

Development and Evaluation of a Prototype of a Novel Clotting Time Test to Monitor Enoxaparin

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ABSTRACT

Purpose Dosing of the anticoagulant enoxaparin may result in bleeding following excessive doses or thrombosis if dose is too low. Rarely, anti-Xa activity is used to assess the dose for enoxaparin, but its utility to predict clotting or bleeding remains uncertain. We aimed to develop a clotting time test to monitor enoxaparin therapy.

Methods A previously developed mathematical model of the coagulation network was used to identify suitable targets for monitoring enoxaparin therapy. *In vitro* experiments were then carried out to demonstrate proof of mechanism of the clotting time test activated by the new target activator.

Results Using the mathematical model, we identified Xa as a plausible activating agent for a clotting time test for enoxaparin. *In vitro* experiments showed a prolongation of the Xa clotting time of 4.6-fold in the presence of enoxaparin (0.5 IU/ml) where 10 nM Xa was used to activate clotting.

Conclusions Using both simulations and *in vitro* experiments, we provide a proof of mechanism for the Xa clotting time (XaCT) test, which can be considered for further development to provide a biomarker of the effect of enoxaparin on the clotting system.

KEY WORDS coagulation network · enoxaparin · low molecular weight heparins · mathematical model · monitoring

ABBREVIATIONS

ACT	activated clotting time
aPTT	activated partial thromboplastin time
AT	antithrombin
INR	international normalised ratio
LMWH	low-molecular-weight heparin
PT	prothrombin time
TF	tissue factor
UFH	unfractionated heparin
XaCT	Xa clotting time

INTRODUCTION

Thrombosis is the pathological activation of the haemostatic system within the vasculature and results in formation of a solid mass of platelets, fibrin and trapped blood cells inside blood vessels. Inappropriate activation of the coagulation process or inappropriate formation of platelet aggregates in blood vessels is treated with anticoagulants or, in a few situations, with medicines that activate fibrinolysis. Careful control of the action of therapeutic anticoagulants is required to prevent the adverse effects of bleeding from excessive anticoagulation or thrombosis and thromboembolism where anticoagulation is inadequate (1).

Enoxaparin is a low molecular weight heparin (LMWH) anticoagulant that is widely used to minimise the risk for thrombosis in patients with acute coronary syndromes, pulmonary embolism or deep vein thrombosis (2–5). Its activity is mediated by increasing the activity of a physiological inhibitor, antithrombin (AT). The outcome

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is enhanced inactivation of Xa and several other activated factors, resulting in inhibition of the coagulation network (6). Despite claims that LMWHs have more predictable dosing requirements than unfractionated heparins, the rates of bleeding and thrombus formation are similar (7–9). Sequelae from bleeding or thrombosis arising as adverse effects from inadequately controlled anticoagulation may be severe and in some cases life threatening.

In contrast to the older anticoagulants, unfractionated heparin (UFH) and warfarin, there is no currently accepted method for monitoring the effect of enoxaparin on coagulation. The need to monitor has been the subject of debate, with some studies suggesting that LMWHs are no safer than UFH and require monitoring (10). This begs the question, could safety and efficacy be improved with appropriate laboratory monitoring of LMWH, either universally or in selected patients? For warfarin and UFH, it is usual to measure the time for a blood sample to clot after stimulation with activating agent(s). The tests used are the standardised prothrombin time (PT), i.e., the international normalised ratio (INR) for warfarin (11) and the activated partial thromboplastin time (aPTT) for UFH (12). Both the PT and the aPTT assess multiple steps in the clotting system and measure the time to form a fibrin clot. These tests identify deficiencies in any of the clotting factors or pathways that may arise and affect the final clot formation. Both PT and aPTT have been found to provide a good prediction of the risk for bleeding, or clotting and thrombosis, when used with warfarin and UFH, respectively.

Currently, there is no multi-step clotting time test for LMWHs such as enoxaparin, and assessment of dose effect is seldom performed. It is possible to measure anti-Xa activity (one of the critical activated precursors for coagulation), but there is no well-accepted target activity value, and dose adjustments are difficult to make (5,13). Neither the PT/INR nor aPTT produce significant dose-response changes with enoxaparin and so do not provide meaningful evaluation of bleeding or thrombotic risks (5,6,12–15). Measurement of anti-Xa activity assesses the activation of only a single component of the clotting system and is not a widely available test. We consider there is a need for an alternative, simple, stable, diagnostic clotting time-based test to monitor treatment with enoxaparin.

There have been various attempts at developing tests to monitor LMWH therapy. The activated clotting time (ACT) is a less sensitive global test of coagulation and is used to monitor the level of anticoagulation in patients undergoing percutaneous coronary interventions or aorto-cardiac bypass surgery who receive bolus doses of UFH (16). Four studies have shown that the ACT is sensitive to LMWH and may be of value in monitoring patients, particularly in relation to percutaneous coronary angioplasty (17–20). Other studies have shown little or no effect

of LMWH on the ACT (21–24). A study involving UFH, enoxaparin and fondaparinux, a synthetic penta-saccharide Xa inhibitor, and their effects on the ACT and aPTT in healthy volunteers found that enoxaparin produced significantly less prolongation of both the ACT and the aPTT than UFH, whereas fondaparinux had no effect on either of these tests (23). Finally, the addition of enoxaparin or fondaparinux to blood containing UFH produced no significant prolongation of the ACT beyond that produced by UFH alone. It was concluded that the ACT and aPTT cannot be used to monitor enoxaparin or fondaparinux (23).

Other clotting tests such as the HepTest and prothrombinase-induced clotting time test were designed as surrogate measures of plasma heparin concentrations and employ excess Xa (25,26). However, they are not specific for measuring heparin effect because the addition of hirudin (which produces an anti-IIa (anti-thrombin) effect) will prolong the clotting time (27). In some clinical conditions, the changes in the concentration of specific markers of blood clotting activation during LMWH administration have been investigated, but assays of prothrombin fragment F1+2, thrombin-antithrombin complexes, and D-dimers have not been found to be sufficiently sensitive and useful in the assessment of antithrombotic efficacy of the anticoagulant therapy (28).

A mechanistic and quantitative model of the coagulation network has been developed (Fig. 1). The model has been shown to accurately describe the time course for activation of coagulation factors both *in vivo* and also *in vitro* in PT and aPTT tests. The model has also been used to predict the concentration-time and effect-time profiles of warfarin, heparins and vitamin K in humans (29), as well as venom-induced consumptive coagulopathy arising from some snake bites (30).

The overarching aim of this study was to identify and evaluate plausible activating agent(s) for a clotting time test to assess the anticoagulant effect of enoxaparin. Four specific objectives were identified: 1) *in silico* assessment of standard clotting time tests (aPTT and PT/INR) when applied to enoxaparin, 2) *in silico* identification of new targets for activating a clotting time test, 3) *in vitro* assessment of Xa as a new target for activating a clotting time test, and 4) *in silico* predictions of the kinetics of activation in the test. These specific objectives were designed to provide a proof-of-mechanism of the clotting time test and to assess whether the test appears to work by a mechanism that we understand and predict. The *in silico* experiments in the study provide the mechanistic framework, and the *in vitro* experiments show a realisation of the mechanism. This study was aimed at exploring a mechanism, and future work will assess the utility of any test identified in this work along the lines of a proof-of-concept.

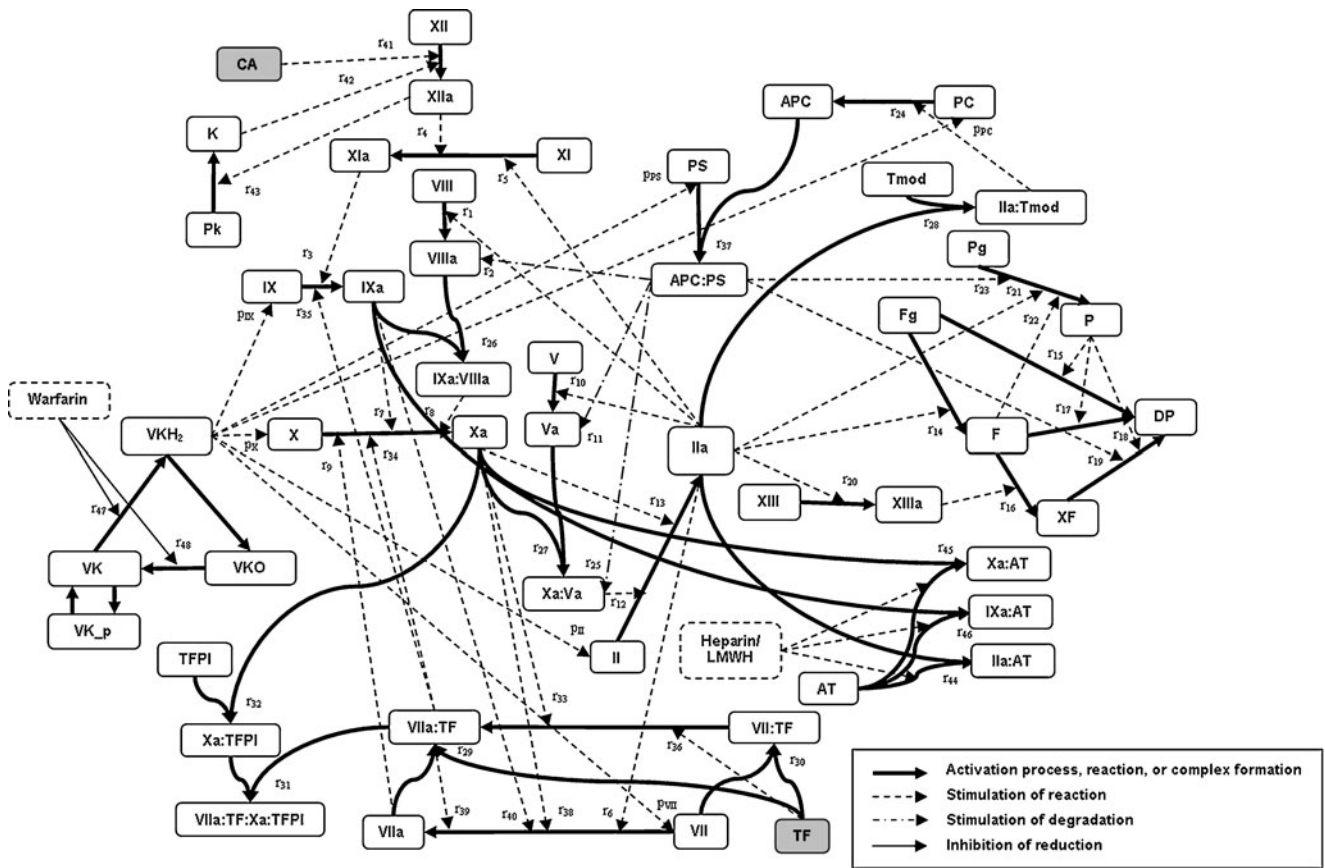


Fig. 1 Schematic of the coagulation network model. The bold solid lines represent activation process, complex formation, reduction or oxidation; the broken lines represent stimulation of reaction or production; the dot-bar lines represent stimulation of degradation; the solid lines represent inhibition of reaction. APC, activated protein C; AT, antithrombin; CA, activator for the contact system; DP, degradation product; F, fibrin; Fg, fibrinogen; II, prothrombin; IIa, thrombin; K, kallikrein; LMWH, low molecular weight heparin; P, plasmin; PC, protein C; Pg, plasminogen; Pk, prekallikrein; PS, protein S; TF, tissue factor; TFPI, tissue factor pathway inhibitor; Tmod, thrombomodulin; VK, vitamin K; VKH₂, vitamin K hydroquinone; VKO, vitamin K epoxide; XF, cross-linked fibrin. (Adapted from Wajima et al. (29)—the one error has been removed, modifications have been made to clarify details, and new subsystems have been added).

MATERIALS AND METHODS

In Silico Assessment of Activation of Standard Clotting Time Tests When Applied to Enoxaparin

A mathematical model of the coagulation system (Fig. 1) published earlier (29) was used to assess current clotting time tests. Simulations using the model were carried out to address why warfarin but not enoxaparin prolongs PT and also why UFH but not enoxaparin prolongs aPTT. All simulations were performed in MATLAB® version 7.10.0 (R2010a), a product of the MathWorks™ Inc. (Natick, USA). Clotting time was defined as the time it takes for a fibrin clot to start to form in plasma after addition of an activating agent to a plasma sample. The criterion for formation of a clot using the mathematical model was based on the integral of fibrin, which determines the area under the fibrin concentration-time curve (fibrin AUC). Using this measure the criterion for clot formation was a

fibrin AUC of 1500 nM.sec (29). This is approximately equivalent to a 30% consumption of fibrinogen in a standard PT/INR test (31).

The PT is the time for plasma to clot after addition of a thromboplastin reagent (tissue factor, calcium and phospholipid). The *in silico* PT test was performed by simulating the clotting time after adding tissue factor (TF) to a plasma sample with the initial TF concentration of 300 nM, which is in excess of VII (10 nM). The TF concentration of 300 nM in the model was chosen arbitrarily and was based on a calibration to a clinically observed PT of 12-15 s in the absence of any anticoagulant. The value used here should not be construed to be necessarily representative of TF concentrations used in actual PT tests. The influences of various lower concentrations of TF were also investigated. The effect of enoxaparin was simulated at a therapeutic concentration of enoxaparin (taken as 0.5 IU/ml of anti-Xa activity). Time courses of X and Xa in the absence as well as presence of enoxaparin (0.5 IU/ml) were also simulated using the model.

The aPTT is the time for plasma to clot after the addition of calcium following pre-incubation with phospholipid and an activator for the contact system (e.g., kaolin). The *in silico* aPTT test was performed by setting the initial XIa and XI concentrations to $0.148 \cdot \text{XI}(0)$ and $0.339 \cdot \text{XI}(0)$, respectively, where $\text{XI}(0)$ is the concentration of XI in a plasma sample (29). The influences of various initial conditions for the aPTT test were also investigated. Finally, the effect of enoxaparin was simulated in a similar way to the *in silico* PT test.

In Silico Identification of New Targets for Activating a Clotting Time Test

The mathematical model was used to identify new targets for monitoring enoxaparin therapy. To identify an activating agent for a clotting time test with enoxaparin, the *in silico* clotting system was activated using a range of activated clotting factors or complexes, including IIa, Va, VIIa, TF, VII-TF, VIIa-TF, VIIIa, IXa, IXaVIIIa, Xa, XaVa, XIa, XIIa, over a range of concentrations, individually and in combination with each other. The simulations assumed that calcium and phospholipid were available in excess. The aim of the simulations was to identify an activating agent, in the form of a clotting factor that

- provides a reasonable clotting time (<60 s) and
- is prolonged by at least two-fold to a reasonable clotting time (<60 s) in the presence of a therapeutic concentration of enoxaparin (0.5 IU/ml).

This would result in clotting times similar to those observed in a PT test.

In Vitro Assessment of Xa as a New Target for Activating a Clotting Time Test

Xa was identified *in silico* (method 2) as the best option as an activating agent for a clotting time test to detect enoxaparin effect. Plasma from a single donor was used for the *in vitro* experiments and was prepared by double centrifugation at 2000 g for 20 min at 20°C to ensure the sample was platelet-free. Xa (100 µL, 0.005–10 nM) was mixed in equal proportion with calcium chloride (100 µL of 25 mM) and kept in a heating block maintained at 37°C for 2–3 min. Plasma (100 µL), pre-warmed at 37°C, was then added to the mixture and the clotting time determined using a digital timer and visual inspection to identify the appearance of fibrin (apparent as a sudden increase in turbidity) (32). The effect of enoxaparin was determined by adding a 1:10 ratio of enoxaparin to plasma at concentrations of 0.1–1.0 IU/ml anti-Xa activity; controls (0 IU/ml activity) had a similar volume of saline buffer added. Clotting times were measured in three different sets of experiments: (I) where

the concentration of Xa was varied in the absence of enoxaparin, (II) where the concentration of Xa was varied in the presence of a therapeutic concentration of enoxaparin (0.5 IU/ml), and (III) where the concentration of enoxaparin was varied in the presence of a specific Xa concentration. Each set of conditions was assessed in triplicate and the mean ± standard deviation determined.

In Silico Predictions of the Kinetics of Activation in the Test

After evaluating various activating agents and choosing Xa as a possible biomarker for enoxaparin activity, it was critical to modify the coagulation network model, as this study aimed to evaluate the influence of AT-Xa-enoxaparin relationship. Due to the behaviour of specific components of the model being tested, further equations were added to the model to account for the influence of variable AT concentrations. The original model (29) of the coagulation network, however, was built with different aims in mind, and at that time it was not necessary to include these equations. The simulated clotting times obtained using the modified mathematical model of the clotting network with Xa as the activating agent were compared to the clotting times obtained with the *in vitro* experiments. Equation (1) describes the concentration of Xa bound with AT in the absence or presence of enoxaparin:

$$\text{Concentration bound (t)} = B_{\max}(t) \times \frac{[Xa]}{(f_{k_D} \times K_{D_{Xa}}) + [Xa]} \quad (1)$$

The parameter $B_{\max}(t)$ describes the maximum binding possible between Xa and AT and was assumed to be time-varying and equal to the concentration of AT in plasma, mean $[AT] = 2.57 \mu\text{M}$ (33). The parameter $K_{D_{Xa}}$ describes the affinity of AT for Xa in the absence of enoxaparin. Because enoxaparin produces its major anticoagulant effect by increasing the activity of AT against Xa (13), a coefficient that describes the increase in the affinity of AT for Xa in the presence of enoxaparin was included in equation (1). The coefficient, f_{k_D} , was described by equation (2) below:

$$f_{k_D} = 1 - \frac{[Enox]}{K_{D_E} + [Enox]} \quad (2)$$

The parameter K_{D_E} in equation (2) describes the overall affinity of enoxaparin for AT. The model was then used to predict the clotting times for the three sets of *in vitro* experiments to provide a comparison of *in silico* predictions with *in vitro* observations. The *in silico* predictions were carried out at a range of values for the parameters $K_{D_{Xa}}$ and K_{D_E} . The final values set in the model were based on the similarity of model predictions to the results from the *in vitro* experiments.

RESULTS

In Silico Assessment of Activation of Standard Clotting Time Tests When Applied to Enoxaparin

PT Test

The *in silico* PT test was performed by simulating the clotting time after adding 300 nM of TF to a plasma sample. At this concentration of TF, a clotting time of 11.8 s was observed which was prolonged to 13.6 s in the presence of 0.5 IU/ml enoxaparin. Simulations of the time course of Xa in the PT test suggested that at a TF concentration of 300 nM and in the absence of enoxaparin, the concentration of Xa present at the time the clot formed (11.8 s) was 0.98 nM (Fig. 2a). In the presence of 0.5 IU/ml enoxaparin, the concentration of Xa present after 11.8 s was 0.37 nM. Although enoxaparin reduced the Xa concentration by 60% at this time, sufficient unbound Xa remained to continue activation of coagulation. This simulation suggests that the concentration of TF used to activate the system is sufficiently high that it results in excess concentrations of Xa that overcome the enoxaparin concentration of 0.5 IU/ml, and hence therapeutic enoxaparin causes only a very slight (15%) prolongation in PT to 13.6 s.

In contrast, changing the TF concentration by a factor of 100,000 to 0.003 nM predicted a clotting time of 2.6 min which was prolonged to 5.9 min in the presence of 0.5 IU/ml enoxaparin. Simulations of the time course

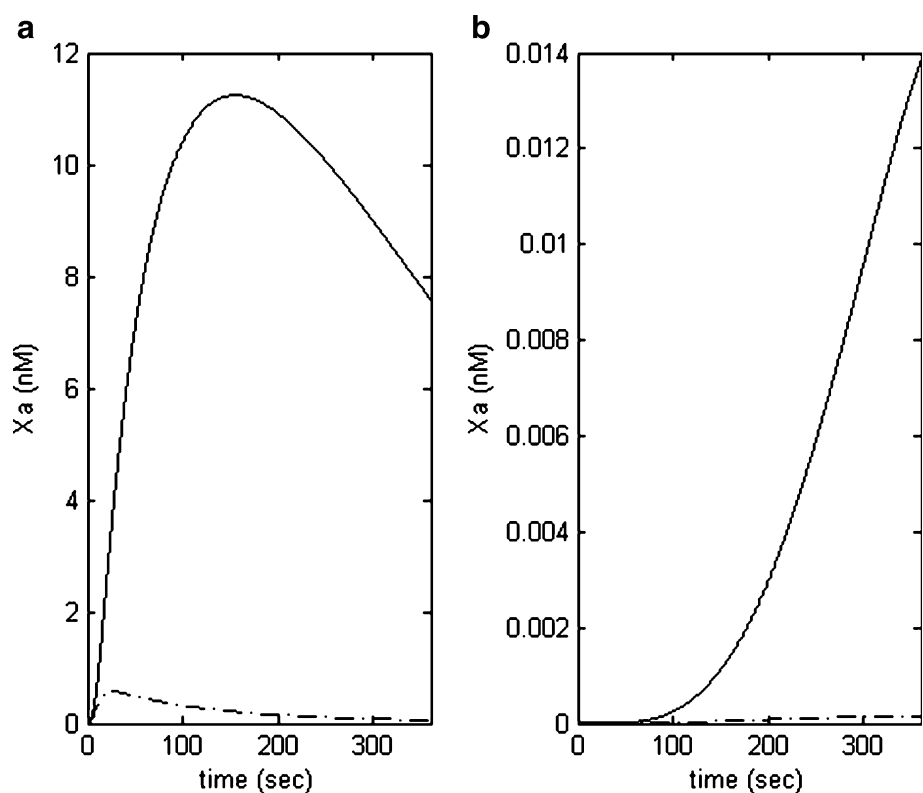
of Xa production, at the TF concentration of 0.003 nM and in the absence of enoxaparin, predicted a concentration of Xa of 1.3 pM at the time the clot formed (2.6 min), which was around 700-fold lower than that formed with 300 nM TF (Fig. 2b). In the presence of 0.5 IU/ml enoxaparin, the concentration of Xa present after 2.6 min was 96% lower (0.05 pM) and resulted in a 2.3-fold prolongation of PT to 5.9 min.

In vitro experiments were carried out at a TF concentration of 1.5 nM, and clotting times greater than 10 min were obtained. Investigation of the effects of the addition of phospholipids appeared to be influential, and further experiments with TF were not continued. Please note that the actual concentration of TF is not able to be determined in these experiments, but rather all predictions are based on the relative concentration (relative to 300 nM).

aPTT Test

The *in silico* aPTT test was performed by setting the initial XIa and XI concentrations to $0.148 \cdot \text{XI}(0)$ and $0.339 \cdot \text{XI}(0)$, respectively, where $\text{XI}(0)$ is the concentration of XI in the plasma sample (29). At an XIa concentration of 1.5 nM, a clotting time of 34.4 s was observed, which was prolonged to 49.3 s in the presence of 0.5 IU/ml enoxaparin. Simulations of the time course of Xa in the aPTT test suggested that at an XIa concentration of 1.5 nM in the absence of enoxaparin,

Fig. 2 Simulated time course of Xa in the PT test. **(a)** Time course of Xa in the PT test when activated by 300 nM of TF *in silico* in the absence (solid line) or presence (dash-dotted line) of 0.5 IU/ml enoxaparin. **(b)** Time course of Xa in the PT test when activated by 0.003 nM of TF *in silico* in the absence (solid line) or presence (dash-dotted line) of 0.5 IU/ml enoxaparin.



the concentration of Xa at the time the clot formed (34.4 s) was 0.06 nM (Fig. 3a). In the presence of 0.5 IU/ml enoxaparin, the concentration of Xa present after 34.4 s was 85% lower (0.0093 nM) and was associated with clot formation at 49.3 s. As with the PT test, the activation condition (concentration of XIa in the case of the aPTT) resulted in concentrations of Xa that largely overcame the enoxaparin concentration of 0.5 IU/ml.

In contrast, at a 100-fold lower XIa concentration of 0.015 nM, a clotting time of 77 s was observed, which was prolonged to 167 s in the presence of 0.5 IU/ml enoxaparin. Simulations of the time course of Xa production at this lower XIa concentration of 0.015 nM and in the absence of enoxaparin predicted that the Xa concentration present at the time the clot formed (77 s) was 0.0063 nM, which was 10-fold lower compared to that formed with 1.5 nM of XIa (Fig. 3b). In the presence of 0.5 IU/ml enoxaparin, the concentration of Xa present after 77 s was 0.0004 nM (94% lower), which resulted in a 2.2-fold prolongation of aPTT to 167 s.

In Silico Identification of New Targets for Activating a Clotting Time Test

Simulations using the mathematical model of the coagulation system identified Xa as a plausible activating agent for a clotting time test to measure the dose effect of enoxaparin (see Table I). A Xa concentration of 0.05 nM gave a clotting time of 13.8 s, which was prolonged 2-fold to 27.2 s in the presence

of 0.5 IU/ml enoxaparin. Activation with Va, VIIa, VII-TF or VIIIa did not result in the formation of a clot, whereas activation with XIIa did not result in a two-fold prolongation in the presence of 0.5 IU/mL enoxaparin. Clotting times on activation with a combination of clotting factors were similar to those with individual factors; individual factors were therefore preferred over combination of factors.

In Vitro Assessment of Xa as a New Target for Activating a Clotting Time Test

Xa was assessed *in vitro* as a plausible activating agent for a clotting time test to measure enoxaparin effect (Xa clotting time (XaCT) test).

Effect of Varying Xa Concentrations on Clotting times in the Absence of Enoxaparin

Concentrations of Xa from 0.005 to 10 nM produced clotting times that ranged from 473 ± 11 to 15.3 ± 0.6 s respectively (Fig. 4).

Effect of Varying Xa Concentrations on Clotting times in the Presence of 0.5 IU/ml Enoxaparin

Using Xa at concentrations of 0.005 to 10 nM with 0.5 IU/ml enoxaparin, produced clotting times that ranged from 2148 ± 49 to 70 ± 5 s. This ranged from 4.5- to 6.8-fold lengthening of the clotting time (Fig. 4).

Fig. 3 Simulated time course of Xa in the aPTT test. **(a)** Time course of Xa in the aPTT test when activated by 1.5 nM of XIa *in silico* in the absence (solid line) or presence (dash-dotted line) of 0.5 IU/ml enoxaparin. **(b)** Time course of Xa in the aPTT test when activated by 0.015 nM of XIa *in silico* in the absence (solid line) or presence (dash-dotted line) of 0.5 IU/ml enoxaparin.

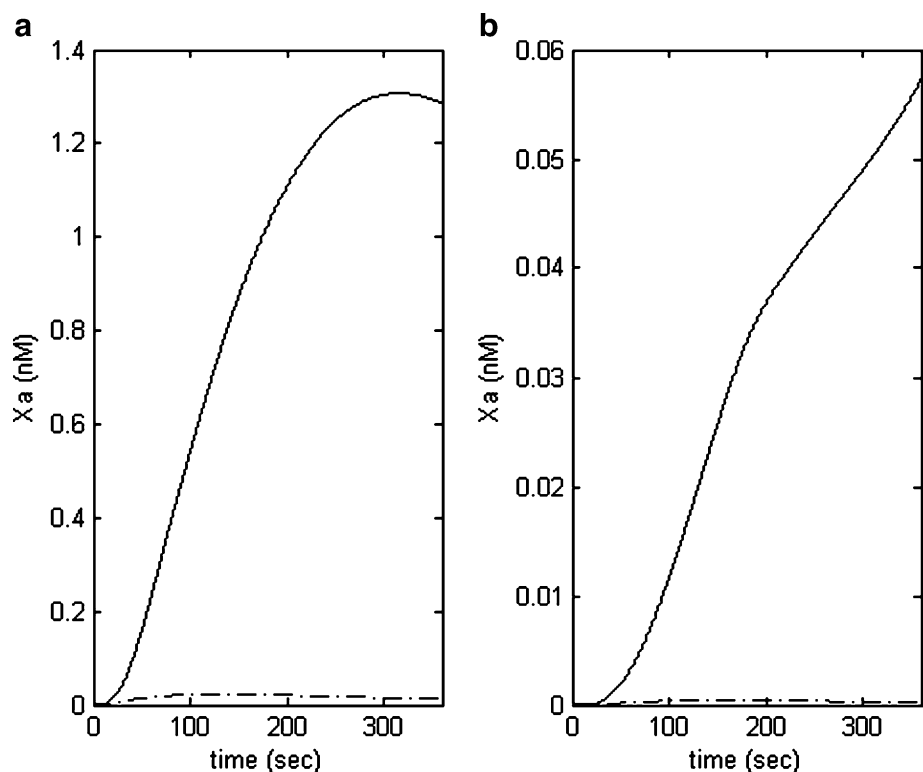


Table I Effect of Various Activating Agents on Clotting Time in the Absence or Presence of Enoxaparin (0.5 IU/mL)

Activating agent (concentration in nM)	CT ₀ = Clotting time when enoxaparin = 0 (seconds)	CT _{0.5} = Clotting time when enoxaparin = 0.5 IU/mL (seconds)	Comments
Individual clotting factors			
Ila (0.00005)	49.2	110.6	CT _{0.5} > 60 s
Va	-	-	No clot seen
VIIa	-	-	No clot seen
TF (0.003)	157.3	353.9	CT ₀ , CT _{0.5} > 60 s
VII:TF	-	-	No clot seen
VIIa:TF (0.0002)	63.3	140.1	CT ₀ , CT _{0.5} > 60 s
VIIIa	-	-	No clot seen
IXa (0.01)	49.6	113.4	CT _{0.5} > 60 s
IXa:VIIIa (0.001)	36.8	86.6	CT _{0.5} > 60 s
Xa (0.05)	13.8	27.2	CT ₀ , CT _{0.5} < 60 s
Xa:Va (0.0000001)	173.0	385.6	CT ₀ , CT _{0.5} > 60 s
XIa (0.015)	77.1	167.2	CT ₀ , CT _{0.5} > 60 s
XIIa (0.000000001)	78.2	100.8	< 2-fold prolongation
Combination of clotting factors			
Xa (0.001) + Ila (0.00005)	37.9	99.4	CT _{0.5} > 60 s
IXa (0.01) + Ila (0.00005)	36.3	68.5	CT _{0.5} > 60 s
Xa (0.01) + IXa (0.01)	23.5	64.3	CT _{0.5} > 60 s
XIa (0.02) + Ila (0.00005)	46.0	90.2	CT _{0.5} > 60 s
XIIa (100) + Ila (0.00005)	40.3	61.5	CT _{0.5} > 60 s
XIa (0.02) + Xa (0.05)	13.8	27.2	CT ₀ , CT _{0.5} similar to those with Xa alone
XIIa (100) + Xa (0.05)	13.8	27.1	CT ₀ , CT _{0.5} similar to those with Xa alone
XIa (0.02) + IXa (0.01)	48.3	100.2	CT _{0.5} > 60 s
XIIa (10) + IXa (0.001)	49.4	72.1	CT _{0.5} > 60 s
Xa (0.05) + Xa:Va (0.0000001)	13.8	27.1	CT ₀ , CT _{0.5} similar to those with Xa alone

Effect of Varying Enoxaparin Concentrations on Clotting Times in the Presence of a Specific Xa Concentration

A clotting time of 15 s, similar to the upper end of the physiological range for the PT, was obtained with a Xa concentration of 10 nM, and this concentration was used to activate the clotting system to assess the effect of varying enoxaparin concentrations (0.1–1.0 IU/ml). In the presence of 1.0 IU/ml enoxaparin, the clotting time was prolonged 10-fold to 153 s; in contrast, 0.1 IU/ml enoxaparin caused only a 1.7-fold prolongation to 26 s, compared to the control with no enoxaparin (see Table II).

In Silico Predictions of the Kinetics of Activation in the Test

After the modification of the mathematical model to include the binding of Xa and AT, as described in equation (1), the model was used to predict the clotting times for the three sets of *in vitro* experiments to provide a comparison of

in silico to *in vitro* observations. Based on the similarity of model predictions to the results obtained in the *in vitro* experiments, the parameters $K_{D_{Xa}}$ and K_{D_E} were set to 0.56 nM and 16.5 nM, respectively. In addition, since the *in silico* system assumes phospholipids and calcium are always in excess, it was found necessary to adjust to a standard concentration of exogenous Xa. The purpose of this *in silico* evaluation was to assess whether the mathematical model supports the findings from the experimental observations on clotting in the presence of varying concentrations of Xa and enoxaparin, i.e., in the *in vitro* testing that were undertaken as a proof-of-mechanism study in method 3 for the “XaCT test.”

Effect of Varying Xa Concentrations on Clotting times in the Absence of Enoxaparin

When the *in silico* clotting system was activated using 0.05 pM to 0.1 nM Xa, a concentration-dependent decrease in simulated clotting times from 3255 s to 15 s

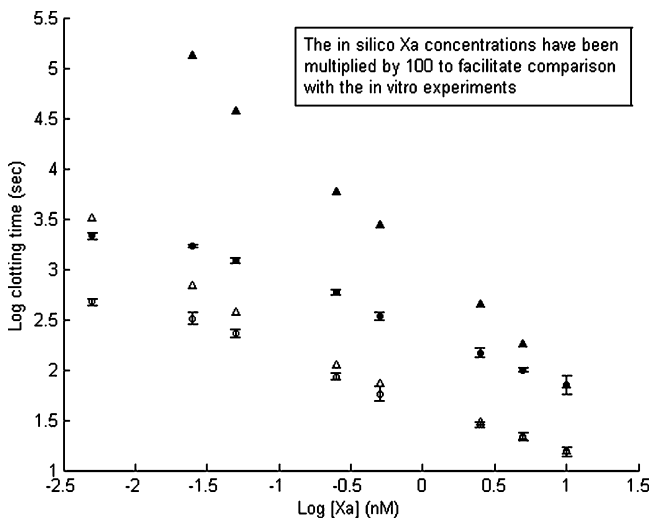


Fig. 4 Effect of varying Xa concentrations on clotting times in the absence or presence of 0.5 IU/ml enoxaparin. *In vitro* clotting times (seconds) after activation by varying Xa concentrations (0.005 to 10 nM) in the absence (\circ) or presence (\bullet) of 0.5 IU/ml enoxaparin using plasma from a single donor. Data are shown as error bars of mean values of triplicate measurements \pm 3SD. *In silico* clotting times (seconds) after activation by varying Xa concentrations (0.05 pM to 0.1 nM) in the absence (Δ) or presence (\blacktriangle) of 0.5 IU/ml enoxaparin. Clotting time for a Xa concentration of 0.05 pM could not be obtained due to the insensitivity of the model for very low Xa concentrations.

was observed (Fig. 4). Similar trends between the *in vitro* and *in silico* experiments were seen, although the *in silico* predictions diverged at lower Xa concentrations. The *in silico* Xa activating concentration in Fig. 4 was adjusted by a factor of 100 for comparison with the *in vitro* experiments.

Effect of Varying Xa Concentrations on Clotting times in the Presence of 0.5 IU/ml Enoxaparin

Clotting times ranged from 37 h to 70 s in the presence of 0.25 pM to 0.1 nM Xa, giving a 4.6- to 193-fold prolongation *in silico*, compared to their respective controls (Fig. 4), in the presence of 0.5 IU/ml enoxaparin. Clotting time for a Xa concentration of 0.05 pM could not be obtained due to the insensitivity of the model for very low

Xa concentrations. Similar to experiment (I), the *in silico* results showed an agreement at 0.1 nM but diverged at lower concentrations of Xa. The model over-predicted the influence of enoxaparin at declining concentrations of Xa.

Effect of Varying Enoxaparin Concentrations on Clotting Times in the Presence of a Specific Xa Concentration

A Xa concentration of 0.1 nM was chosen to activate the system *in silico*, as this concentration gave the same clotting time of 15 s, as was observed with the *in vitro* results. Presence of 0.1 to 1 IU/ml of enoxaparin *in silico* resulted in a prolongation of clotting time from 26 s to 145 s, *i.e.* 1.7- to 9.5-fold (see Table II). The model and *in vitro* results agree closely.

DISCUSSION

Using both simulations from an *in silico* model and *in vitro* experiments, we show that a Xa clotting time test has the potential to be used to assess the effect of enoxaparin on the clotting system. The *in silico* simulations showed that only clotting tests activated by low concentrations of Xa were sensitive to the effects of enoxaparin and produced clotting times which are likely to be manageable in the clinic. We then used *in vitro* experiments to demonstrate proof-of-mechanism of the Xa clotting time test and compared clotting times from these experiments to *in silico* simulated clotting times. These provide the basis for the Xa clotting time test being useful for monitoring enoxaparin.

Our *in silico* model of the coagulation system was initially used to investigate the reason for enoxaparin not having a significant prolonging effect on the common clotting time tests, PT and aPTT. The *in silico* PT and aPTT experiments suggested that the extent of PT or aPTT prolongation in the presence of enoxaparin depends on the concentration of the activating agent used in the test (TF for the PT and XIa for the aPTT). Routine PT tests use

Table II Effect of Varying Enoxaparin Concentrations on Clotting Times in the Presence of a Specific Xa Concentration

Enoxaparin (IU/ml)	Results from <i>in vitro</i> experiments (triplicates), (Xa = 10 nM)		Results from <i>in silico</i> experiments (Xa = 0.1 nM)	
	Mean clotting time (seconds)	Fold increase in clotting time by enoxaparin	Clotting time (seconds)	Fold increase in clotting time by enoxaparin
0	15	0	15	0
0.1	26	1.7	26	1.7
0.25	42	2.7	41	2.7
0.5	71	4.6	70	4.6
0.75	105	6.8	106	6.9
1	153	10	145	9.5

much higher concentrations of thromboplastin than might be expected under physiological conditions (34) and achieve high concentrations of Xa that overcome the effect of enoxaparin. The simulations suggested that at lower TF concentration, an appreciable prolongation in PT is observed with a therapeutic concentration of enoxaparin (0.5 IU/ml) because there is a much larger reduction in Xa concentrations. Similarly, the use of a lower concentration of XIa in the *in silico* aPTT test caused an appreciable prolongation in aPTT in the presence of enoxaparin. This is because a higher concentration of the activating agent used to activate the system results in excess concentrations of Xa that overcome therapeutic enoxaparin concentrations of 0.5 IU/ml. Hence, therapeutic enoxaparin causes only a small prolongation in currently manufactured versions of the PT and aPTT. The use of lower TF concentrations in a PT test would result in therapeutic concentrations of enoxaparin to cause an appreciable prolongation in the PT that would provide a diagnostically useful quantitation. Similarly, the use of lower concentrations of the activator (silica or kaolin) in the aPTT test reagents would also result in an appreciable prolongation in aPTT by therapeutic enoxaparin (data not shown). However, these activating agents would result in longer clotting times in the order of minutes and so limit the clinical usefulness of a modified aPTT or PT to evaluate enoxaparin effect.

We believe our findings could be applied to other anticoagulants as well. Samama *et al.* used commercially available PT and aPTT kits and showed that fondaparinux did not prolong PT and aPTT (27). The authors related the response of the drug on this system to its mechanism of action by stating that fondaparinux inhibits Xa activity by acting on free Xa instead of the prothrombinase complex and is thus less efficient at inhibiting the activation of prothrombin to thrombin. We suggest that the reason for fondaparinux not prolonging the PT and aPTT to an appreciable extent was due to the high concentration of TF or silica/kaolin, respectively, present in the PT or aPTT kits used in the study.

Commercially available PT test kits contain a mixture of agents including TF, phospholipids and calcium. Because a PT test is primarily used to monitor warfarin therapy, the PT test kits also include a heparin neutralizer to minimise effects on the PT from any heparins in the patient plasma samples. For example, Innovin[®] (recombinant human TF) and Thromborel[®] S (human placental thromboplastin), products of Dade Behring, Liederbach, Germany are insensitive to heparin concentration up to approximately 2 IU/ml and 0.6 IU/ml, respectively (http://www.medcorp.com.br/medcorp/upload/downloads/Innovin_Thromborel_PT_2006317143540.pdf). Therefore, besides a high concentration of TF used in PT test reagents, a

further reason that LMWHs do not produce appreciable prolongation with currently available PT test kits is the addition of heparin neutralizers.

Clotting times of the order of a few seconds permit efficient use of diagnostic laboratory facilities when testing large batches of patient samples and are a driver for the high concentrations of activators used in PT and aPTT test kits. Our study shows that using Xa as an activating agent for a test with enoxaparin results in a clotting time in the order of seconds, and this is prolonged with increasing amounts of enoxaparin. A Xa concentration of 10 nM gave a physiological clotting time of 15 s when used as an activating agent in the *in vitro* experiments involving plasma from a single healthy volunteer. An appreciable prolongation of this clotting time to 26 s was observed in the presence of 0.1 IU/ml of enoxaparin compared to 153 s when 1.0 IU/ml of enoxaparin was present. The clotting time prolongation of 1.7- to 10-fold in the presence of 0.1-1.0 IU/ml of enoxaparin now needs to be assessed for consistency of performance with normal and patient plasma samples and ultimately for overall clinical utility.

Current tests for enoxaparin (and fondaparinux) dose effect involve assessment of anti-Xa activities and may be poor measures of patient outcomes (5,13). We suggest that a new test, which we have called the XaCT test, which uses Xa to activate plasma clotting, will ultimately provide an effective and simple means for assessing enoxaparin concentration in plasma and provide greater certainty around clinical treatment where this is appropriate.

We have provided a proof-of-mechanism study which has shown that our mathematical model of the coagulation system can be used to predict targets for blood coagulation tests and the general behaviour of these targets when used in an *in vitro* clotting time test. There was good agreement between the *in silico* and *in vitro* results for parts I and III of the fourth experiment after scaling for Xa concentration. Although scaling of Xa concentrations between *in silico* and *in vitro* was an important issue, we believe the main results are represented in the time course and concentration-response of activity rather than the specific factor concentrations. The clotting network model does not account for calcium and phospholipids, which are assumed to be in excess during all *in silico* versions of both *in vivo* and *in vitro* simulations. Hence, we would expect the *in silico* model should provide the lower bound of the likely *in vitro* concentrations. We also note that the model over-predicted the clotting time due to activation by variable concentrations of Xa under conditions of fixed enoxaparin concentration, while providing accurate predictions of the effect of variable enoxaparin-induced clotting time prolongation for a fixed Xa concentration. Again, we believe this discrepancy for variable Xa concentrations may in part arise from assumptions about phospholipids and calcium, which becomes more evident for longer clotting

time values when the concentrations of Xa are low and hence the system is more sensitive to phospholipid and calcium concentrations.

We also believe that the choice of the criteria for clot formation may not be reflective of slow-forming clots. In the *in silico* experiments, a fibrin AUC of 1500 nM.sec was used as a criterion for clot formation. Formation of a clot, however, is likely to be much more complex than this criterion suggests, since a very slow production of fibrin to achieve the same AUC may result in clot formation at a different critical value. Therefore, this critical value of the fibrin AUC to cause clot formation only relates to rapid formation of fibrin and we do not know how time will affect interpretation of this value. We believe this may have affected the model predictions at lower Xa concentrations when clotting times were in the order of minutes. In addition, the model neither accounts for binding of AT with clotting factors other than Xa nor for non-specific binding of Xa that may be involved. The value of 100 as a scaling factor was therefore chosen empirically so that the *in vitro* predictions at 10 nM match with the *in silico* predictions obtained at 0.1 nM. In all cases, we expect the model to predict the lower bound of any concentration range for activation of the system, since the model does not include non-specific binding nor does it account for a lack of phospholipids, both of which may occur in *in vitro* experiments. Also, the current coagulation network model was based on literature parameter values (29), and it is likely that some of these values may need to be modelled to get estimates that better reflect observed data. Despite these discrepancies, the model provided acceptable estimates of clotting times for the clinically important outcomes presented in Table II.

Xa is used extensively all over the world for anti-Xa measurements to get enoxaparin concentrations. The concentrations of Xa used in our study are similar to those in standard anti-Xa kits, and we do not perceive an issue with using Xa as an activating agent for clot formation. Therefore, the next stage of the development of the prototype “XaCT Test” will be a proof-of-concept study that aims to explore the clinical utility of the test. Validation studies will use plasma from a wide range of healthy volunteers to evaluate the reproducibility of the test. A successful proof-of-concept study would mean that this XaCT test could then be evaluated in patients receiving therapeutic LMWH to assess the amount of variance in anticoagulation achieved and the potential for reduced risk of thrombotic and bleeding events where control is optimised. We suggest that the XaCT test will provide a missing direct link for dose optimisation of drugs like LMWHs and fondaparinux. However, we would point out that there are other factors that need to be considered as well, which would not necessarily benefit from multiple subjects such as non-specific binding and assumption in the model that a clot forms at 1500 nM.sec for all values of time.

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