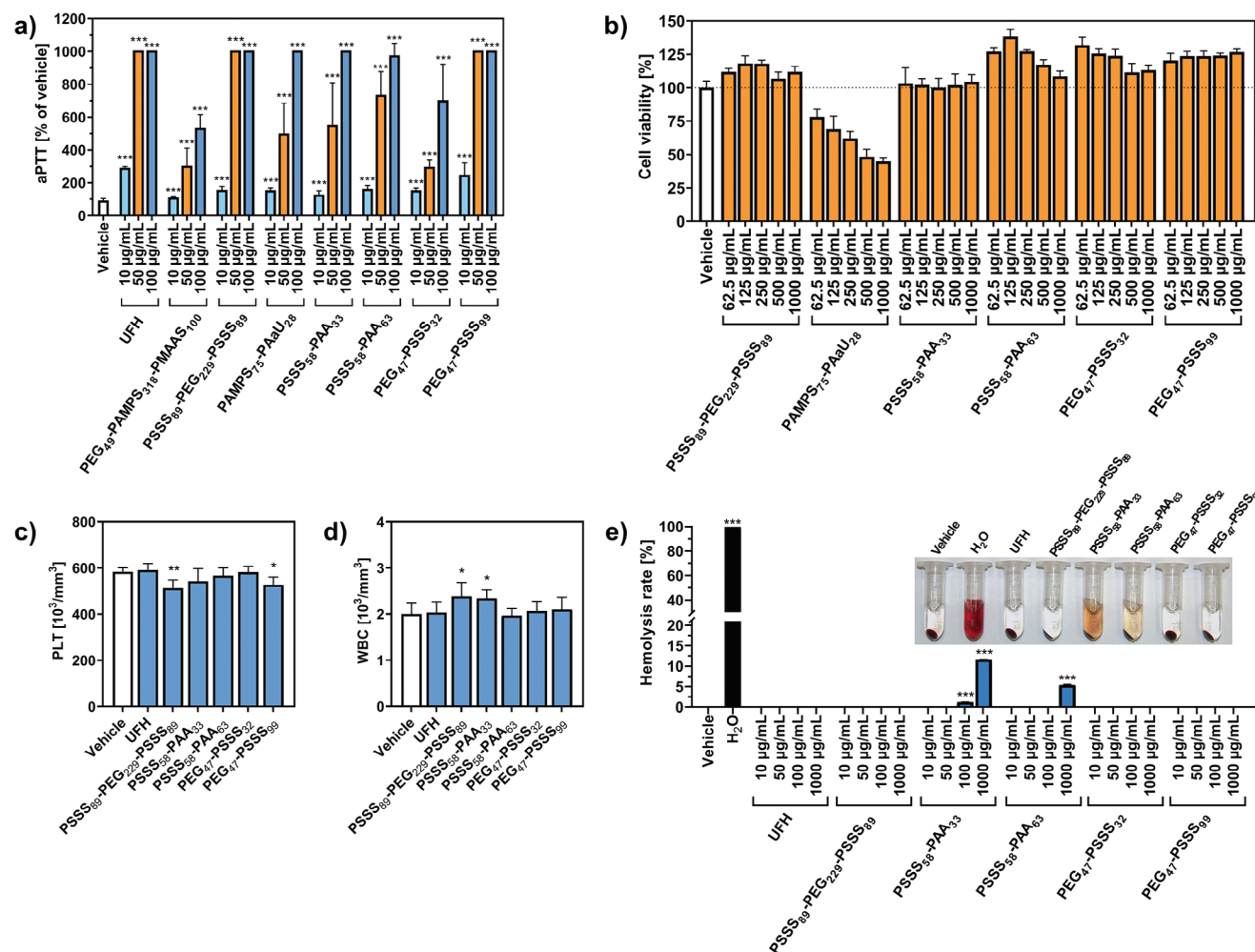


Figure 2. a) Effect of the copolymers and unfractionated heparin (UFH) on activated partial thromboplastin time (aPTT) in rat plasma. Results are presented as the percentage of the vehicle (0.9% NaCl) activity. 1006% of vehicle activity was ascribed as a maximum value. *** $p < 0.001$ versus vehicle, Student t -test. Results are shown as mean $\pm$ SD, $n = 6$. b) Effect of copolymers on the viability of rat embryonic myoblasts H9C2(2-1) (ATCC® CRL-1446™) cells. Results are shown as mean $\pm$ SD. c) Platelet (PLT) count and d) white blood cell (WBC) count after incubation of rat blood with the copolymers or UFH solutions at the final concentration of 100 $\mu\text{g mL}^{-1}$. * $p < 0.05$, ** $p < 0.01$ versus vehicle, Student t -test. Results are shown as mean $\pm$ SD, $n = 3$ –5. e) Effect of copolymers on the mean percentage of total hemolysis in rat blood in comparison to negative control (vehicle, 0.9% NaCl) and positive control (pure H₂O), and the photo of the hemolytic effect induced by copolymers at the concentration of 1,000 $\mu\text{g mL}^{-1}$. *** $p < 0.001$ versus vehicle, Student t -test. Results are shown as mean $\pm$ SD, $n = 3$.

conditions. The AR chip is coated with type I collagen combined with tissue thromboplastin, which serves the dual purpose of activating both platelets and the coagulation system. The PL chip is coated solely with type I collagen, focusing on platelet adhesion and aggregation without the coagulation cascade activation.^[43,44] In the AR and PL chip tests, PEG₄₇-PSSS₃₂ demonstrated high activity at the concentration of 50 $\mu\text{g mL}^{-1}$ (Figure 4a,b). We observed a right shift of the waveform in the AR chip test (Figure 4c), indicating delayed occlusion. PEG₄₇-PSSS₃₂ at the highest concentration strongly and similarly to UFH inhibited AR chip occlusion. These results indicate high anticoagulant activity *ex vivo* of PEG₄₇-PSSS₃₂. The right shift of the waveform in the PL chip test (Figure 4d) indicated delayed occlusion at 25 $\mu\text{g mL}^{-1}$ as a result of alternation in the thrombus formation process and a complete inhibition of occlusion at 50 $\mu\text{g mL}^{-1}$. In con-

trast to the AR chip, UFH showed no activity in the PL chip test.

2.8. Targeting Coagulation Cascade Pathways: Prothrombin Time (PT), Thrombin Time (TT), and Fibrinogen Concentration Assays *in vitro*

To elucidate the mechanism of action of copolymer PEG₄₇-PSSS₃₂, we examined its impact on both intrinsic (aPTT test, Figure 2a), extrinsic (PT test, Figure 5a), and common (TT test, Figure 5b) clotting pathways, as well as its influence on fibrinogen concentration (Figure 5c). Except for prolonging aPTT, PEG₄₇-PSSS₃₂ has also prolonged PT and TT, indicating interference with all coagulation cascade pathways, and decreased in the concentration of fibrinogen in rat plasma.

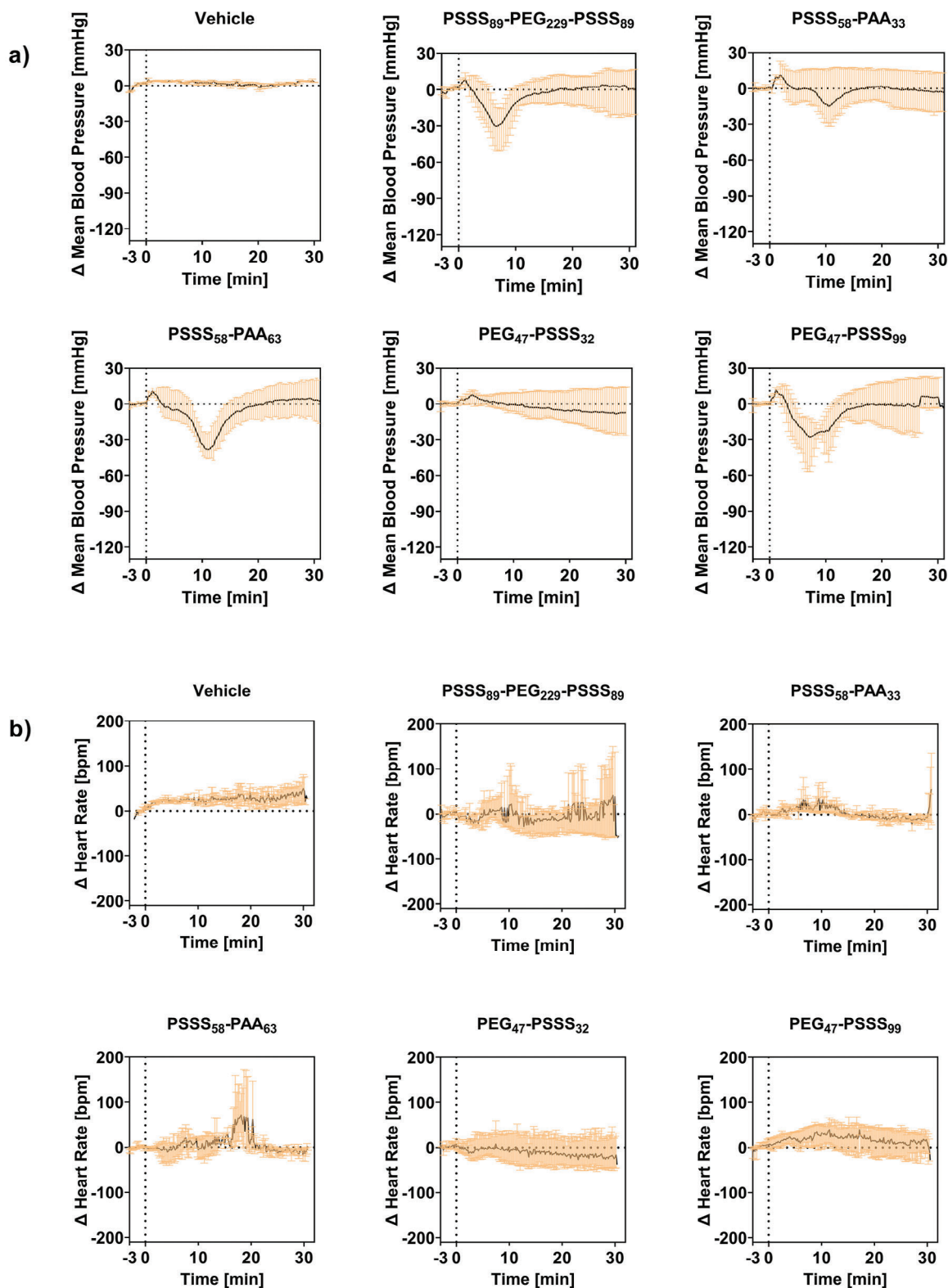


Figure 3. Effect of vehicle or copolymers (10 mg kg^{-1}) on a) delta mean blood pressure, and b) delta heart rate. The course of tested parameters was registered for 30 min after intravenous injection of vehicle or copolymers. Results are shown as median (line), with min-max values, $n = 3-4$. Millimeter of mercury (mmHg), beats per minute (bpm).

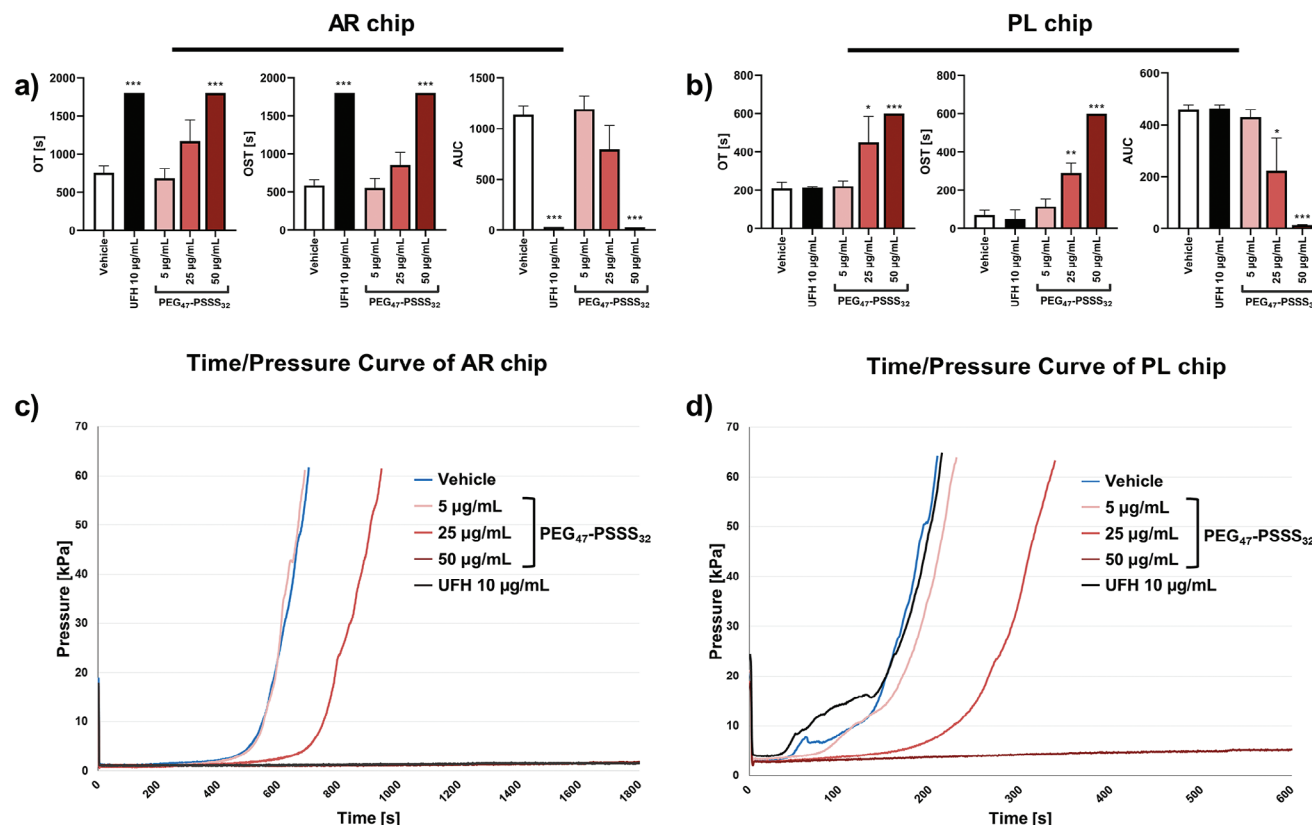


Figure 4. Effect of PEG₄₇-PSSS₃₂ or unfractionated heparin (UFH) on total thrombus formation measured in a) AR chip and b) PL chip, and corresponding pressure-time curves of c) AR chip and d) PL chip using rat blood. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus vehicle, Student t-test. Results of occlusion time (OT), occlusion start time (OST), and area under the curve (AUC) are presented as mean ± SD, *n* = 3.

2.9. Isothermal Titration Calorimetry (ITC) Measurements

We have examined the thermodynamic aspects of interactions between copolymer PEG₄₇-PSSS₃₂ and human AT, to compare its binding affinity to that of UFH. We have also investigated PEG₄₇-PSSS₃₂ interaction with human fII. Although these interactions were moderately weak, ranging between 10–100 µM, they were observable with both proteins (Figure 5d, Table 2). Both interactions could be successfully analyzed according to the model of one class of binding sites. Because the calorimetric data analysis was performed assuming that the ligand is a protein, the apparent stoichiometry suggests binding of 1 to 3 protein molecules per copolymer molecule. The reaction was driven by a favorable enthalpy change compensated largely by an unfavorable entropy change for both systems studied.

2.10. The Influence on Coagulation Factors

To investigate which clotting factor is affected by PEG₄₇-PSSS₃₂, we measured the prolongation of clotting time (aPTT or PT) by PEG₄₇-PSSS₃₂ in plasma with relevant deficient factor versus by vehicle in plasma with relevant deficient factor.^[45] If the clotting times were prolonged equally, the coagulation factor of interest was not influenced by PEG₄₇-PSSS₃₂. If the clotting time after incubation with PEG₄₇-PSSS₃₂ was prolonged compared to the

vehicle group, the coagulation factor of interest was inhibited by PEG₄₇-PSSS₃₂.

PEG₄₇-PSSS₃₂ inhibited mainly fXII, and to a lesser extent fX, fIX, fVIII, and fII (Figure 5e).

2.11. in vitro Neutralization of the Lead Copolymer by HBC

To evaluate the neutralization of the lead copolymer, we incubated PEG₄₇-PSSS₃₂ with rat or human plasma to prolong aPTT, and then HBC was added. Effective neutralization resulted in a reduction in aPTT prolongation. The prolongation of aPTT after incubation with PEG₄₇-PSSS₃₂ was more pronounced in human plasma (286 s ± 30) than in rat plasma (223 s ± 62) at the same concentration (100 µg mL⁻¹) (Figure 5f,g). We have found that HBC completely neutralized PEG₄₇-PSSS₃₂ activity at the mass ratio of 1:1 in the aPTT assay, both in the rat (Figure 5f) and human plasma (Figure 5g).

2.12. Arterial Thrombosis Induced by Electrical Stimulation in Rats

To confirm whether PEG₄₇-PSSS₃₂ possesses in vivo antithrombotic activity, we investigated its effect in the arterial thrombosis rat model. This model simulates thrombus formation under controlled conditions after significant common carotid artery injury

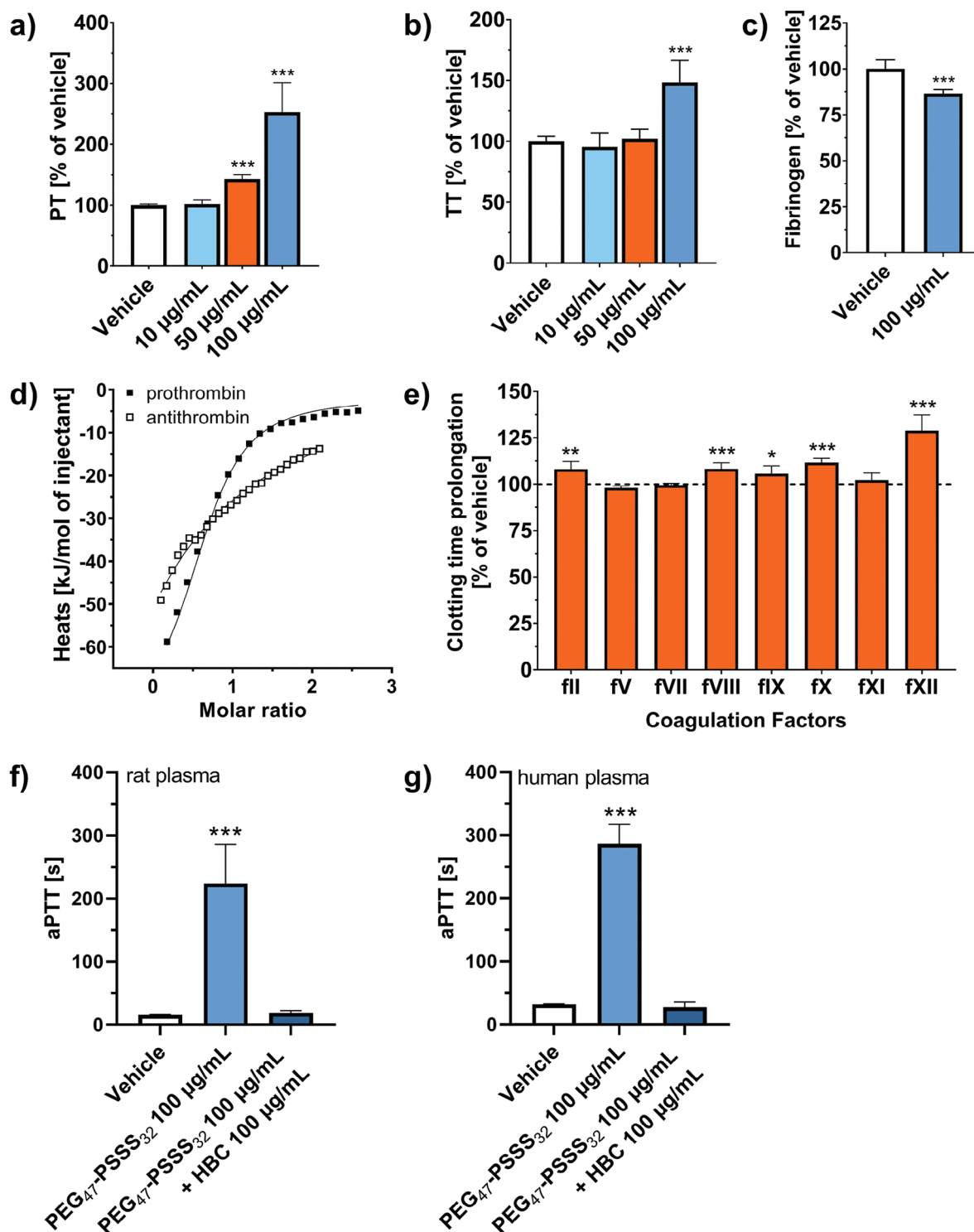


Figure 5. Effect of PEG₄₇-PSSS₃₂ on a) prothrombin time (PT), b) thrombin time (TT), and c) fibrinogen concentration (Clauss) in vitro in rat plasma. Results are presented as the percentage of the vehicle activity. *** $p < 0.001$ versus vehicle, Student t -test. Results are shown as mean $\pm$ SD, $n = 5$. d) Representative calorimetric isotherms of the binding of PEG₄₇-PSSS₃₂ copolymer to human antithrombin and prothrombin. The lines represent the best fit of the one class of binding sites model to the experimental data. e) Effect of PEG₄₇-PSSS₃₂ at the concentration of 50 µg mL⁻¹ on the aPTT (for fII, fV, fVIII, fIX, fX, fXI, fXII) or PT (for fVII) prolongation in factor deficient human plasmas. Results are presented as the percentage of the vehicle activity. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle activity (100%), Student t -test. Results are shown as mean $\pm$ SD, $n = 5$. f) Reversal activity of Heparin Binding Copolymer (HBC) on PEG₄₇-PSSS₃₂ at the mass ratio of 1:1 in vitro in rat and g) human plasma, shown as the prolongation of aPTT. *** $p < 0.001$ versus vehicle, Student t -test. Results are shown as mean $\pm$ SD, $n = 5$.

Table 2. Thermodynamic parameters of the interactions between PEG₄₇-PSSS₃₂ copolymer and human antithrombin (AT) and prothrombin (fII).

	Stoichiometry	K_a [$\times 10^3$ M ⁻¹]	ΔH_a [kJ/mol]	ΔS_a [J/mol/K]	ΔG_a [kJ/mol]	K_d [μ M]
PEG ₄₇ -PSSS ₃₂ /AT	3.3 $\pm$ 1.4	5.3 $\pm$ 2.7	-163 $\pm$ 23	-476 $\pm$ 78	-21.3 $\pm$ 1.3	190 $\pm$ 96
PEG ₄₇ -PSSS ₃₂ /fII	1.5 $\pm$ 0.1	366 $\pm$ 59	-50 $\pm$ 3	-62 $\pm$ 9	-31.8 $\pm$ 0.4	2.7 $\pm$ 0.5

by electrical stimulation. Measuring thrombus weight, bleeding time, and coagulation and platelet activity parameters allows for assessing the effectiveness of tested compounds such as PEG₄₇-PSSS₃₂ on thrombosis. Administration of PEG₄₇-PSSS₃₂ at the dose of 10 mg kg⁻¹ resulted in a significant decrease in the weight of arterial thrombus (Figure 6a) and in significant prolongation of the tail bleeding time (Figure 6b). Rats treated i.v. with PEG₄₇-PSSS₃₂ at the doses of 1, 3, and 10 mg kg⁻¹ exhibited signifi-

cant anticoagulant activity, measured as the prolongation of activated clotting time (ACT) (Figure 6c), aPTT (Figure 6d), and PT (Figure 6e) when compared to the vehicle groups. No significant differences in the activity against fIIa and fXa were observed in the rats treated with PEG₄₇-PSSS₃₂ compared to the vehicle groups (Figure 6f,g), contrary to rats treated with UFH. In rats treated with the copolymer, a dose-dependent elevation in total fII and fIIa concentration was observed, whereas no such increase

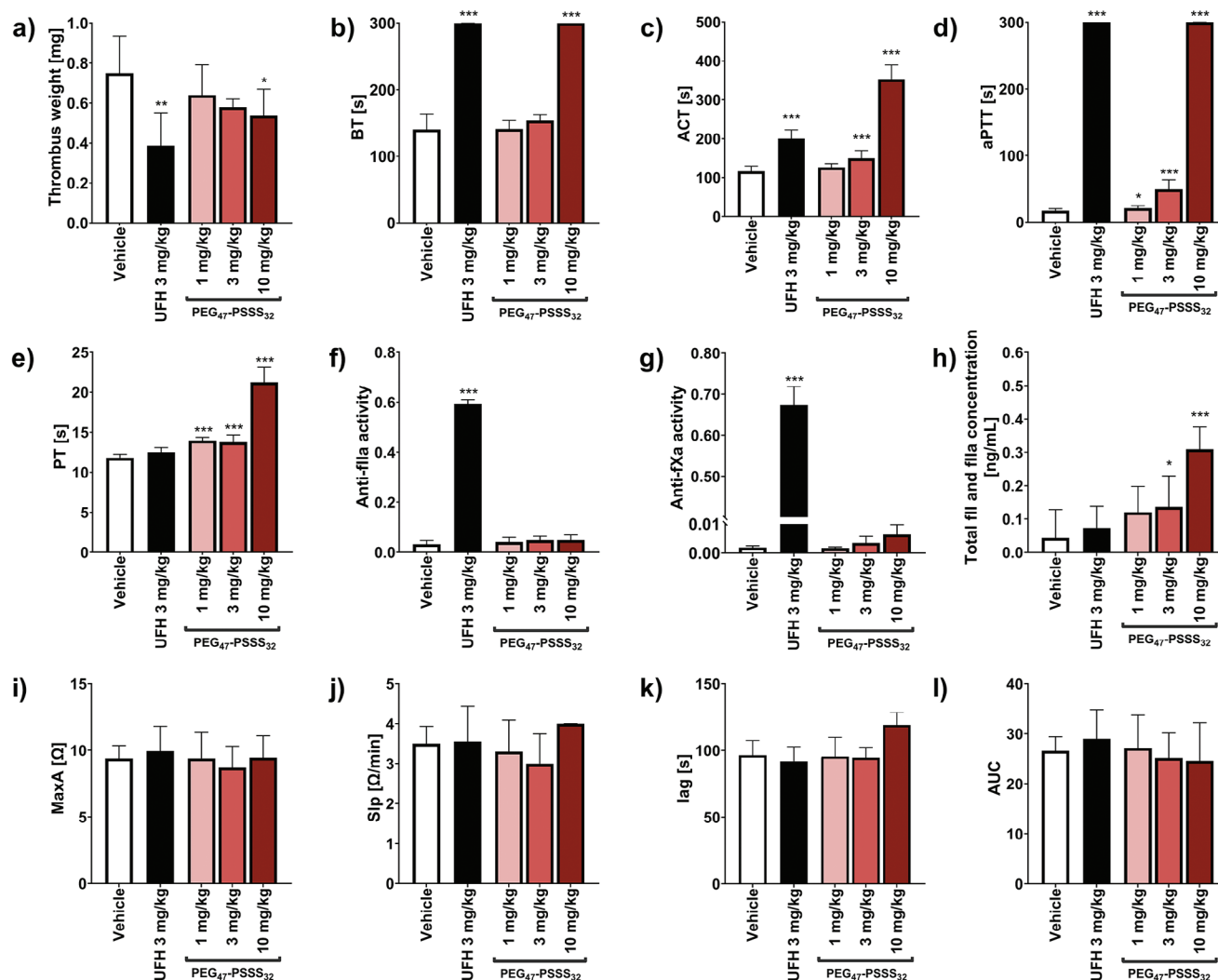


Figure 6. Anticoagulant (a–h) and antiplatelet (i–l) activity in rats treated intravenously with PEG₄₇-PSSS₃₂, unfractionated heparin (UFH), or vehicle after arterial thrombosis induced by electrical stimulation. Bleeding time (BT), activated clotting time (ACT), activated partial thromboplastin time (aPTT), prothrombin time (PT), prothrombin (fII), and thrombin (fIIa). Platelet aggregation after collagen addition was assessed in whole blood by measuring maximal extension (MaxA), the slope of platelet aggregation (Slp), lag phase (Lag), and area under the curve (AUC). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus vehicle, Student *t*-test. Results are shown as mean $\pm$ SD, *n* = 9–10.

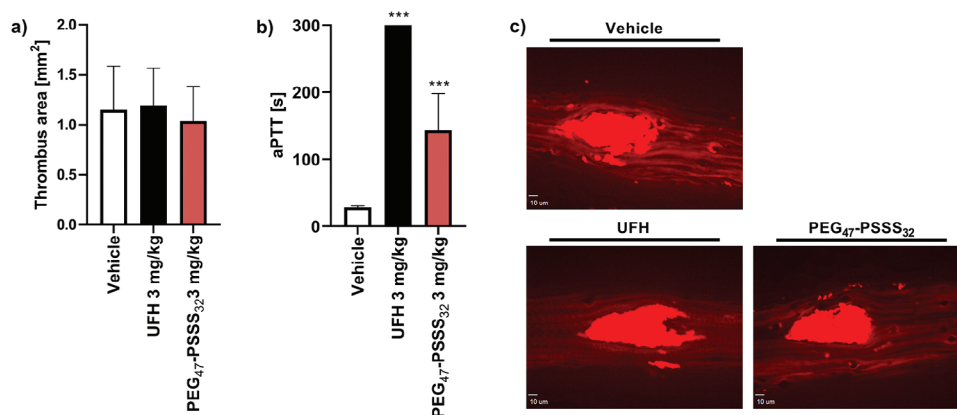


Figure 7. a) Thrombus area and b) aPTT prolongation in mice treated intravenously with PEG₄₇-PSSS₃₂ (3 mg kg⁻¹), UFH (3 mg kg⁻¹), or vehicle after laser-induced thrombosis in mice mesenteric vein. c) Representative thrombi obtained in the presence of PEG₄₇-PSSS₃₂, unfractionated heparin (UFH), or vehicle. ****p* < 0.001 versus vehicle, Student *t*-test. Results are shown as mean ± SD, *n* = 13–16 (thrombus area), or *n* = 6 (aPTT).

was observed in the UFH group (Figure 6h). This increase may be due to fIIa reduced ability to bind fibrinogen, leading to the accumulation of total fII and fIIa concentration in the blood of rats. This hypothesis was supported by subsequent in vitro experiments, which demonstrated a decrease in fibrinogen levels after incubation with PEG₄₇-PSSS₃₂. Rats treated i.v. with PEG₄₇-PSSS₃₂ and UFH represented no increase at the maximal extension, the slope of platelet aggregation, lag phase, and area under the curve (Figure 6i–l). Both PEG₄₇-PSSS₃₂ at 1, 3, and 10 mg kg⁻¹, and UFH at 3 mg kg⁻¹ increased white blood cell count, but no other blood count parameter was affected (Table S3).

2.13. Laser-Induced Thrombosis in Mice Mesenteric Vein

A laser-induced thrombosis model was developed to measure the early phase of platelet activation, focusing on a small area of endothelial damage and using fluorescent labeling of glycoprotein GP1Bβ as a platelet activation marker. This methodology enables the evaluation of antiplatelet rather than anticoagulant activity. In this experiment, neither UFH nor PEG₄₇-PSSS₃₂ inhibited platelet accumulation in the area of laser-injured endothelium of the mesenteric vein, determined by using antibodies against platelet glycoprotein GP1Bβ labeled with DyLight649 (Figure 7a,c). At the same time, UFH and PEG₄₇-PSSS₃₂ prolonged aPTT in blood samples taken from mice at the end of this experiment (Figure 7b).

2.14. Anticoagulant Activity after s.c. Administration of PEG₄₇-PSSS₃₂ in Mice

To investigate whether PEG₄₇-PSSS₃₂ exhibits anticoagulant activity after s.c. administration, it was administered in such a manner to mice at the dose of 25 mg kg⁻¹. Blood samples were collected at set time points, and aPTT was measured to evaluate the anticoagulant activity. The result showed prolongation of aPTT 1 and 4 h after the administration of PEG₄₇-PSSS₃₂, indicating its anticoagulant activity (Figure 8).

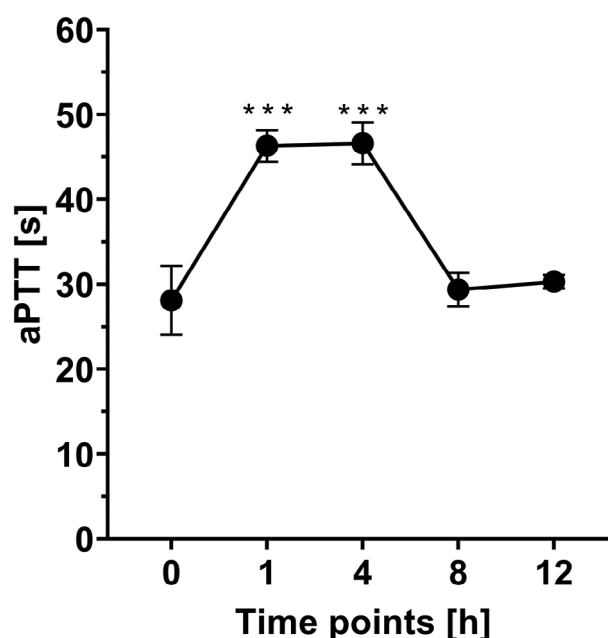


Figure 8. The prolongation of aPTT after subcutaneous administration of PEG₄₇-PSSS₃₂ at the dose of 25 mg kg⁻¹ in mice. ****p* < 0.001 versus aPTT time at starting point (0), Student *t*-test. Results are shown as mean ± SD, *n* = 3 for each time point.

3. Discussion

The primary objective of this study was to evaluate the anticoagulant activity of sulfonate-based copolymers in vitro and in vivo, with particular emphasis on their safety profile, including the possibility of being neutralized. Considering the challenges associated with UFH,^[46–48] we synthesized sulfonate-based copolymers using RAFT to achieve precise control over their composition, molecular weight, and sulfonic group content. We excluded compounds with questionable safety profiles, and we focused on the copolymer PEG₄₇-PSSS₃₂, which exhibited the most favorable activity-to-safety ratio. Its anticoagulant activity was completely reversed with a cationic block copolymer, HBC. In the final step,

we investigated the anticoagulant and antiplatelet activity of the lead copolymer in animal models and explored potential targets of action.

The selected polymers were representatives of different block copolymer classes: a di- and triblock hydrophilic neutral-strong polyelectrolyte (PEG-PSSS and PSSS-PEG-PSSS), a triblock hydrophilic neutral-strong polyelectrolyte-weak polyelectrolyte (PEG-PAMPS-PMAAS), a diblock hydrophilic strong polyelectrolyte-weak polyelectrolyte (PSSS-PAA), and a diblock strong polyelectrolyte-weak polyelectrolyte, which becomes amphiphilic at pH<8 and consequently self-organizes into nanometer-sized objects.^[40] In all these polymers the blocks responsible for anticoagulant activity were sulfonated blocks (PSSS and PAMPS) and to a lesser extent blocks containing carboxylic groups (PAA, PMAAS, and PAaU). The neutral PEG block was used to decrease the polymer toxicity.

The anticoagulant activities of the copolymers were evaluated at concentrations of 10, 50, and 100 $\mu\text{g mL}^{-1}$ in pooled rat plasma by measuring the prolongation of aPTT, commonly used for monitoring UFH therapy.^[49] UFH comprises repeating units of uronic acid and D-glucosamine and is characterized by a heterogeneous degree of sulfation pattern among its chains.^[46] The high-affinity pentasaccharide sequence with a specific 3-O-sulfated glucosamine residue binds AT, the major regulator of blood clotting.^[50,51] PSSS-based copolymers demonstrated greater activity compared to those based on PAMPS, and the differences between copolymers and UFH in the prolongation of a PTT could stem from their structural differences.

While anionic polymers are generally considered to be biocompatible in vitro,^[52–54] certain studies conducted on animals suggest their potential toxicity.^[39,55] The results of the hemolysis study indicated that PSSS-PAA copolymers induced hemolysis while substituting PAA with PEG effectively eliminated this adverse effect. Furthermore, non-PEGylated PAMPS-PAaU copolymer reduced the cell viability of cardiomyocytes. The significant benefit of PEGylation was previously demonstrated in the rat safety study, with a direct comparison between the non-PEGylated and PEGylated polymers.^[39] Regarding alternations in blood count, copolymers containing PAA blocks affected red blood cells in the in vitro hemolysis test only at a very high concentration of 1,000 $\mu\text{g mL}^{-1}$, which is unlikely to be achieved with the doses administered in our in vivo studies in rats, including the highest dose of 10 mg kg^{-1} . Therefore, despite the observed hemolysis, we did not exclude these polymers at this stage. No alternation in red blood cell count was observed in the in vivo experiment. The decrease in platelet count was observed in vitro only at the highest concentration of copolymers PSSS₈₉-PEG₂₂₉-PSSS₈₉ and PEG₄₇-PSSS₉₉. Some studies have shown the potential of charged compounds to activate platelet aggregation,^[45,56–60] but, on the other hand, the antiplatelet activity of sulfonated compounds was also described.^[61,62] PEG₄₇-PSSS₃₂ was the only copolymer that did not decrease platelet count both under in vitro and in vivo conditions, which suggests that the overall charge could play a crucial role not only in the inhibition of the coagulation cascade but also in the activation of platelets. The potential interaction of PEG₄₇-PSSS₃₂ with fibrinogen, which is involved in the aggregation of platelets,^[63,64] could also serve as an explanation. We have observed a slight increase in white blood cell count in vitro in rat blood after incubation with PSSS₈₉-PEG₂₂₉-

PSSS₈₉ and PSSS₅₈-PAA₃₃ at the highest polymer concentrations. This finding aligns with previous reports, in which anionic polymers elevated white blood cell count, with the effect depending on the molecular weight and charge of the polymers.^[65,66] Similarly, UFH induced an increase in white blood cell count, where sulfation, particularly 6-O-sulfation, played a crucial role.^[67] The mechanism may involve the blockage of lymphocyte homing, the release of thymocytes and neutrophils from bone marrow, and the inhibition of L-selectin-, P-selectin-, and chemokine CXCL12-mediated leukocyte trafficking.^[68]

Given the absence of animal safety data on these copolymers, we evaluated cardiorespiratory parameters in rats. Disturbances were observed following the administration of all copolymers at the concentration of 10 mg kg^{-1} , with the least deviations from vehicle noted after the administration of PEG₄₇-PSSS₃₂, thus we selected it as our lead copolymer. Disturbances in blood pressure and heart rate were also seen after UFH administration in rats.^[19] In the T-TAS system, the PL chip mimics the initial steps of hemostasis, where platelets adhere to exposed collagen at the site of vascular injury and form aggregates to initiate clot formation. The similar results obtained between the polymer and UFH in the AR chip but different in the PL chip are probably related to the differences in their mechanisms of action. Results comparable to those of UFH in AR and PL chips were previously observed in dogs after the treatment with dalteparin.^[69] The different outcomes between the PL chip and the collagen-induced platelet aggregation in the rat model could stem from the conditions under which these assays are conducted. The latter measures platelet aggregation in response to collagen in a stirred environment. It might not capture the same platelet behavior under flow conditions as the PL chip, which measures explicitly platelet activity without generating fIIa. Additionally, fibrinogen levels were shown to impact the PL measurements.^[70] The results of both tests were shown to be rarely correlated with each other.^[70] To investigate the specific targets of the lead copolymer, we measured the prolongation of clotting times in human plasmas with deficient factors of interest. PEG₄₇-PSSS₃₂ decreased mainly the activity of fXII, and to a lesser extent of fX, fVIII, and fII. Selectively inhibiting fXII in human plasma holds considerable potential for improving the safety profile of anticoagulation. There is an ongoing interest in developing inhibitors of fXII or fXI,^[71–75] as they provide control over thrombosis while sparing hemostasis,^[76–79] thus mitigating bleeding risks. Despite fXII central function in thrombosis, its deletion in mice and rats did not disrupt hemostasis in vivo.^[80] These observations are consistent with clinical findings of individuals with fXII deficiency maintaining normal hemostatic function without spontaneous bleeding, despite prolonged aPTT.^[81–83] Also, in murine models fXIIa inhibition was found to reduce abdominal aortic aneurysm and atherosclerosis and to stabilize atherosclerotic plaques.^[84] Moreover, fXII inhibitors could mitigate complications related to blood exposure to artificial biomaterials,^[85–87] as seen in patients undergoing procedures such as cardiopulmonary bypass surgery,^[88] or hemodialysis.^[89] On the other hand, fXII deficiency does not provide significant protection against stroke, venous thromboembolism, myocardial infarction or deep vein thrombosis in humans.^[90–93] Hence, the action of PEG₄₇-PSSS₃₂ which includes residual inhibition of fX, fVIII, and fII may be beneficial and helpful in overcoming this limitation. Also, targeting

fibrinogen is another approach that allows for normal fIIa generation and preservation of hemostasis in high-risk settings, such as in patients with recurrent venous thromboembolism, or with constitutively elevated fibrinogen or hyperfibrinogenemia.^[94] The relationship between fibrinogen and anionic compounds was also explored before. For instance, sulfonated polyisoprenes demonstrated the ability to impede the *in vitro* conversion of fibrinogen to fibrin monomer, inhibiting the polymerization of the fibrin monomer, and presenting potent anticoagulant action of these compounds.^[45] The adsorption of fibrinogen to sulfonate surfaces was also observed,^[95–97] and induced hypofibrinogenemia in mice was protective against venous thrombosis while preserving hemostasis.^[98]

In the arterial thrombosis rat model, we have observed significant anticoagulant activity of the copolymer at the dose of 3 mg kg^{−1} (shown as a prolongation of aPTT and ACT) without concurrent excessive bleeding (no differences in bleeding time compared to the vehicle). Bleeding complications stand out as the primary drawback of anticoagulants,^[99] emphasizing the significance of our copolymer's potential in mitigating such risks. The sulfonate groups imparted anticoagulant activity to the lead polymer by partially mimicking the structure of UFH. However, they did not appear to be as potent as the combination of the presence of functional groups and complex structures found in UFH. However, while UFH more effectively prolonged aPTT and ACT and decreased thrombus weight, it also induced more severe bleeding in rats. The differences between UFH and lead polymer in the results of PT, and factors IIa and Xa activities in this model indicated distinct mechanisms involved in the inhibition of coagulation by sulfated polysaccharides and PSSS-based copolymers. What is more, this study revealed an unexpected mild increase in the total fII and fIIa concentration in the PEG₄₇-PSSS₃₂ group. Such an increase would suggest a prothrombotic tendency, but prominent anticoagulant activity contradicts this association. It could be attributed to fIIa inability to effectively bind fibrinogen, leading to the accumulation of total fII and fIIa concentration in the blood of rats. Subsequent *in vitro* results supported our hypothesis, revealing reduced fibrinogen concentration after incubation with PEG₄₇-PSSS₃₂. This interference with fibrinogen might lead to fIIa accumulation. Strong interactions with fibrinogen have been previously observed with sulfonated compounds.^[45] In contrast to the experiment in the PL chip in T-TAS, we did not observe platelet activation in rats developing arterial thrombosis 1 h after *i.v.* administration of PEG₄₇-PSSS₃₂. The lack of antiplatelet activity in the arterial thrombosis model might have been influenced by the duration of the experiment and sample collection (≈65 min). We considered the possibility that PEG₄₇-PSSS₃₂ could exhibit rapid but short-duration antiplatelet activity. Thus, we conducted laser-induced thrombosis in mice to measure platelet activation right after administration of the copolymer. It is important to note that the efficacy of anticoagulants can vary across different laser-induced injury models. These models exhibit differences in the mechanism, extent, and timing of injury, which result in varying biological effects.^[100] Our model was particularly designed to measure the early phase of platelet activation, focusing on a small area of endothelial damage and employing fluorescent labeling of glycoprotein GP1Bβ as a platelet activation marker. Due to the small area of exposed subendothelial matrix in our model,

the extent of irreversible platelet activation was minimal. Consequently, the thrombus primarily comprised platelets lacking phosphatidylserine, which had not undergone irreversible activation, unlike phosphatidylserine-positive platelets, which are irreversibly activated and contribute to fibrin formation and the mechanical stability of the thrombus core.^[101] The results indicated that PEG₄₇-PSSS₃₂, similar to UFH, did not influence the initial platelet response to the endothelial damage. However, they exhibited an anticoagulant effect, as evidenced by the prolongation of the aPTT in blood samples collected from mice at the end of the experiment. Despite sharing this feature, our experimental findings indicate that these anticoagulants primarily demonstrate differences in their anticoagulant mechanism of action. While both compounds interacted with AT, the significance of this interaction differs. For UFH, binding to AT is essential, as it forms a complex with AT and amplifies its ability to inhibit fIIa and fXa. Copolymer PEG₄₇-PSSS₃₂ interacted with AT with lower affinity than with fII, which exhibited relatively high affinity, dissociation constant of ≈3 μM, and similar enthalpy and entropy contributions. The affinity between UFH and AT varies with experimental conditions and the specific assay used but typically falls within the nanomolar range.^[46,102] For our polymer, both interactions were weak and probably involved a non-specific binding. The low affinity for AT may be attributed to the lack of specific 3-O-sulfated glucosamine residues, which have been shown to enhance the binding ability of UFH to AT.^[50,51] What is more, UFH is known to prolong TT significantly. Our polymer did not prolong TT up to 50 μg mL^{−1}, possibly due to a lack of carboxyl groups, which have been previously shown to play a crucial role in TT prolongation.^[52]

Our study showed that the mechanism of anticoagulant activity of PSSS-based copolymers differs from that of UFH, possibly due to structural differences (the absence of polysaccharide sequences in PSSS essential for AT binding).^[103] Heparin mimetics, such as dermatan sulfate or fucoidan, which have structural similarities to UFH, inhibit fIIa through AT and heparin cofactor II.^[104,105] Some sulfated glycopolymers can inhibit fIIa via both AT-dependent and AT-independent mechanisms,^[106,107] while others inhibit fIIa through heparin cofactor II, but not AT. Additionally, they inhibit fIXa and fVIIIa and bind fibrinogen.^[108] Sulfated cellulose derivatives displayed anticoagulant properties that depend on the sulfate content at the C2-position, which mimics the interaction between UFH and AT. Sulfate groups provided the most significant anticoagulant activity among other substituents, such as phosphates or quaternary ammonium. Sulfated cellulose derivatives inhibited fIIa rather than fXa, suggesting a mechanism similar to UFH.^[109–111] Low molecular weight sulfated chitosan derivatives demonstrated anticoagulant activity via increased anti-Xa activity, similar to LMWHs. The activity was mediated through heparin cofactor II.^[112,113] In contrast, an HMP-modified sponge inhibited coagulation by inhibiting fXI and fIIa, unlike UFH.^[8] Microspheres composed of acrylic acid and PAMPS effectively bound fVIII, fIX, and fXI, preventing clotting in both the tubing and hemodialyser.^[9] Anticoagulant activity of PAMPS-based polymers involved interaction with AT, fIIa, and prostacyclin-mediated inhibition of platelets.^[29,39,114,115] In contrast, potassium-3-sulfopropyl acrylate-based polymers showed anticoagulant activity by prolonging aPTT and TT but were less effective than heparins and did not inhibit fIIa and fXa.^[29,114]

Also, polyglycerol sulfates significantly prolonged aPTT and TT, though their effectiveness was not clearly dependent on the molecular weight. Potassium-3-sulphopropyl methacrylate-based copolymers, despite containing SO_3^- groups, exhibited minimal anticoagulant activity.^[29,114] The mechanisms of action for other heparin mimetics are not yet fully understood, even though studies suggest they differ from those of UFH.^[116–119] The prolongation of aPTT by PSSS-based polymers involves intrinsic and common coagulation pathways, potentially through electrostatic interactions.^[29,114] Also, a mild fXII binding was observed.^[120] When merged with PAA, bearing carboxyl groups, aPTT and TT were prolonged significantly, which resembled the behavior of UFH, likely due to the presence of both SO_3^- and carboxyl groups in the copolymer.^[52] Although PSSS has demonstrated the ability to mimic UFH in various contexts – such as exhibiting antiproliferative activity,^[121] stabilizing basic fibroblast growth factor FGF-2,^[122] promoting myotube formation,^[123] and enhancing fibroblast proliferation^[124] – our PSSS-based copolymer showed a low affinity for AT. This could be attributed to the absence of the glucosamine moiety with 3-O-sulfation, which increases the affinity for AT.^[125]

Based on the recent discoveries in the field of anticoagulant neutralization, having access to reverse the effects of novel anticoagulants should be a necessity, not just an option. To evaluate the ability to control hemorrhages, we proposed to reverse the activity of PEG₄₇-PSSS₃₂ by HBC, a cationic antidote for heparins developed by our team.^[19,20] Due to a more complementary structure of both block copolymers, we demonstrated a complete neutralization in a mass ratio of 1:1, which was twice more effective than the neutralization of UFH and LMWHs by HBC.^[13,14] Complete neutralization of PEG₄₇-PSSS₃₂ anticoagulant activity in human plasma highlights its potential as a safe therapeutic option for managing bleeding complications associated with anticoagulant therapy in patients. Furthermore, the possibility to administer PEG₄₇-PSSS₃₂ via both *i.v.* and *s.c.* routes offer an advantage over the conventional approach, which typically involves the use of separate anticoagulant agents, such as UFH or LMWHs. This approach not only simplifies treatment regimens but also offers potential benefits such as improved patient compliance, reduced healthcare burden, and potential cost savings.

The anticoagulant activity and the safety profile of sulfonate-based copolymers with precise composition and molecular properties were evaluated. PEG₄₇-PSSS₃₂ emerged as a lead polymer, demonstrating significant and predictable anticoagulant effects *in vitro* and *in vivo*, after both *i.v.* and *s.c.* administration. It does not act through AT binding but instead inhibits factors within the coagulation cascade, particularly fXII, fX, fVIII, fII, and fibrinogen. Additionally, its anticoagulant activity can be fully reversed using the cationic block copolymer, HBC. In conclusion, this study demonstrates that PEG₄₇-PSSS₃₂ is a promising alternative to traditional anticoagulants.

4. Experimental Section

Characterization of the Studied Copolymers: Poly(ethylene glycol)₄₉-*block*-poly(sodium 2-acrylamido-2-methylpropane sulfonate)₃₁₈-*block*-poly(sodium methyl methacrylate)₁₀₀ (PEG₄₉-PAMPS₃₁₈-PMAAS₁₀₀),^[34] poly(sodium styrenesulfonate)₈₉-*block*-poly(ethylene glycol)₂₂₉-*block*-poly(sodium styrenesulfonate)₈₉ (PSSS₈₉-PEG₂₂₉-PSSS₈₉),^[35] poly(sodium 2-acrylamido-2-methylpropane sulfonate)₇₅-*block*-

poly(sodium 11-acrylamidoundecanoate)₂₈ (PAMPS₇₅-PAAU₂₈),^[36] poly(sodium styrenesulfonate)₅₈-*block*-poly(acrylic acid)_m (PSSS₅₈-PAA_m, $m = 33$ and 63),^[37] and poly(ethylene glycol)₄₉-*block*-poly(sodium styrenesulfonate)_n (PEG₄₇-PSSS_n, $n = 32$ and 99)^[38] were prepared via RAFT polymerization. The degree of polymerization and number-average molecular weight (M_n) values of each block copolymer were estimated from ¹H NMR spectroscopy using the integral intensity ratio of the signals. The ¹H NMR spectra were recorded using a JEOL (Tokyo, Japan) ECZ400 spectrometer. Molecular weight distribution (M_w/M_n) values were determined using gel-permeation chromatography measurements performed using a Shodex GF-7 M HQ column (Tokyo, Japan, exclusion limit, $\approx 10^7$) and Tosoh RI-8020 refractive index detector (Tokyo, Japan) using a phosphate buffer (pH 8.0, 50 mM) containing 9.0 vol% acetonitrile as an eluent.

Animals and Human Plasma: Male Wistar rats and male BALB/c mice were obtained from the Centre of Experimental Medicine in the Medical University of Białystok. Animals were housed with a 12 h light/dark cycle in temperature (22 ± 2 °C) and humidity ($55 \pm 10\%$) controlled room, grouped into cages as appropriate, and allowed to have *ad libitum* access to sterilized tap water and standard chow. All the procedures involving animals were approved by the Local Ethical Committee on Animal Testing in Olsztyn, Poland (Permit Numbers: 6/2021 and 04/2024) and conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals, Animal Research: Reporting of *In Vivo* Experiments guidelines, and national laws. All animals were euthanized by exsanguination at the end of the experiments.

Human plasma samples were obtained from 8 healthy adult volunteers (Bioethics Committee of the Medical University of Białystok, Poland, Permit Number APK.002.530.2023) into trisodium citrate (9:1 v/v) vacuettes (Vacuette, Greiner Bio-One GmbH, Austria). The group consisted of four women and four men who hadn't been taking any medications at the time of sample collection.

***In Vitro* Anticoagulant Activity Measured by Activated Partial Thromboplastin Time (aPTT) Assay:** Blood samples from healthy rats were collected from the heart and drawn into 3.13% trisodium citrate (Sigma-Aldrich, Germany) in a volume ratio of 9:1. Plasma was obtained by centrifugation of whole blood at $3500 \times g$ for 20 min at 4 °C. Pooled plasma (200 μL) was incubated with copolymers solutions (20 μL) at the final concentrations of 10, 50, and 100 $\mu\text{g mL}^{-1}$. The control samples were incubated with the 0.9% NaCl solution (saline, vehicle) (Fresenius Kabi, Spain). The anticoagulant activity of all solutions was analyzed by measuring aPTT in Coag Chrom 4000 (Bio-Ksel, Poland) according to the manufacturer instruction (Bio-Ksel, Poland). The maximal values were denoted as 300 s, which was the maximal range of the apparatus. The results were expressed as a percentage of the control samples.

Viability of H9c2(2-1) Cells After Exposure to Copolymers: Viability studies of the copolymers were performed on H9c2(2-1) (ATCC® CRL-1446™) *Rattus norvegicus*, rat, heart/myocardium cells. Cells were cultured on Petri dishes in a culture medium (Dulbecco's Modified Eagle's Medium – high glucose) supplemented with 10% v/v fetal bovine serum (FBS), at 37 °C in a humidified atmosphere containing 5% v/v CO₂. The cells were then seeded in 96-well plates in 100 μL culture medium supplemented with 10% fetal bovine serum and cultured for 24 h. After this time, for experiments without fetal bovine serum, the medium was changed. Later cells were treated with a solution of copolymers in PBS at different concentrations and incubated for 24 h. For FBS conditions only the second step was performed. Next, 10 μL of solution of MTT in PBS was added to each well and kept in the dark for 4 h at 37 °C. Then, the MTT solution was gently aspirated and 100 μL of DMSO/methanol 1:1 v/v was added to each well. After dissolving the crystals of purple formazan, the absorbance at 570 nm was measured using a plate reader. Measurements were made in triplicate.

Blood Cell Count: Rat blood samples were taken from the heart into test tubes containing 3.13% sodium citrate. Citrate-anticoagulated blood (900 μL) was added to saline (100 μL) containing different concentrations of copolymers (10, 50, and 100 $\mu\text{g mL}^{-1}$) or UFH (Heparinum WZF 5000 IU mL^{-1} , Polfa Warszawa, Poland) (10, 50, and 100 $\mu\text{g mL}^{-1}$, an equivalent to 1, 5, and 10 IU mL^{-1} , respectively), and mixed manually. The control

samples were incubated with the saline (vehicle). After 10 min of incubation at room temperature, the blood cell count was measured using the Animal Blood Counter (ABC Vet, Horiba, Germany).

Hemocompatibility: The hemolysis assay was evaluated to assess the hemocompatibility of copolymers solutions. Rat blood samples were taken from the heart into test tubes containing 3.13% sodium citrate. Further sample preparation and hemolytic assay were performed as described previously.^[126]

Cardiorespiratory Safety in Rats: Nineteen male Wistar rats weighting 220.0 ± 18.3 g were divided into six groups ($n = 4$ for PEG₄₇-PSSS₃₂), anesthetized by an intraperitoneal injection of pentobarbital (45 mg kg^{-1}), and placed in a supine position on a heated operation table. Copolymers (10 mg kg^{-1} , 1 mL kg^{-1}) or vehicle (saline, 1 mL kg^{-1}) were administered into the right femoral vein. Mean blood pressure, heart rate, blood perfusion of paw vessels, peak CO₂, respiratory rate, and blood oxygen saturation were measured using the PhysioSuite Physiological Monitoring Modular System (Kent Scientific Corporation) for 30 min after copolymers or vehicle administration as described previously.^[127] Blood samples were taken from the heart at the end of the experiment for measurement of blood cell count.

Ex Vivo Total Thrombus Formation (T-TAS System): T-TAS01 Instrument (Zacros, Tokyo, and Japan) is an automated microchip-based flow chamber system designed to analyze thrombus formation in semi-physiological conditions, using microchips as artificial blood vessels.^[128,129] The AR chip is coated with both type I collagen and tissue thromboplastin, simultaneously triggering platelets activation and the coagulation system. The PL chip measures platelets thrombus formation under physiological conditions on a collagen-coated surface. This coating induces platelets attachment to collagen, and platelets thrombus formation. The PL assay measures overall primary hemostatic function, which represents the totality of platelet activation pathways stimulated under arterial shear conditions across a collagen-coated surface.^[129,130] The assessment of thrombus formation in both chips can be made using the following variables: occlusion start time, occlusion time, and area under the curve.^[131] In the experiment with AR chip, rat blood samples were taken from the heart into test tubes containing 3.13% sodium citrate. Citrated rat blood ($750 \mu\text{L}$) was mixed with saline ($750 \mu\text{L}$) containing PEG₄₇-PSSS₃₂ (at the final concentration of 5, 25, and $50 \mu\text{g mL}^{-1}$) or UFH (at the final concentration of $10 \mu\text{g mL}^{-1}$, an equivalent to 1 IU mL^{-1}), and mixed manually. The control samples were incubated with the saline (vehicle). After 1 h of incubation at room temperature, thrombus formation was evaluated as described previously.

In the experiment with the PL chip, rat blood samples were taken from the heart into test tubes containing BAPA (Fujimori Kogyo Co., Ltd, Tokyo, Japan). Anticoagulated rat blood ($750 \mu\text{L}$) was mixed with saline ($750 \mu\text{L}$) containing PEG₄₇-PSSS₃₂ (at the final concentration of 5, 25, and $50 \mu\text{g mL}^{-1}$) or UFH (at the final concentration of $10 \mu\text{g mL}^{-1}$, an equivalent to 1 IU mL^{-1}), and mixed manually. The control samples were incubated with the saline (vehicle). After 1 h of incubation in room temperature, thrombus formation was evaluated as described previously.

Targeting Coagulation Cascade Pathways: In Vitro Prothrombin Time (PT), Thrombin Time (TT), and Fibrinogen Concentration Assays: In the experiment, blood samples from healthy rats were collected from the heart and drawn into 3.13% trisodium citrate in a volume ratio of 9:1. Plasma was obtained by centrifugation of whole blood at $3500 \times g$ for 20 min at 4°C . The anticoagulant activity of PEG₄₇-PSSS₃₂ was analyzed by measuring the prolongation of PT and TT, and the fibrinogen activity (Clauss method) according to the manufacturer instruction. PT, TT, and fibrinogen measurements were made using Bio-Ksel reagents in Coag Chrom 4000 (Bio-Ksel, Poland). Pooled plasma ($200 \mu\text{L}$) was incubated with PEG₄₇-PSSS₃₂ solutions ($20 \mu\text{L}$) at the final concentrations of 10, 50, and $100 \mu\text{g mL}^{-1}$ for PT and TT, and $100 \mu\text{g mL}^{-1}$ for fibrinogen assay. The control samples were incubated with the saline solution (vehicle). The results were expressed as a percentage of the vehicle.

Isothermal Titration Calorimetry Measurements: ITC was used for the assessment of thermodynamic parameters of the interactions of PEG₄₇-PSSS₃₂ with human AT and fII. ITC measurements were performed using a VP-ITC instrument (MicroCal, USA). All experiments were conducted in

duplicate in phosphate-buffered saline (PBS) at 25°C . The protein solutions were dialyzed in PBS, and PEG₄₇-PSSS₃₂ was dissolved in a dialysis buffer. All solutions were degassed for 5 min under vacuum before the experiments. Typically, $10 \mu\text{L}$ aliquots of PEG₄₇-PSSS₃₂ ($200 \mu\text{M}$) were added as 25–30 injections into a calorimeter cell of $1435.5 \mu\text{L}$ volume filled with protein solution (10 or $20 \mu\text{M}$). The injection rate was $0.5 \mu\text{L s}^{-1}$, and the interval between injections was 4 min. To ensure proper mixing after each injection, a constant stirring speed of 300 rpm was maintained throughout the experiment. Data analysis was performed globally for duplicates with shared stoichiometry, enthalpy, and binding constant values according to one class of binding site model using MicroCal Origin software.

Coagulation Factors Activity: To determine the influence of PEG₄₇-PSSS₃₂ on other coagulation factors, a modified method presented by Y. Tamada et al. was used.^[45] Coagulation factor deficient plasmas were purchased from Protolix (USA).

The amount of normal plasma was first determined that must be added to $20 \mu\text{L}$ of PEG₄₇-PSSS₃₂ plasma in order to achieve the same clotting time as $20 \mu\text{L}$ of normal plasma alone. It is determined that adding $190 \mu\text{L}$ of normal human plasma to $20 \mu\text{L}$ of PEG₄₇-PSSS₃₂ plasma (for aPTT) and adding $100 \mu\text{L}$ of normal human plasma to $50 \mu\text{L}$ of PEG₄₇-PSSS₃₂ plasma (for PT) cancels the influence of PEG₄₇-PSSS₃₂ on clotting factors. By diluting the plasma, conditions were created where the only source of the relevant clotting factor was the initial PEG₄₇-PSSS₃₂ plasma. Next, normal human plasma ($20 \mu\text{L}$) containing PEG₄₇-PSSS₃₂ ($50 \mu\text{g mL}^{-1}$) or vehicle and coagulation factor deficient human plasma ($190 \mu\text{L}$) were mixed and incubated at 37°C for 3 min. Under these conditions, the only source of the relevant clotting factor was from the initial $20 \mu\text{L}$ of PEG₄₇-PSSS₃₂ plasma, but all other clotting factors were present in abundance. aPTT was measured for fII, fV, fVIII, fIX, fX, fXI, and fXII according to the manufacturer instructions. Human plasma ($100 \mu\text{L}$) containing PEG₄₇-PSSS₃₂ ($50 \mu\text{g mL}^{-1}$) or vehicle and coagulation factor deficient human plasma ($50 \mu\text{L}$) were mixed and incubated at 37°C for 3 min, and PT was measured for fVII according to the manufacturer instruction.

In Vitro Neutralization of the Lead Copolymer by HBC: The neutralization of PEG₄₇-PSSS₃₂ was analyzed by measuring the prolongation of aPTT in citrated rat and human plasma as described previously.^[39] Pooled rat or human plasma ($200 \mu\text{L}$) was mixed with PEG₄₇-PSSS₃₂ ($10 \mu\text{L}$, $100 \mu\text{g mL}^{-1}$). After 150 s of incubation, HBC ($10 \mu\text{L}$, $100 \mu\text{g mL}^{-1}$) was added. After another 150 s of incubation, the prolongation of aPTT was measured.

Arterial Thrombosis Induced by Electrical Stimulation in Rats: Forty-nine male Wistar rats weighing 191.0 ± 24.0 g were divided into five groups, anesthetized by an intraperitoneal injection of pentobarbital (45 mg kg^{-1}), and placed in a supine position on a heated operation table. PEG₄₇-PSSS₃₂ (1 , 3 , 10 mg kg^{-1} , 1 mL kg^{-1}) or UFH (3 mg kg^{-1} , an equivalent to 300 IU kg^{-1} , 1 mL kg^{-1}) were administered into the right femoral vein. Vehicle (saline, 1 mL kg^{-1}) treated animals served as a control group. Arterial thrombosis was induced by electrical stimulation (1 mA) of the common carotid artery according to the method described previously.^[132] 60 min after stimulation the segment of the common carotid artery with the formed thrombus was clipped at both sides, dissected, opened lengthwise, and the thrombus was completely removed, air-dried at 37°C , and weighted after 24 h. Immediately bleeding time was measured from the moment the tail was surgically cut until the bleeding stopped completely (no rebleeding within 30 s). If bleeding was still present at the end of the 300 s observation period, a value of 300 s was ascribed for the sake of statistical analysis. Bleeding time was expressed in seconds. Next, blood samples were taken from the heart and immediately ACT was measured by Hemochron Junior Signature device (ITC Corporation, USA) in collected blood samples. To obtain sera, blood samples were put into tubes without anticoagulants and allowed to coagulate, then centrifuged at $10\,000 \times g$ at 20°C for 5 min. The sera were collected and deep-frozen (-70°C) until further assays could be performed. The remaining blood samples were drawn into 3.13% trisodium citrate in a volume ratio of 9:1, and blood cell count was assessed with an Animal Blood Counter (ABC Vet, Horiba, Germany). Then, platelet aggregation after collagen addition was assessed in

whole blood by measuring electrical impedance with Chrono-log aggregometer (Chrono-log Corporation, USA) as described previously.^[39] The results were described using the maximal extension, slope of platelet aggregation, lag phase, and area under the curve. The rest of the citrated blood samples were centrifuged at $3500 \times g$ at 4°C for 20 min and 1 mL aliquots of plasma were deep-frozen (-70°C) until further assays could be performed. aPTT, PT, anti-fXa, and anti-fIIa activities were measured as described previously.^[39] Total fII and fIIa concentration was analyzed with an enzyme-linked immunosorbent assay (ELISA) in a 96-well plate using a microplate reader (Dynex Technologies, USA) at 450 nm according to the instructions of the kit's manufacturer (Novus Biologicals, USA).

Laser-Induced Thrombosis in Mice Mesenteric Vein: In the experiments involving laser-induced thrombosis a fixed-stage microscope was used, the Zeiss Axio Examiner Z1 (Carl Zeiss Microscopy GmbH, Germany), a confocal scanner unit (CSU-X1, Yokogawa Electric Corporation, Japan), and water immersion objective W Plan-Apochromat $20 \times /1.0$ (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Twenty-four male BALB/c mice weighting 25.7 ± 2.9 g were anesthetized with ketamine and xylazine (120 mg kg^{-1} , intraperitoneally, Ketamina 10%, Biowet, Poland; 12.5 mg kg^{-1} , intraperitoneally, Xylapan, Biowet, Poland). PEG₄₇-PSSS₃₂ (3 mg kg^{-1} , 4 mL kg^{-1}) or UFH (3 mg kg^{-1} , an equivalent to 300 IU kg^{-1} , 4 mL kg^{-1}) were administered into the left femoral vein 5 min before the endothelial injury. Vehicle (saline, 4 mL kg^{-1}) treated animals served as a control group. Antibody DyLight649 ($0.1 \mu\text{g g}^{-1}$, $100 \mu\text{L g}^{-1}$) (Emfret Analytics, Germany, ref: X-649) was injected into the right femoral vein of mice 5 min before the endothelial injury, to visualize a platelets marker, GPIIb/IIIa, of the murine platelet/megakaryocyte-specific GPIIb-V-IX complex.^[133] Then a midline laparotomy incision was made, and the mesentery of the ileum was pulled out of the abdomen and placed around a plastic mound. The mesentery vein was identified and examined microscopically. The mesentery was continuously perfused with prewarmed (37°C) PBS to prevent the drying up of the vessels. The mesentery vein wall was injured using a 532-nm argon ion ablation laser (Ablate, Intelligent Imaging Innovations, Inc., USA). The induction and progression of thrombosis were recorded for 3 min. One record was automatically divided into 700 time points. At each time point, the area of the thrombus was calculated by SlideBook 6.0 (Intelligent Imaging Innovations, Inc., USA). In this study, the sum of the values of the thrombus area from each time point was referred to as the thrombus area.

Blood samples were taken from the heart at the end of the experiment for aPTT measurement. The citrated blood samples were centrifuged at $3500 \times g$ at 4°C for 20 min and 1 mL aliquots of plasma were deep-frozen (-70°C).

Anticoagulant Activity after s.c. Administration of PEG₄₇-PSSS₃₂ in Mice: Fifteen male BALB/c mice weighting 23.3 ± 1.4 g were divided into 5 groups, and PEG₄₇-PSSS₃₂ (25 mg kg^{-1} , 0.4 mL kg^{-1}) or vehicle (0.4 mL kg^{-1}) were administered s.c. over the right flank of mice. After 1, 4, 8, or 12 h, mice were anesthetized with a mixture of isoflurane and oxygen (3% v/v), and placed in the anesthetic induction chamber, equipped with an individual mask providing a mixture of isoflurane and oxygen (1.5–2% v/v). The blood from the heart was collected in tubes containing trisodium citrate in a volume ratio of 9:1, centrifuged at $3500 \times g$ at 4°C for 20 min, and plasma was deep-frozen (-80°C) until further assays could be performed. The activity of PEG₄₇-PSSS₃₂ was assessed by measuring the prolongation of aPTT.

Statistical Analysis: For each test, the experimental unit was an individual animal. The Shapiro–Wilk test was used for data distribution analysis. The Gaussian data were presented as mean with SD using the parametric Student t-test. The data was analyzed and graphically presented with GraphPad Prism 9 (GraphPad Software, USA). P-values less than 0.05 were considered significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This research was funded by the Medical University of Białystok, grant number B.SUB.24.331.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

anionic block copolymers, heparin mimetics, novel anticoagulants, sulfonate groups, thrombosis

Received: June 14, 2024
Revised: September 20, 2024
Published online:

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