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In Vitro And In Vivo Characterization Of A Reversible Synthetic Heparin Analogue

Matthew F. Whelihan[1,4], **Brian Cooley**[2,4], **Yongmei Xu**[3], **Rafal Pawlinski**[1,4], **Jian Liu**[3], and **Nigel S. Key**[1,2,4]

[1]Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC

[2]Department of Pathology and Laboratory Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC

[3]Division of Chemical Biology and Medicinal Chemistry, The University of North Carolina at Chapel Hill, Chapel Hill, NC

[4]McAllister Heart Institute, The University of North Carolina at Chapel Hill, Chapel Hill, NC

Abstract

Background—The global supply of unfractionated heparin (UFH) and all commercially available low molecular weight heparins (LMWH) remain dependent on animal sources, such as porcine intestine or bovine lung. Recent experience has shown that contamination of the supply chain (with over-sulfated chondroitin sulfates) can result in lethal toxicity. Fondaparinux is currently the only commercially available synthetic analogue of heparin. We recently described a new class of chemoenzymatically synthesized heparin analogues. One of these compounds (S12-mer) is a dodecasaccharide consisting of an antithrombin-binding moiety with repeating units of IdoA2S-GlcNS6S and two 3-O-sulfate groups that confer the ability to bind protamine.

Objective/Methods—We sought to further characterize this new compound *in vitro* using biochemical and global coagulation assays and *in vivo* using thrombosis and hemostasis assays.

Results—The anticoagulant activities of the Super 12-mer (S12-mer) and Enoxaparin in anti-factor Xa and plasma-based thrombin generation assays were roughly equivalent with a 50% reduction in peak thrombin generation occurring at approximately 325 nM. When protamine was titrated against a fixed concentration of S12-mer in plasma or blood, the S12-mer displayed a

Address correspondence to: Dr. Nigel S. Key, 1079 Genetic Medicine Building, CB #7035, 120 Mason Farm Rd., Chapel Hill, NC 27599, Nigel_key@med.unc.edu, 919-966-3311.

Authorship Contributions

MFW aided in experimental design, performed all *in vitro* experiments and wrote the manuscript. BC participated in experimental design, performed all *in vivo* experiments and helped write the manuscript. YX performed all chemical synthesis of S12-mer compounds. JL, RP and NSK aided in experimental design and helped write the manuscript.

Conflicts of Interest Disclosure

MFW, NSK, BC and RP have no conflicts of interest to declare.

significant restitution of thrombin generation and clot formation. *In vivo*, S12-mer inhibited venous thrombosis to a similar extent as Enoxaparin, with similar bleeding profiles.

Conclusions—These data show that the S12-mer has almost identical efficacy to Enoxaparin in terms of FXa inhibition, while displaying significant reversibility with protamine. Taken together with the ability to ensure purity and homogeneity from batch to batch, the S12-mer is a promising new synthetic heparin analogue with a potentially enhanced safety profile.

Introduction

Heparin-based anticoagulants are considered to be standard therapy for the prevention and treatment of arterial and venous thromboembolism (VTE) [1]. Natural heparin is physiologically synthesized in several tissues by a series of enzymes that link together and modify the basic disaccharide backbone of iduronic or glucuronic acid and glucosamine residues, resulting in a mixture of molecules with an average molecular weight of 14,000 Da [2, 3]. Unfractionated heparin (UFH) is administered as a mixture of sulfated glycosaminoglycans of variable lengths and molecular weight and is a natural product obtained from a relatively crude preparation of bovine and/or porcine mucosal tissue [4–6]. UFH has a propensity to bind to plasma proteins, platelets, macrophages and endothelial cells, and as a result, its bioavailability, pharmacological properties and anticoagulant effects may be unpredictable [7–9]. UFH exerts its anticoagulant effect by functioning as a potent cofactor in the inactivation of several coagulation enzymes, including factors IIa (thrombin), VIIa, IXa, Xa and XIa by antithrombin (AT) [9–15]. Unfortunately, UFH is susceptible to problems with the supply chain; consequently, a string of high profile incidents has resulted in serious concerns about its quality control practices and safety profile [16–19].

In the last few decades, UFH has been largely supplanted by low molecular weight heparins (LMWHs) in many clinical scenarios [20, 21]. LMWHs, which are derived from depolymerized heparin, have several advantages over UFH. They bind less avidly to plasma proteins, endothelial cells, macrophages, and platelets, and therefore possess a more predictable bioavailability profile [22, 23]. Currently, LMWHs are the most widely prescribed heparin in many countries and remain the standard of care for VTE prevention and treatment [20]. LMWHs, however, are still a fairly heterogeneous mixture with molecular weights that range from 3,500 to 6,000 Da [21, 23]. Due to their reduced chain length, LMWHs are poorly and variably reversed with protamine, restricting their clinical utility to scenarios with acceptably low bleeding risk [24, 25].

More recently, the synthetic heparin analogue Fondaparinux has found clinical utility [26, 27]. Fondaparinux is a construct of the naturally occurring sulfated pentasaccharide core sequence found in all anticoagulant heparins, and is responsible for their binding to and potentiation of AT [28, 29]. Reduced sulfation of molecular species in LMWH preparations or the shorter polysaccharide chain size has been identified as the cause of the relative lack of protamine reversal [30]. Whereas LMWHs inactivate both Factor Xa -- and to a lesser extent thrombin -- when complexed with AT, Fondaparinux is a short fragment with specificity for FXa only [29, 31]. However, despite its high degree of sulfation, the

relatively short chain length precludes Fondaparinux from any capacity for reversal by protamine [32].

In this study, we undertook a complete evaluation of the *in vitro* anticoagulant and *in vivo* antithrombotic profiles of a chemoenzymatically synthesized heparin analogue. This homogenous molecule, deemed “Super 12-mer” (S12-mer), has been engineered with the key 3-O-sulfation at sites needed to preserve its (indirect) anti-factor Xa activity and a longer 12-sugar chain length to conserve its interaction with protamine for effective reversal (Figure 1) [33]. These experiments establish the feasibility of future studies aimed at advancing this compound to further pre-clinical evaluation.

Materials and Methods

Materials

HEPES, polyethylene glycol MW 8,000 and calcium chloride were purchased from Fisher (Waltham, MA). 1-palmitoyl-2-oleoyl-phosphatidyl serine (PS), and 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Recombinant TF 1–263 was purchased from Haematologic Technologies Inc. (Essex Junction, VT) and was re-lipidated in PCPS (25% PS, 75% PC) vesicles as previously described [34, 35]. Corn trypsin inhibitor (CTI) was prepared as previously described [36] and qualified via aPTT assay with a commercial standard. Plasma derived FXa and AT were purchased from Haematologic Technologies Inc. (Essex Junction, VT). UFH was obtained from Hospira Inc. (Lake Forest, IL). Fondaparinux sodium (Arixtra™) was purchased from Cardinal Heath (Dublin, OH). Enoxaparin (Lovenox™) was from Sanofi-Aventis (Bridgewater, NJ). Protamine sulfate was obtained from APP Pharmaceuticals (Schaumburg, IL). The synthetic dodecasaccharide (S12-mer) was synthesized from a glucuronide-based monosaccharide, with a series of elongation, epimerization, and O-sulfation steps catalyzed with recombinant enzymes, as previously described [33]; a dodecasaccharide (C12-mer) was synthesized in parallel without 3-O-sulfation to generate a non-anticoagulant control. The purity and structures of both S12-mer and C12-mer were confirmed by nuclear magnetic resonance (NMR) and mass spectrometry as described previously [33]. Rhodamine 6G was from Sigma/Aldrich. A monoclonal fibrin-specific antibody was isolated from ascites derived from a clone kindly provided by Dr. Marschall S. Runge; this antibody was labeled with Alexa Fluor-647 (Invitrogen).

Methods

Blood collection

Healthy individuals were recruited by advertisement under a protocol that was approved by the Institutional Review Board for human subjects at the University of North Carolina. After discarding the first 3 mL, blood samples were obtained by clean venipuncture of an antecubital vein with a 21Ga. butterfly needle into syringes preloaded with 3.2% sodium citrate (1:9). Contact pathway-inhibited citrate plasma was prepared by drawing blood into 3.2% sodium citrate containing 0.1mg/mL CTI. Platelet poor plasma was prepared via 2 successive centrifugations at 2,500g.

Anti-FXa assay

Various heparins (UFH, Fondaparinux, Enoxaparin, S12-mer or C12-mer) were titrated with 200 nM human antithrombin (final) in hepes-buffered saline with 2 mM $CaCl_2$ and 0.1% PEG 8000 (HBS/PEG/Ca^{2+}) in the presence or absence of 2μM Protamine sulfate and allowed to incubate for 2 minutes at room temperature in a 96-well microplate. 5 nM human FXa was added to the wells and allowed to incubate for 1 minute. The reaction was initiated by the addition of 200 μM (final) FXa chromogenic substrate (Pefachrome FXa) and amidolytic activity at 405 nm was measured in a Molecular Devices Spectromax microplate reader.

TEG

Thromboelastography was performed in a TEG 5000 coagulation analyzer (Haemonetics, Niles, IL). Whole blood was drawn via antecubital venipuncture and anticoagulated with 0.1 mg/mL of corn trypsin inhibitor; 350μL of this sample was immediately added to TEG cups containing 5 pM recombinant TF 1–263 and varying amounts of heparin and/or protamine sulfate.

Thrombin generation assays

Plasma-based thrombin generation assays were performed essentially as described [37] with some modifications. Heparins and/or protamine were added to selected wells of a 96-well microplate followed by 80μL of pooled (10 donors) citrate plasma. Plasmas were re-calcified (15 mM $CaCl_2$) for 3 minutes in the presence of 416 μM Z-GGR-AMC substrate prior to activation with a 5 pM rTF / 4 μM PCPEPS stimulus. Control plasmas that were re-calcified for the 3-minute incubation period and received phospholipids without tissue factor, generated no thrombin over the 1-hour course of the assay. Substrate hydrolysis was monitored in a Biotek Synergy H1M fluorometer, and thrombin generation was calculated based on a thrombin standard curve.

In Vivo Study Design

All studies were performed in mice under an IACUC-approved protocol at the University of North Carolina, following PHS guidelines for laboratory animal care and use. Adult male wild-type C57Bl/6 mice, ages 8–12 weeks were used in all studies, under ketamine and xylazine anesthesia (intraperitoneal administration, 100 and 10 mg/kg body weight, respectively). Four experimental groups were used for both thrombosis and hemostasis assays: controls (vehicle infused), S12-mer (0.6 mg/kg), S12-mer + protamine (0.6 mg/kg and 15 mg/kg, respectively) and Enoxaparin (1 mg/kg). These compounds were infused by jugular vein immediately preceding both *in vivo* assays; S12-mer preceded protamine administration by 5 minutes, using separate injections.

Thrombosis Assay

A model of venous thrombosis was used, as previously described [38]. Briefly, rhodamine 6G (platelets) and Alexa Fluor-647-labeled anti-fibrin antibody were injected through the external jugular vein. The femoral vein was surgically exposed and an electrolytic injury (30 seconds, 1.5 volts) was applied to a 75-micron diameter area on the vessel surface. The

vessel injury site was shutter-illuminated with 532-nm and 650-nm defocused lasers, with fluorescence emission capture via time-lapse digital video over 60 minutes. Relative intensity of each fluorophore was quantitated, normalizing for animal body weight and amount of injected fluorophore.

Hemostasis Assay

A saphenous vein bleeding assay was applied, slightly modified from that previously described [39]. The saphenous vein was exposed and transected: upon cessation of bleeding and a 30-second observation, the hemostatic clot was physically dislodged and the site was observed for repeat hemostasis, repeating this process over the course of 30 minutes. The total number of hemostatic events over 30 minutes was recorded.

Statistical Analysis

Heparin IC_{50} concentrations were calculated using Graph Pad Prizm software. Analysis of variance was applied to relative intensities of both platelet and anti-fibrin accumulation at thrombosis sites at the 60-minute time point and on the number of hemostatic events for the bleeding assay. Posthoc Fisher LSD tests were done for between-group comparisons. A p-value of 0.05 was used to assign significance for initial analysis of variance and for the posthoc tests.

Results

Heparin anti-FXa efficacy

Five heparins including Fondaparinux, UFH, Enoxaparin, S12-mer and Control C12-mer, were analyzed in this study. As UFH and Enoxaparin are inherently heterogeneous mixtures, we used the average molecular weights (14,500 and 4,500 Da for UFH and Enoxaparin, respectively) associated with the functional sulfated polymer forms to calculate approximate molarity. Both S12-mer and C12-mer are structurally homogeneous dodecasaccharides synthesized through a chemoenzymatic approach that have definite molecular weights (3,483 and 3,387 Da for Super and Control 12-mer respectively); thus, molarity was precisely quantified. Anti-FXa activity was evaluated (Figure 2) to compare the activity between the four different heparins and IC50 values were calculated (Fondaparinux 7.2±0.5 nM, UFH 39.8±4.8 nM, Enoxaparin 147±19.8 nM and S12-mer 99.7±12.9 nM, Control 12-mer ~4 uM).

When protamine was added to the reaction, all three sulfated heparins (UFH, Enoxaparin and S12-mer) showed significant reversibility of their respective anti-FXa activities, and shifted their IC50 values approximately one order of magnitude higher. As expected, protamine had little effect on the anti-FXa capabilities of fondaparinux or the control 12-mer (data not shown).

S12-mer's effects on thrombin generation in human plasma

TF-initiated thrombin generation in contact pathway-inhibited platelet poor plasma was assessed in the presence of S12-mer and Enoxaparin (Figure 3A and B respectively). Given the relatively similar specific anti-FXa activity, plasma half-life and potential reversal with

protamine, enoxaparin was chosen as a standard for S12-mer comparison [33]. The displayed concentration range (150–600 nM) was chosen as it displayed the best dose response in terms of thrombin generation inhibition for both S12-mer and Enoxaparin. The relevant thrombin generation parameters are summarized in Table 1. Compared to Enoxaparin, S12-mer shows a similar dose response in all thrombin generation parameters (lag, peak thrombin and time to peak thrombin). In contrast to the anti-FXa activity in a purified system, Enoxaparin shows slightly higher efficacy in plasma, which is not unexpected as Enoxaparin retains some anti-thrombin as well as anti-FXa activity. However, consistent with the anti-Xa data, both heparins reduced peak thrombin generation by half at approximately 150 nM, with nearly identical areas under the curve.

The ability of protamine sulfate to reverse the anticoagulant effect of S12-mer was evaluated at two different concentrations (Figure 4). Protamine sulfate is a mix of polypeptides of heterogeneous molecular weights. As with UFH and Enoxaparin, we used the average molecular weight of the functional peptide (5,000 Da) for a molar conversion. Analysis of thrombin generation in the presence of protamine proved to be problematic as protamine itself is an inhibitor of coagulation [40]. Specifically, titration of protamine in this plasma system (Figure 4A) resulted in a dose dependent increase in lag and time to peak thrombin as well as a decrease in peak thrombin. At both S12-mer concentrations tested (Fig 4B: 337 nM and Fig. 4C: 168 nM), protamine reversibility was only achieved at near equal molar concentration of S12-mer and protamine. Interestingly, while peak thrombin and the area under the curve were reconstituted, both the lag time and time to peak thrombin remained unchanged from those values seen with either concentration of S12-mer alone.

S12-mer effects on ex vivo clot formation in whole blood

Thromboelastography was used to examine the effects of S12-mer on clot formation in the whole blood of three healthy volunteers (Figure 5A and Table 2). As with the plasma-based thrombin generation assays, a 5 pM TF stimulus (in the presence of 0.1 mg/mL of CTI to inhibit contact activation) was used to initiate the reaction. When S12-mer was titrated in whole blood (Figure 5A), a dose-dependent increase in R (clot time) was observed. Consistent with an increase in R, the α-angle (rate of clot formation) and maximum amplitude (MA, maximal clot firmness) of the reaction both decreased in a dose-dependent manner that plateaued between 300 and 600 nM.

The ability of protamine to reverse the effects of S12-mer on clot formation was also investigated (Figure 5B and Table 3). Maximal protamine reversibility was achieved at an equal molar quantity of heparin and protamine with an approximate 87% reconstitution in R-time; however, the α-angle and MA were not proportionately reconstituted. In terms of clot formation, the observation that the S12-mer is not completely reversible with protamine, likely is due to protamine's inherent anticoagulant effects on cofactor activation demonstrated in the figure 4A. Similar to what was displayed in plasma, protamine alone had significant dose-dependent anticoagulant effects on all TEG parameters (data not shown).

In vivo thrombosis assay

The intravital fluorescence assay generally shows a peak in platelet accumulation at 20–30 minutes, with a subsequent reduction by 60 minutes, whereas the fibrin accumulation achieves a sustained peak at slightly later times. Platelet accumulation was relatively similar among groups in our study, with some observable reduction in the group that received Enoxaparin and slight variations among the other groups (Figure 6A), although this did not achieve statistical significance in any case. In contrast, analysis of variance indicated significance among groups for fibrin accumulation ($p<0.001$) as detected by the anti-fibrin antibody. There was a reduction in fibrin accumulation at 60 minutes (Figure 6B) for S12-mer and Enoxaparin in comparison to the control group (vehicle), achieving significance for all between-group comparisons ($p<0.001$). Of note, protamine administration following S12-mer treatment returned fibrin formation to control levels ($p<0.001$ vs. S12-mer without protamine). Time-lapse videos for representative thrombus development are shown for vehicle, S12-mer, S-12-mer plus protamine, and Enoxaparin treatments (Supplemental Videos 1–4), showing fibrin and platelet accumulation over 60 minutes.

In vivo hemostasis Assay

The bleeding assay (Figure 7) also revealed statistical significance through analysis of variance ($p<0.05$). There were comparable numbers of hemostatic events over 30 minutes for mice treated with vehicle, (18 ± 2; mean and standard deviation); a greater number of hemostatic events equates with better hemostatic activity. The S12-mer and Enoxaparin both produced significantly fewer hemostatic events in the same time period (10 ± 2 and 10 ± 2 respectively; $p < 0.05$ vs. the control group in posthoc tests), indicative of a bleeding risk. Protamine effectively restored hemostatic activity in S12-mer-treated animals (17 ± 2 hemostatic events; $p < 0.05$ vs. S12-mer without protamine).

Discussion

Pharmaceutical grade heparins are mostly isolated in bulk from the mucosa of porcine intestines, a process that is frequently not performed under Good Manufacturing Practice (GMP) guidelines. Recently, the presence of an over-sulfated chondroitin sulfate impurity in unfractionated heparin was the direct cause of at least 90 deaths in the recipients of the contaminated UFH [16, 18, 41]. Indeed, this number could have been a gross underestimate of the true number of fatalities, given the known infrequency with which drug adverse events are reported to Regulatory agencies. This 18kD impurity has also been documented to be present in LMWHs [42]. Other concerns have been raised with naturally occurring heparins, such as the theoretical risk of contamination by the BSE agent in heparins of bovine origin. However, since no documented cases of variant Creutzfeld-Jakob disease transmitted by heparin have been documented, and in response to the need to diversify the source of naturally occurring heparins, the re-introduction of bovine heparins has recently been proposed [43]. These problems have therefore accentuated the need to develop synthetic heparins for human usage. At present, the only completely synthetic heparin analogue available is Fondaparinux sodium (Arixtra™). Despite its advantages, Fondaparinux also has certain drawbacks; its short chain polysaccharide structure lacks some of the other ‘off target’ pharmacologic benefits of longer chain heparins, and its long

half-life and strong dependency on renal excretion, as well as the lack of a specific anticoagulation reversal agent have limited its use in certain clinical situations.

In this study, we characterized the anticoagulant and antithrombotic profile of a homogenous synthetic heparin analogue 'S12-mer', the structure of which we have previously described [33]. Compared to Enoxaparin, S12-mer displayed superior anti-FXa activity in a purified system with a similar anticoagulant activity in plasma. Differences in activity in global coagulation assays are most likely due to the fact that LMWHs are a heterogeneous mixture that maintain some anti-thrombin as well as anti-FXa activity. Nonetheless, *in vivo*, S12-mer effectively inhibited venous thrombosis to a similar extent as Enoxaparin, with a similar bleeding profile.

This compound was designed in part to have a sufficient length that would promote high protamine binding; previous work by our group showed that a minimum of 12 saccharide residues are needed for effective reversal with protamine [33]. It is somewhat paradoxical that heparin's antidote protamine is itself an anticoagulant. Specifically, protamine appears to exert its anticoagulant affect by inhibiting the activation of Factor V [40]. Thus, accurate dosing of protamine in an exact molar ratio to the specific heparin is essential, as excess protamine could lead to further anticoagulation. These data suggest that dosage of an equal molar ratio (1:1) of protamine to S12-mer will effectively reverse its anticoagulant effects. The reversibility of the S12-mer was demonstrated *in vivo* both in thrombosis and hemostasis assays; in a venous thrombosis assay, reduction in fibrin formation was seen with S12-mer administration, which was restored to near control levels by protamine administration (Figure 5B). Similarly, in a bleeding assay, the hemostatic response was restored with protamine administration following S12-mer treatment (Figure 6). There was a slight beneficial effect of S12-mer plus protamine on reducing platelet accumulation compared with S12-mer alone; though not statistically significant, this may indicate a profound effect of protamine on platelet accumulation in a venous system.

The S12-mer described herein offers the advantages of Fondaparinux, a single molecule compound with anticoagulation efficacy, but with protamine reversibility. Another potential advantage of the S12-mer lies in its short length compared to UFH species, for which longer lengths are considered to cause heparin-induced thrombocytopenia (HIT), a serious complication of heparin therapy [44]. Like Fondaparinux, for which the short length is reasoned to preclude formation of large molecular complexes with platelet factor-4, subsequently attracting antibodies that stimulate platelet activation and clearance [44, 45], the S12-mer should have a minimal risk for inducing HIT. However, this potential advantage requires further investigation.

Annually in the United States, it is estimated that 300,000–900,000 people are affected by VTE [46, 47]. Almost half of all events occur within 24 hours of a hospital stay or surgery, and prophylaxis with LMWHs or Fondaparinux has become the standard of care [21, 48]. Despite the advent of the direct oral anticoagulants that target factor Xa or thrombin, the need for injectable rapid acting, shorter half–life reversible heparins with a reliable and reproducible manufacturing source is likely to persist.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights for Review

- This paper characterizes the *in vitro* and *in vivo* anticoagulant properties of a novel, synthetic homogenous heparin, referred to here as '12-mer'
- *In vitro*, the molecule has similar anti-Xa activity as the comparator LMWH, Enoxaparin.
- *In vivo*, S12-mer inhibited venous thrombosis to a similar extent as Enoxaparin, with similar bleeding profiles
- The 12-mer displays significant reversibility with protamine *in vitro* and *in vivo*.

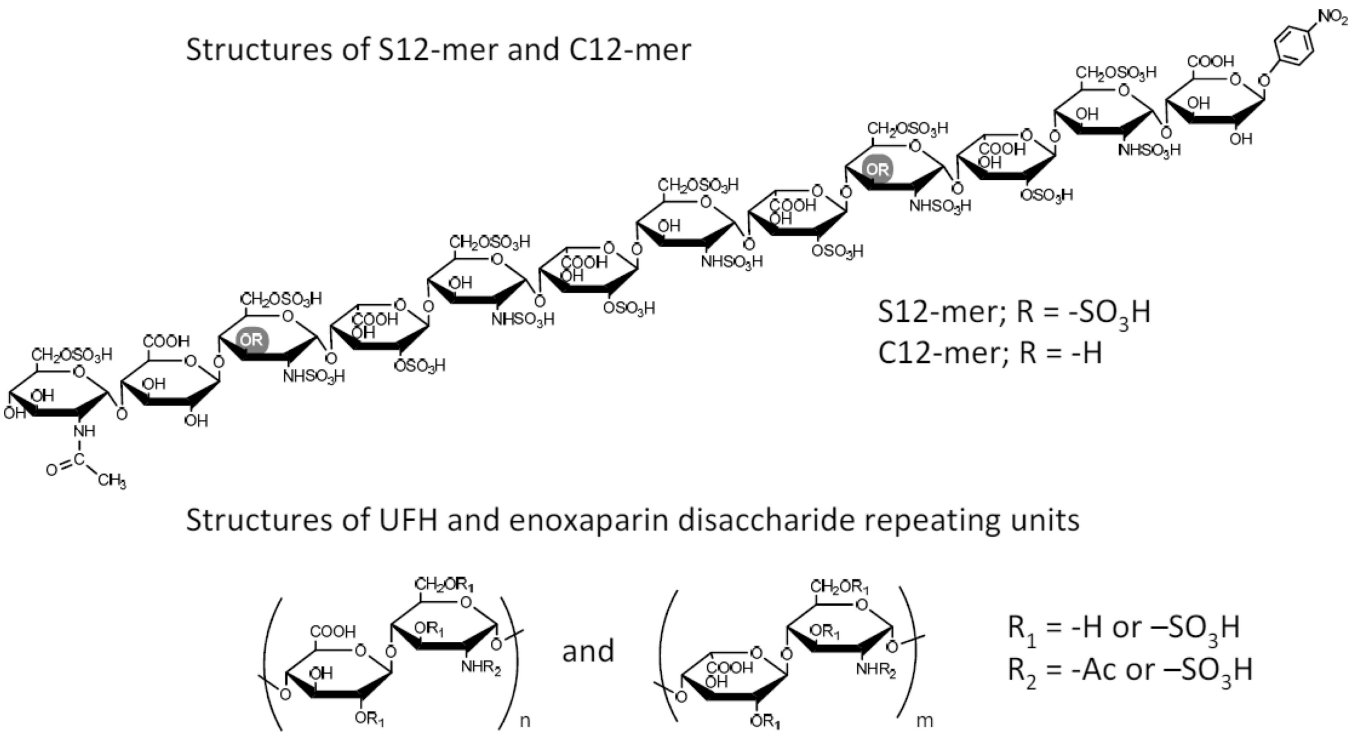


Figure 1. Chemical structures of synthetic 12-mers, enoxaparin and unfractionated heparin

Panel A shows the structures of Super (S)12-mer and control (C)12-mer. Panel B shows the representative structures of disaccharide repeating unit of enoxaparin (N+M ≈ 9) and unfractionated heparin (M+N ≈ 32).

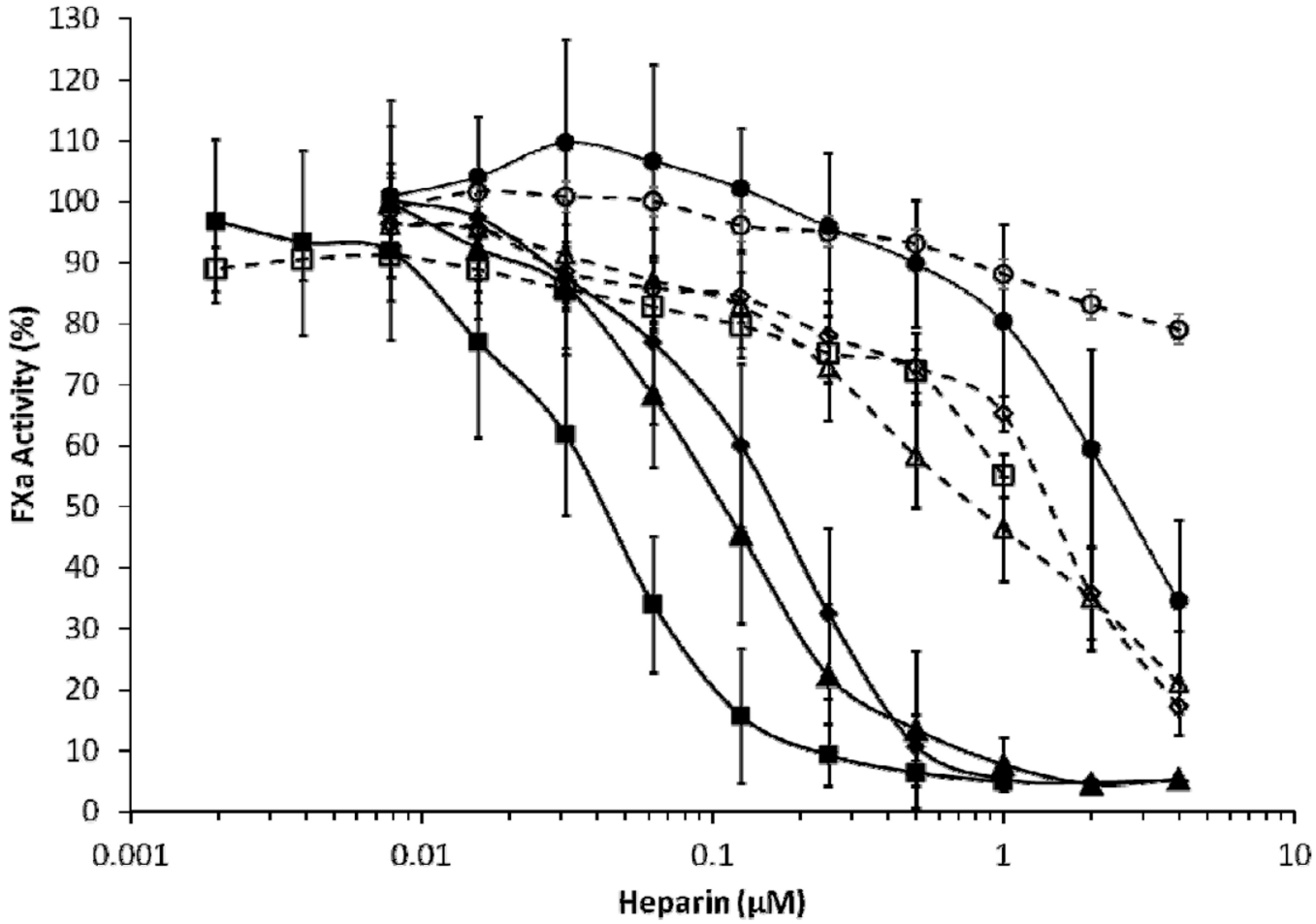
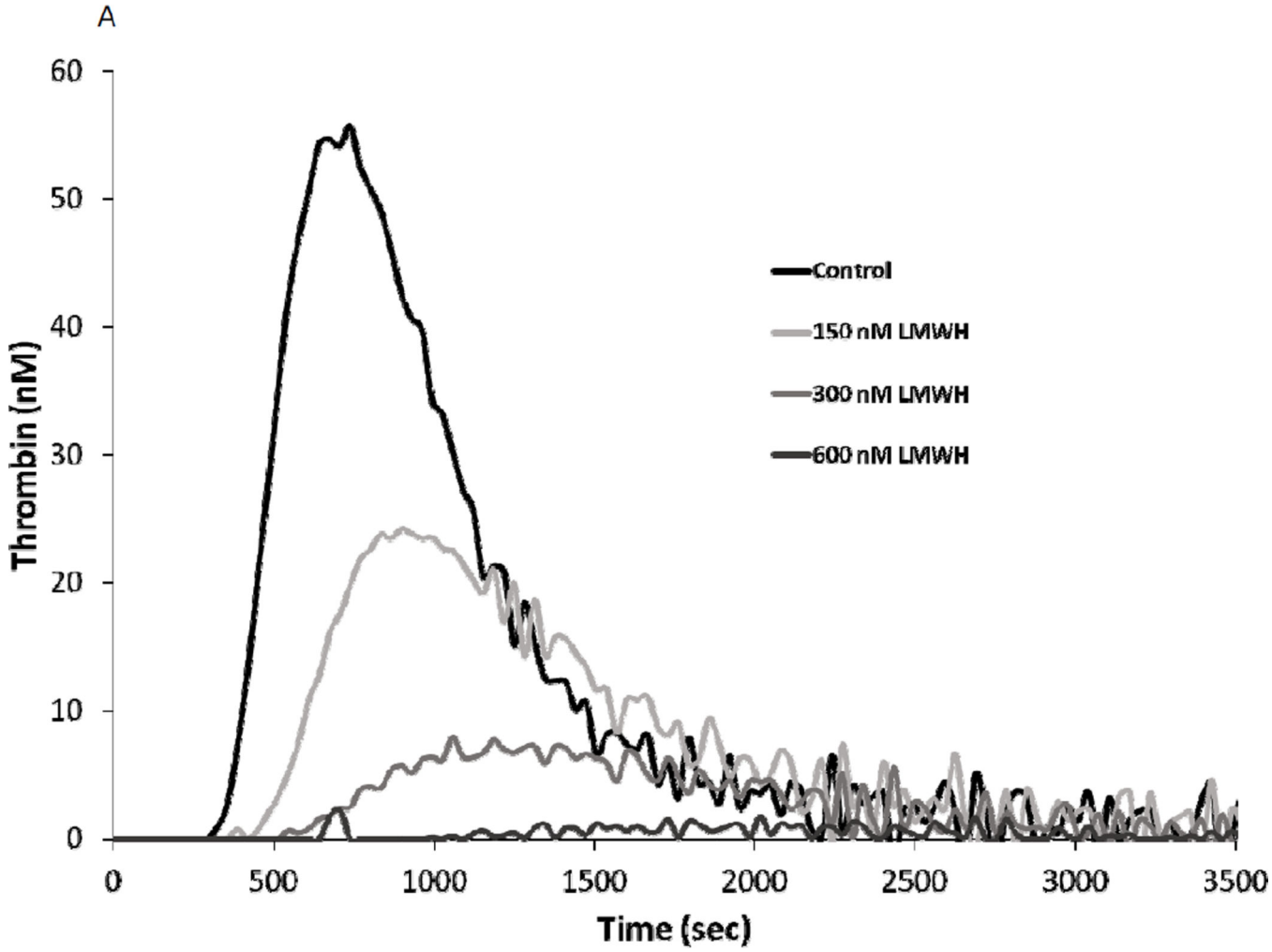


Figure 2. Anti-factor Xa activity of various heparins

Human antithrombin (200 nM) was incubated with varying amounts of Fondaparinux (*), UFH (■), Enoxaparin (◆), S12-mer (▲) or control 12-mer (●) with (open symbols) or without (closed symbols) 2 μM protamine sulfate in HBS/PEG/Ca^{2+} buffer for 5 minutes at room temperature followed by the addition human FXa (5 nM). The reaction was initiated by the addition of FXa substrate (200 μM). FXa activity was monitored as a function of amidolytic cleavage of the FXa substrate and plotted as % FXa activity *vs*. control. Data are shown as the mean ± SD (N=3).



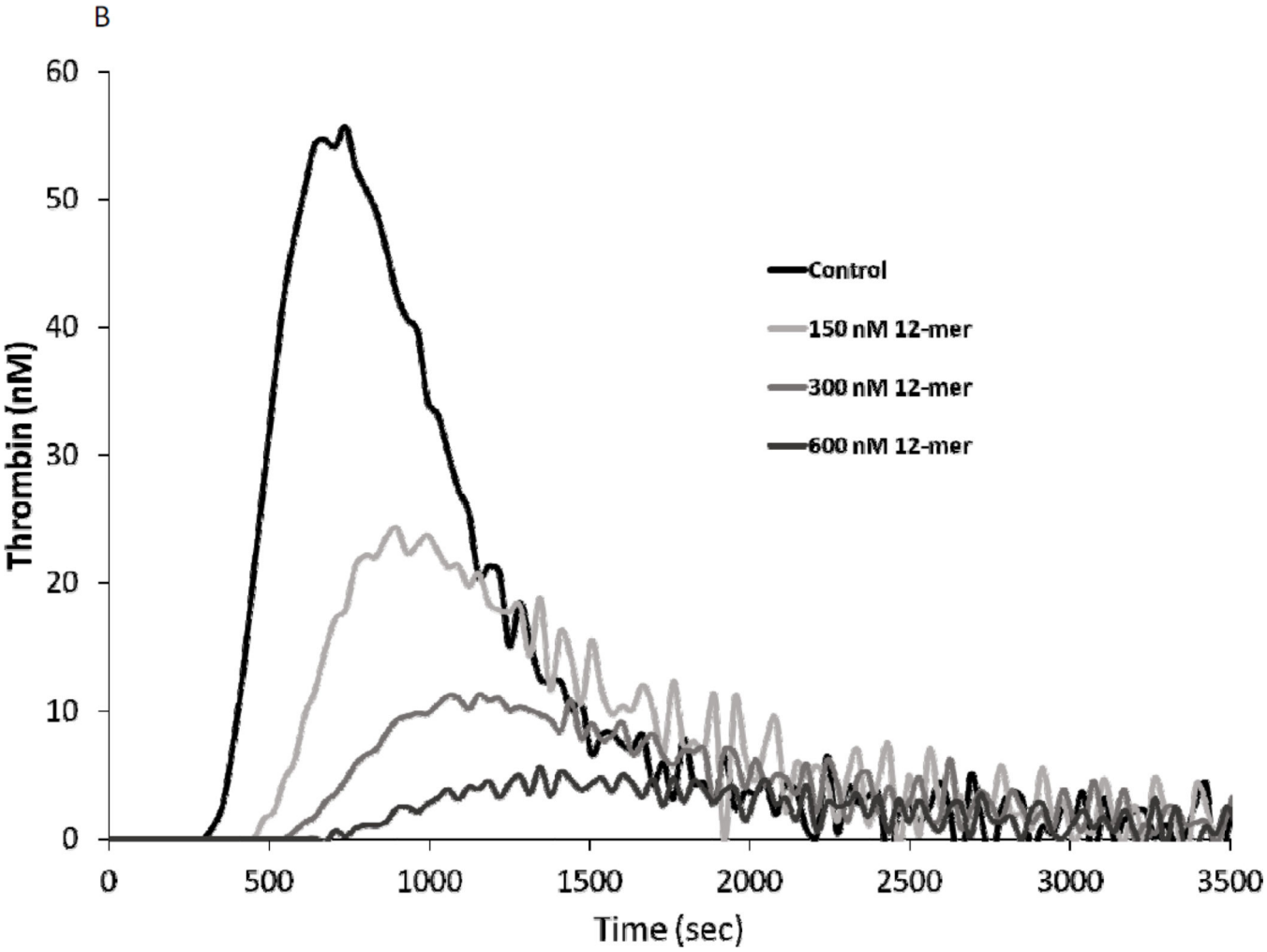
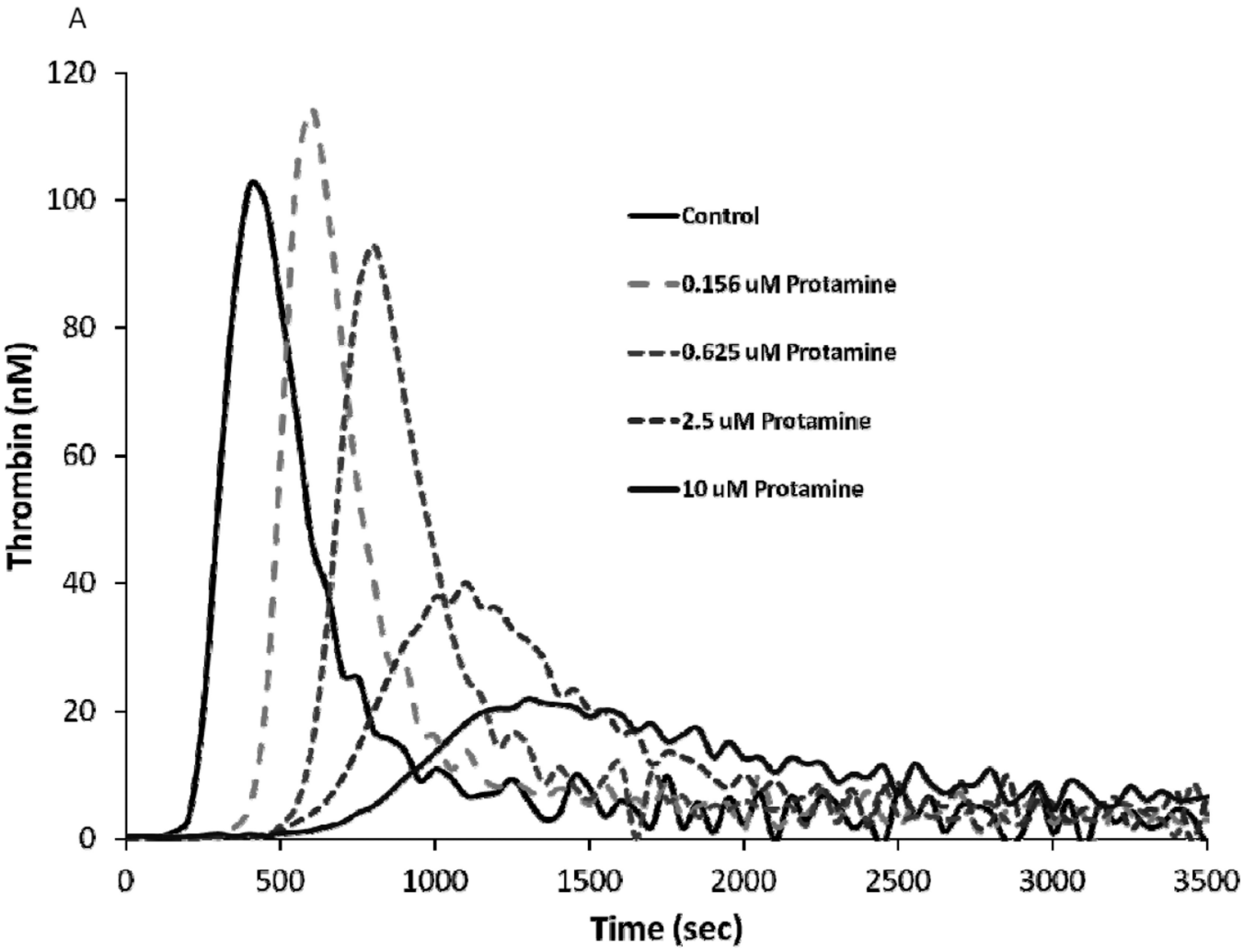
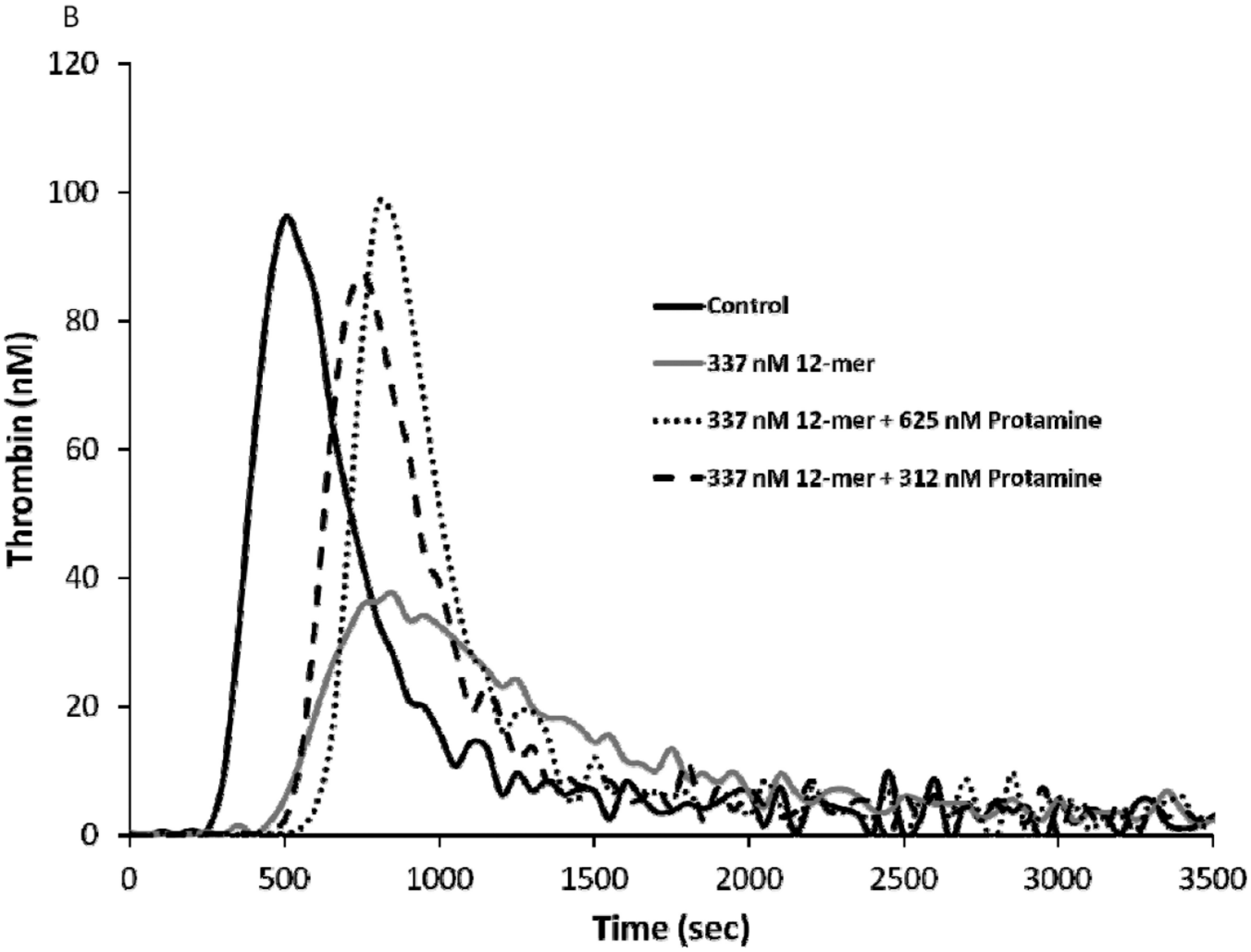


Figure 3. Effects of S12-mer and LMWH (Enoxaparin) on thrombin generation in platelet poor plasma

Varying amounts of S12-mer (**A**) or LMWH (**B**) were added to contact pathway-inhibited and citrated platelet poor plasma. Thrombin generation was initiated with TF (5 pM) and phospholipid (4 μM) and monitored by cleavage of the fluorogenic substrate Z-GGR-AMC (416 μM). Data are shown as the mean of three independent measurements.





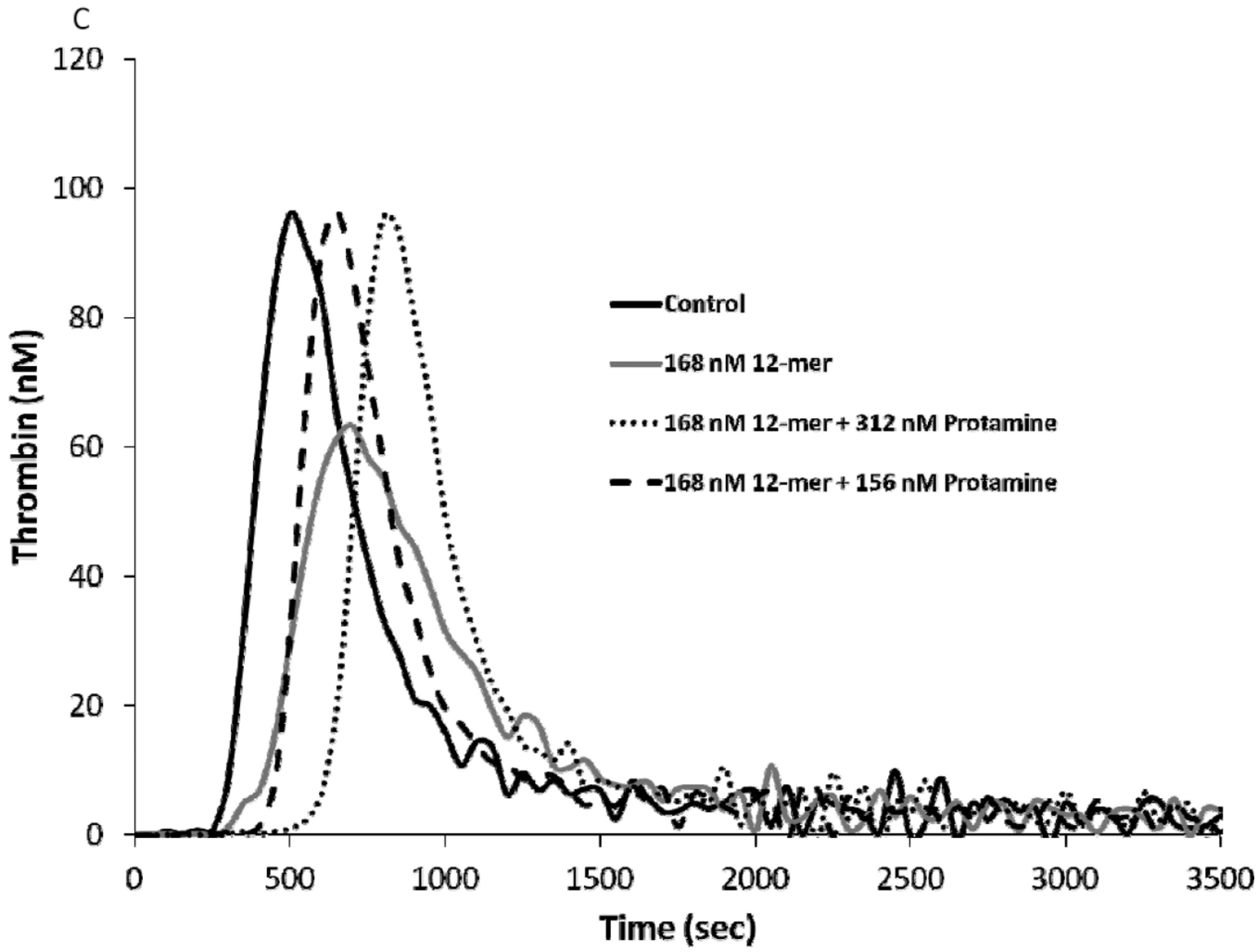
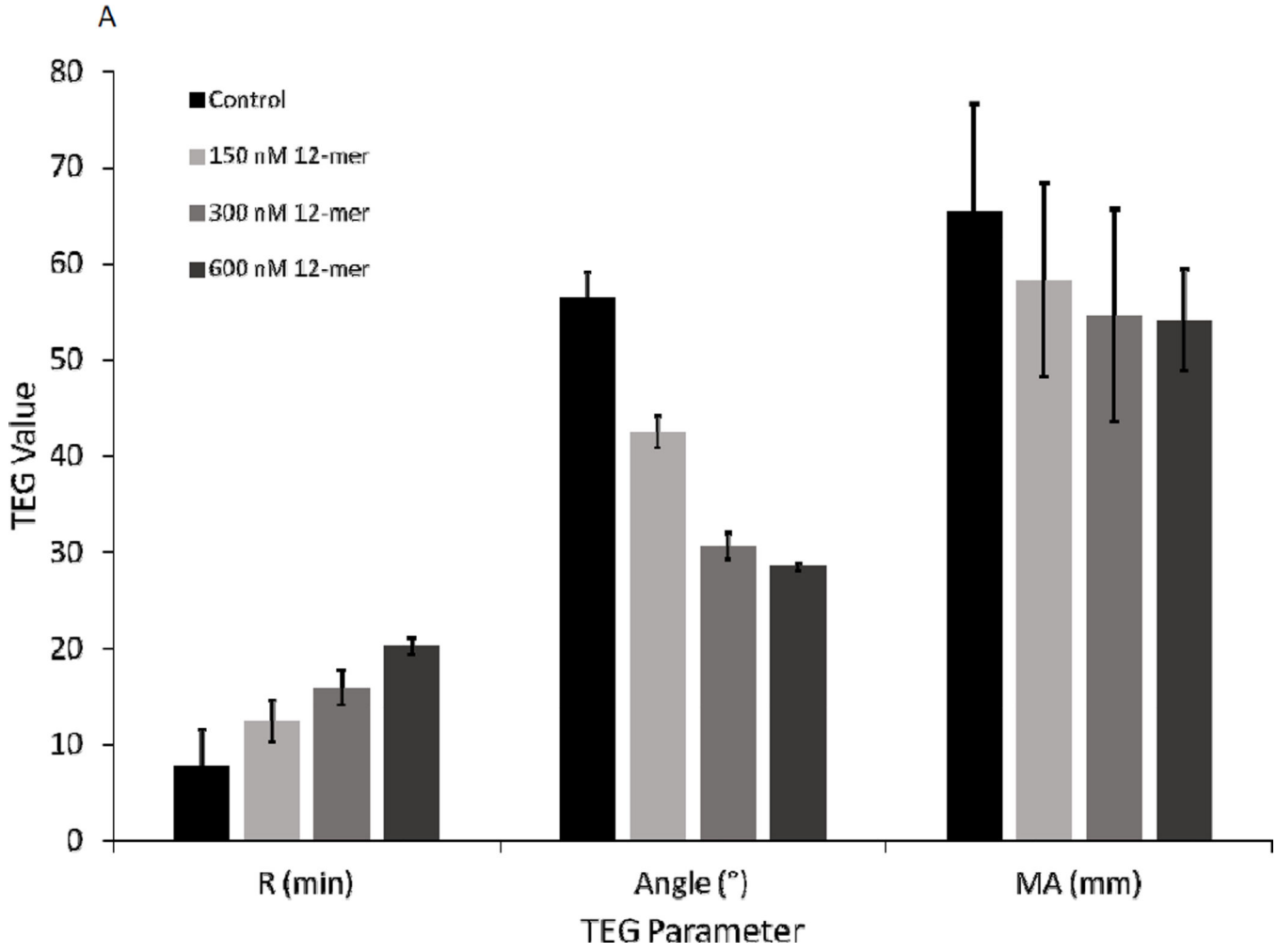


Figure 4. Protamine reversibility of S12-mer in platelet poor plasma

Platelet poor plasma containing protamine alone (**A**) or two concentrations of 12-mer (**B**) 337 nM, **C**) 156 nM that were incubated with an equimolar or 2-fold molar excess of protamine. Thrombin generation was initiated with TF (5 pM) and phospholipid (4 μM) and monitored via cleavage of the fluorogenic substrate Z-GGR-AMC (416 μM). Data are shown as the mean of three independent measurements.



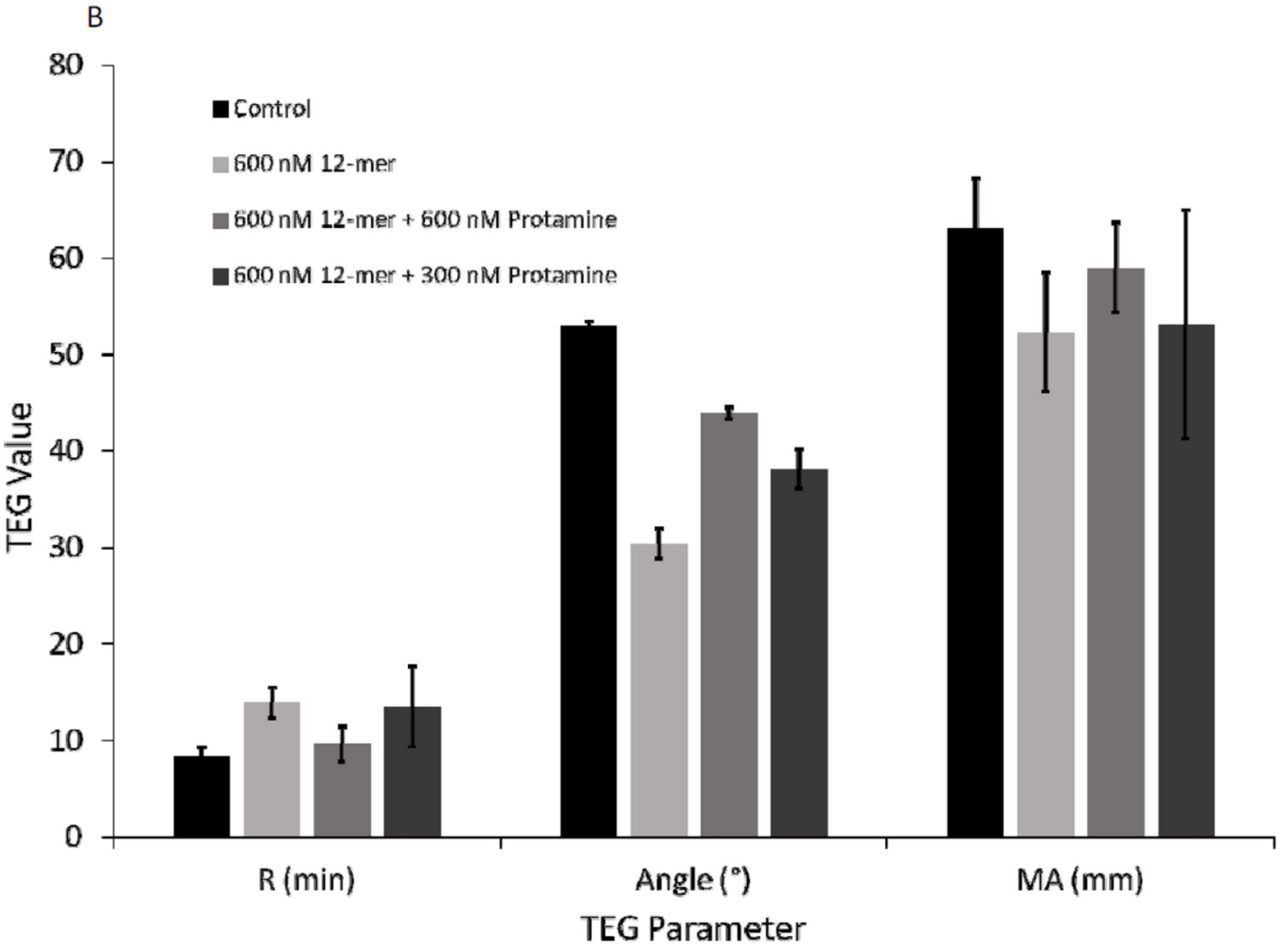
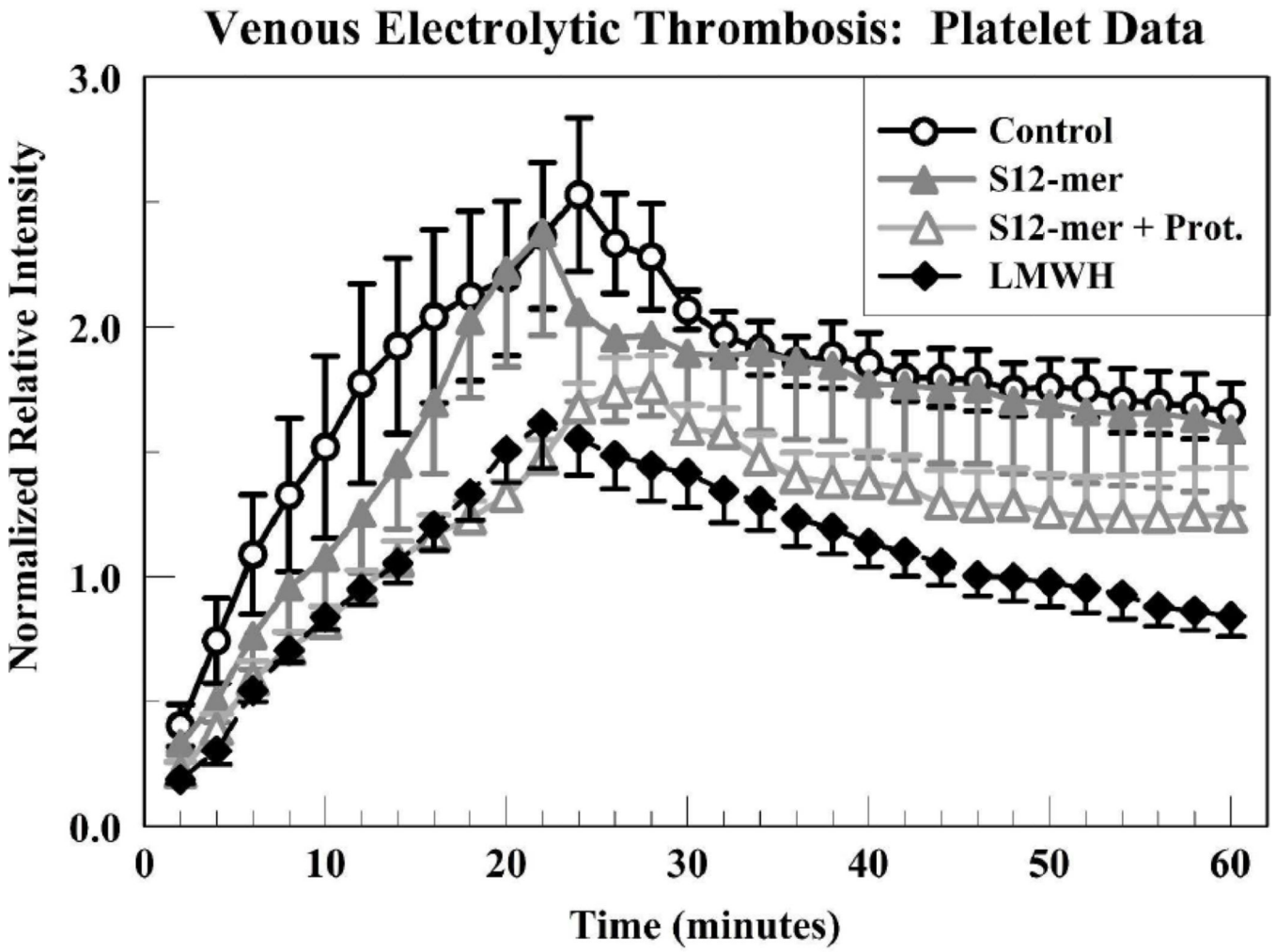


Figure 5. S12-mer effects on clot formation

S12-mer was titrated in the absence **(A)** or presence of an equal molar or 2-fold molar excess protamine (**B**) in contact pathway inhibited whole blood and subjected to thromboelastographic analysis after a 5pM TF stimulus. Data are presented as the mean ± SD (N=3 individuals).

A



B

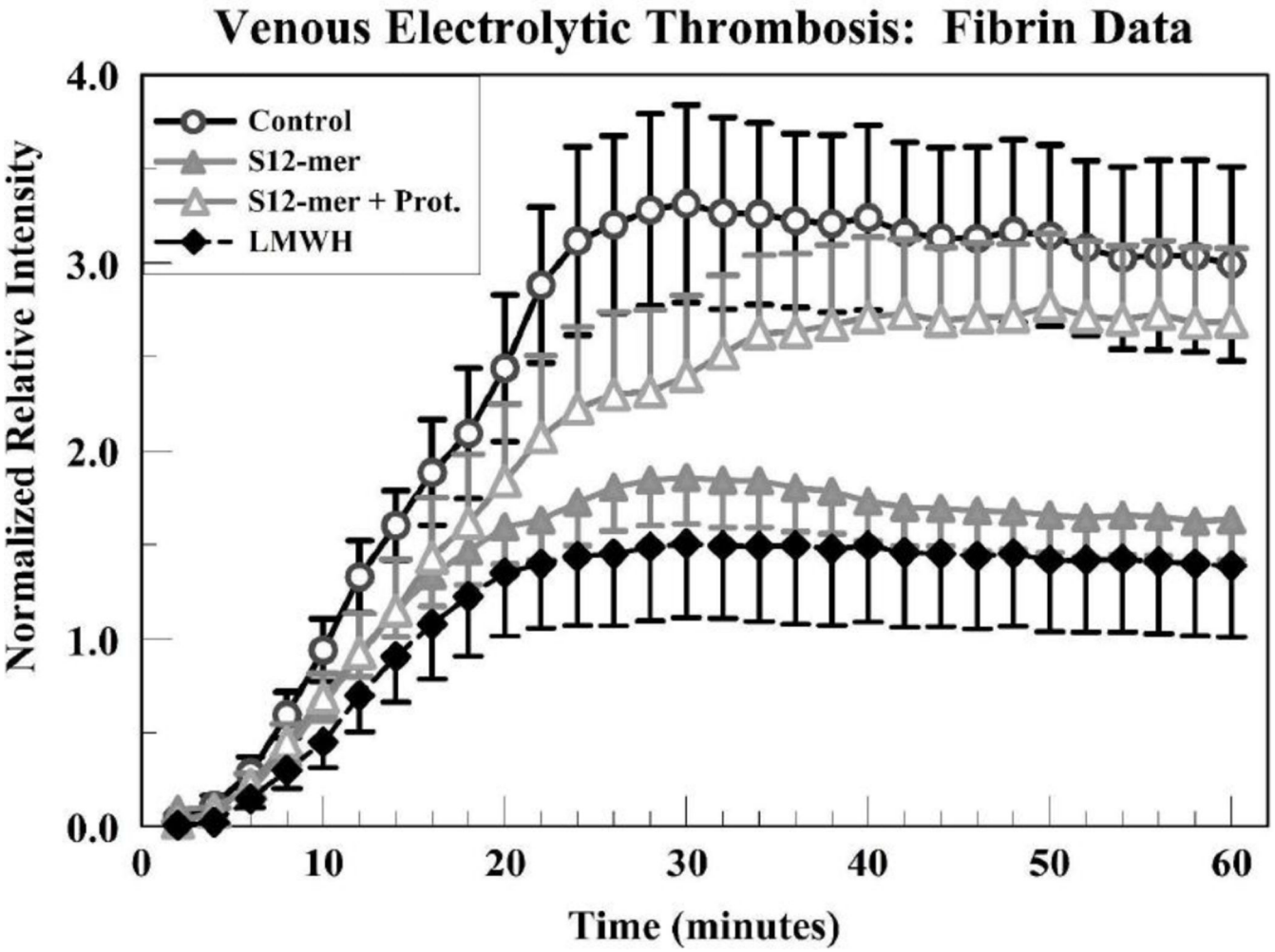


Figure 6. In vivo thrombosis assay

Graphs of normalized relative intensities for fluorescently labeled (**A**) platelets and (**B**) fibrin in a murine femoral vein electrolytic injury model of thrombosis, measuring thrombus localized fluorescence every 2 minutes over a 60-minute course after thrombus induction; n = 6 animals per treatment group. Each line represents the average of 6 experimental runs; error bars are standard deviations. For the fibrin data (**B**), the lower two lines (S12-mer and LMWH (Enoxaparin)) showed statistical reductions in fibrin accumulation at 60 minutes ($p < 0.001$), in comparison to the other groups (controls and S12-mer + protamine). Abbreviations in legends are as defined in the text; Prot = protamine.

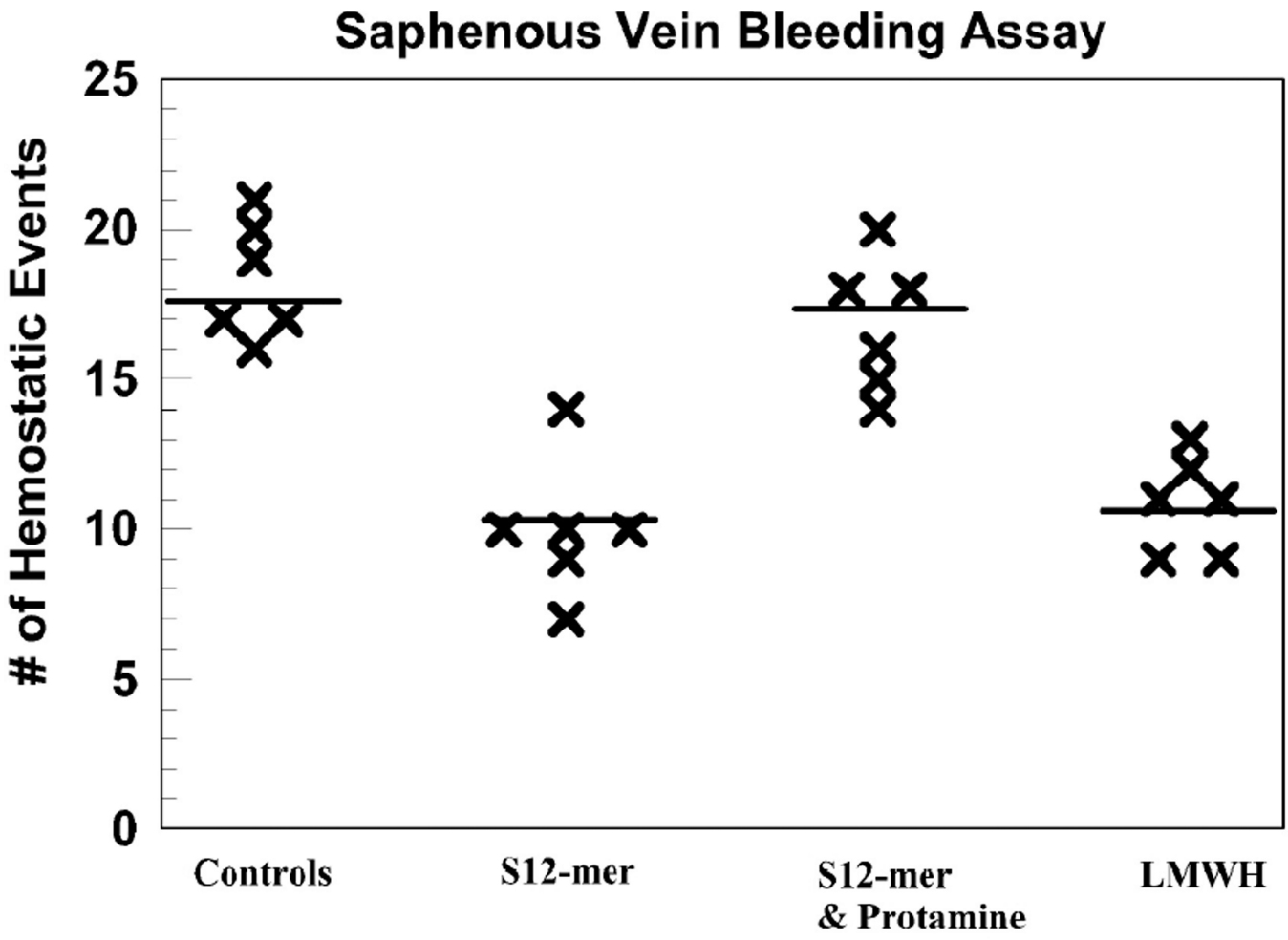


Figure 7. In vivo hemostasis assay

Graph of the number of hemostatic recurrences following repeated clot dislodgement from the transected saphenous vein in mice, over a 30-minute interval; n = 6 animals per treatment group. S12-mer and LMWH (Enoxaparin) were found to have similar increased numbers of hemostatic events in comparison to the control (vehicle) group and to the S12-mer group treated with protamine ($p<0.05$). Abbreviations in legends are as defined in the text; horizontal bars represent the average for each group.

Table 1

Thrombin generation parameters for the effects of S12-mer and LMWH (Enoxaparin) on thrombin generation in platelet poor plasma.

	Lag Time (sec)				Peak Thrombin (nM)				Time to Peak Thrombin (sec)			
Heparinoid (nM)	0	150	300	600	0	150	300	600	0	150	300	600
Enoxaparin	323	355	547	955	55.7	24.1	7.9	1.47	739	899	1059	1731
Super 12-mer	323	483	579	771	55.7	24.3	11.2	5.58	739	899	1155	1347

Table 2

TEG parameters for S12-mer effects on clot formation.

Super 12-mer (nM)	R (min)	Angle (°)	MA (mm)
600	20.2 ± 3.8	28 ± 11	54 ± 10
300	15.9 ± 2.2	31 ± 10	55 ± 11
150	12.4 ± 1.8	42 ± 11	58 ± 9
0	7.8 ± 0.9	56 ± 5	65 ± 6

Table 3

TEG parameters for protamine reversibility heparinoid effects on clot formation.

Super 12-mer (12-mer)	R (min)	Angle (°)	MA (mm)
Control	8.4 ± 0.9	53 ± 5	63 ± 6
600 nM 12-mer	13.9 ± 1.6	30 ± 6	52 ± 5
12-mer + 600 nM Protamine	9.6 ± 1.8	44 ± 5	59 ± 4
12-mer + 300 nM Protamine	13.5 ± 4.15	38 ± 12	53 ± 7