Comment

Exploring the limits of modelling thrombus formation Comment on "Modeling thrombosis in silico: Frontiers, challenges, unresolved problems and milestones" by A.V. Belyaev et al.

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Thrombosis is not only the most important cause of death and inability, it is also likely to be one of the most complicated pathogenic mechanisms. Where other mechanisms might involve equally complex biochemical networks and comparably composite interactions between cells, in thrombosis such scenarios play at the interface of the vessel wall and streaming blood, which adds an extra dimension to the difficulties.

And difficulties there are! One of the admirable features of the accompanying article [1] is that it stresses time and again that the solutions that are being found are at the best partial solutions that, before being of any practical (and *a fortiori* medical) use, will have to be checked against real life situations.

Another aspect that is expertly brought forward in this text, is how fascinating an intellectual game simulation is. In a way it is like chess, with its own rules, its own results and its own addicts. (Also, in this sense chess can be considered as the simulation of a battlefield.)

This being said, it is no use not to address directly the main and burning question: *In how far simulation of thrombus formation has already been developed to a stage that is useful in (medical) practice.*

In this matter we dare to express an opinion only in so far as our own experience goes – which lies in the field of the interaction of blood clotting factors and blood platelets that results in the generation of thrombin and fibrin.

The mechanism of blood coagulation is complicated enough: a set of chemical equations that attempts at realism will easily contain some 20 reactants and a hundred reaction constants [2,3]. Under the tacit (but, as we will see, unrealistic) assumption of a perfectly stirred reaction medium, this leads to a set of ordinary differential equations that can be numerically solved. Such solutions will pertain to physiological reality only if *a*: the set of chemical equations is a correct representation of what happens *in vivo* and *b*: we can assign realistic values to each of the concentrations and constants.

As recognised by the authors of ref. [1]: We are far from sure that the reaction mechanisms that we think to be responsible for thrombin generation have been exhaustively explored; there may remain essential reactions that are as yet unknown, or – if known – are not taken into account. Recently e.g. it has been demonstrated that activated factor V

is a strong inhibitor of factor X activation by the TF-FVII-PPL complex [4], a feature that has not been taken into account by any of the reaction mechanisms that underpin the available simulation programs.

This shows problem 1:
The reaction mechanisms leading to thrombin and fibrin formation are only partially represented in the models used.

As to the reactant concentrations and reaction constants: fair estimates are available for most of the concentrations, be it that some reactants are, and remain surface bound (e.g. tissue factor (TF) and thrombomodulin (TM)) so that they are difficult to be represented by bulk concentrations. But the reaction constants? There are values known from the literature, but they have mostly been obtained from experiments with purified factors in buffers of various compositions rather than under the conditions prevailing in clotting plasma. And kinetic constants are known to vary with pH, ionic strength, ion-composition, viscosity etc.

Hence *problem 2: Uncertainty about the reaction constants.*

Then, at least six important reactions do not occur in free solution but at the interface of the solution with a membrane of procoagulant phospholipids (PPL): • The conversion of prothrombin into thrombin; • the activation of factor X by tissue factor (TF) and factor VII; • the activation of factor IX by tissue factor (TF) and factor VII; • the activation of factor X by factor IXa and factor VIII; • the inactivation of factor V by activated protein C together with protein S; • the inactivation of factor VIII by activated protein C together with protein S and factor V.

In each of these reactions the kinetic constants are dependent upon the composition and the physical form of the membrane and the concentration of the "helper protein".

The prothrombinase complex e.g. consists of factor Xa and factor Va adsorbed on a PPL-liquid interface [5]. Like any enzymatic reaction it is characterised by a k_{cat} and a Km. The k_{cat} is dependent upon the concentration of FVa [6]. FVa arises from the action of the product, thrombin, on Factor V (positive feedback). In real life k_{cat} therefore changes during the reaction. The Km of prothrombinase on the other hand, is entirely dependent upon the physical form and the composition of the phospholipid surface [7]. In the form of small vesicles, a phospholipid preparation will have a Km that is about a hundred times higher than in the form of a large surface [8]. The mole % of phosphatidyl serine heads on the surface is a determinant for the procoagulant properties but the anticoagulant properties (protein C dependent reactions) require phosphatidylethanolamine (PE) as well [9].

Intact platelets do not show procoagulant phospholipids on their exterior. These phospholipids are located at the inside leaf of the bilayer membrane. They become available by (*a*) disruption of the platelets (mechanical, osmotic etc.) and (*b*) by a mechanism specific for the platelets, that we call the membrane flip-flop. Membrane flip-flop translocates procoagulant phospholipids (mainly phosphatidylserine) in the intact platelet from the inside to the outside. Thus, the intact platelet becomes procoagulant through a gradual change of the phospholipid composition of its membrane [10].

In parallel with the flip-flop mechanism platelets shed procoagulant microparticles. So both the composition and the physical form of the procoagulant membrane surfaces change during hemostasis and thrombus formation. Consequently, in real life, the kinetic "constants" change continuously in time as well as in space.

Problem 3: *The concept "reaction constants" remains ill-defined.*

A fourth problem is that we are far from sure that the reaction rates are set by chemical processes. The bulk of thrombin (98%) generates in plasma that is captured in a web of fibrin fibres. We have evidence that under these conditions the reaction rate is diffusion controlled rather than chemically controlled [11]. Thrombin that is adsorbed onto fibrin is to a certain extent protected from antithrombins. The thickness of fibrin fibres is determined by the velocity with which thrombin generates, hence the amount of thrombin adsorbed on the fibres is higher when thrombin forms fast, so fast forming thrombin is better protected from antithrombins than slow forming thrombin is. Simulations, however, adopt the decay constants found in free solution.

Problem 4: *Thrombin generation is to a large extent determined by physical and not by chemical transport processes.*

We conclude that at this moment realistic simulation of thrombin generation is illusionary and will remain so in the foreseeable future.
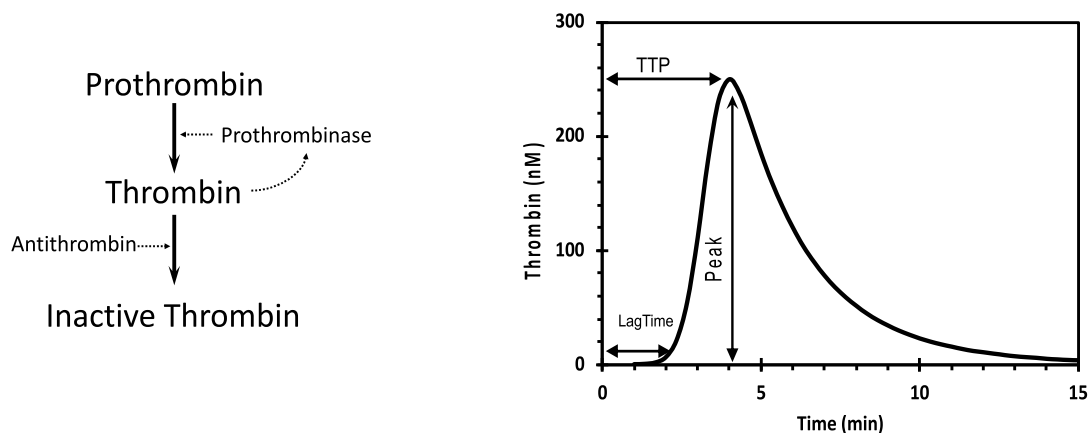
Fig. 1. A minimal schema of thrombin generation and a normal thrombin generation curve.

Nevertheless, in any simulation of thrombus formation thrombin generation (TG) is an essential component and needs to be accommodated. Belyaev et al. circumvent the problem by using a simplified reaction mechanism of thrombin generation to produce a limited set of differential equations that results in curves that have a certain similarity with real thrombin generation curves (Fig. 2 in ref. [1]).

That a limited set of differential equations based on a simplified clotting scheme can produce curves that cannot be distinguished from an experimentally obtained TG we have demonstrated before [12]. In that article we showed that TG-curves, obtained after injection of factor VIII into a haemophiliac can be perfectly simulated by a clotting mechanism in which factor VIII does not even participate. We concluded that even perfect resemblance of a simulated curve with an experimental curve does not validate the assumptions (reaction mechanisms and parameters) on which the simulation is based.

For the purpose of simulating thrombus formation this property can be used to our benefit: Creating TG-like curves with a limited set of differential equations, based on a fake mechanism, as proposed by Belyaev et al. is a perfectly legitimate practical shortcut.

There are two reasons why, once this approach is adopted, the smallest possible mechanism that yields useful and realistic TG-curves should be sought (Ockham's razor). In the first place, as long as the proposed mechanism suggests a basis in biological reality it may be mistaken for representing that reality. Some authors pretend that imperfect simulation can help to understand pathological thrombin generation [3,13,14]. We beg to differ. There is even a definite danger in extrapolating from differential equations to medical practice because physicians are unlikely to appreciate the problematic basis (i.e. problems 1–4 cited above) of the resulting insights and will mistake simulation for reality.

In the second place, any mechanism larger than the minimal one necessarily contains superfluous constants that blur the picture and introduce ambiguity. Then of course there is the additional benefit that small systems require less computational effort.

The simplest clotting scheme that intuitively explains the thrombin generation mechanism is shown in Fig. 1. We call it the "washbasin" model because the kinetics of the concentration of thrombin are those of the level of fluid in a washbasin in which a pail of water is quickly emptied. The inflow is the generation of thrombin from prothrombin by the action of prothrombinase. The velocity of outflow is determined by the level of the water, that, just like the inactivation of thrombin, can in good approximation be described by a first order exponential decay.

An essential feature of the blood coagulation mechanism, not included in the washbasin model *per se*, is the thrombin-mediated feedback on prothrombinase activity. Prothrombinase is a complex of factor Xa and factor V on phospholipid [5]. Its activity is critically dependent upon the feedback activation of factor V by thrombin [15]. This phenomenon is responsible for the lag-time that occurs before the thrombin burst. During the lag-time an incomplete TG-mechanism produces sub-nanomolar amounts of thrombin that cause activation of factor V (and of other factors, on purpose omitted from this minimal scheme). These first traces of thrombin thus allow the complete mechanism to produce the bulk of thrombin. In our model the incomplete starting mechanism is represented by a minimal amount of thrombin present at zero-time.

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Table 1
Characteristic values of thrombin generation in a normal population.

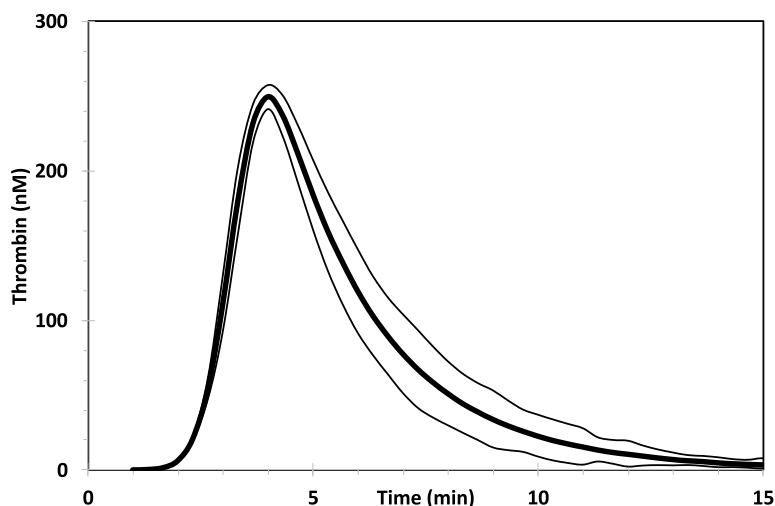|	ETP (nM.min)	Peak (nM)	TTP (min)
TF 1 pM	860 ± 140	140 ± 40	6 ± 1
TF 5 pM	900 ± 140	240 ± 40	$3 \pm 0,5$

Fig. 2. The normal thrombin generation curve and its boundaries. Thick line: Average TG-curve of 127 normal plasmas. Thin lines: Average plus and average minus one SD.

The model results in the following set of differential equations:

$$dP(t)/dt = -k_1 E(t) P(t)/(P(t) + K_m)$$
$$dE(t)/dt = k_1 E(t) P(t)/(P(t) + K_m) - k_2 E(t)$$
$$dT(t)/dt = k_2 E(t) - k_3 T(t)$$

To validate our simulations, we obtained TG-curves from 127 normal individuals at two different concentrations of trigger (tissue factor); at 5 pM the factors VIII, IX and XI are not involved, at 1 pM they are.

The values for the Endogenous thrombin potential (ETP, i.e. the area under the TG-curve) and peak are shown in Table 1.

In Fig. 2 we show the average curve and plus and minus one standard deviation. The data have been corrected for the effect of the thrombin substrate used to determine the curves, which inhibits thrombin decay and thereby reduces the values of ETP and peak as well as prolonging the lag-time and the time-to-peak by a factor of about 1.5. Before calculating the average, we have lined up the individual curves so as to have the same time to peak, otherwise shifts along the abscissa would have been interpreted as differences along the ordinate. In Figs. 3 and 4 we have shown the plus and minus 1 SD boundaries so as to enable the reader to judge the goodness of fit.

It appears that it suffices to postulate

  i: Michaelis–Menten kinetics of the formation of thrombin from prothrombin by prothrombinase, taking into account substrate consumption, i.e. not initial-rate Michaelis–Menten kinetics.
 ii: decay of thrombin proportional to its concentration.
iii: prothrombinase activity that is proportional to the amount of thrombin present (positive feedback) and that decays proportional to its concentration, with a decay constant independent of that of thrombin.

Another minimal set of differential equations that will yield a TG-like result we found to be.
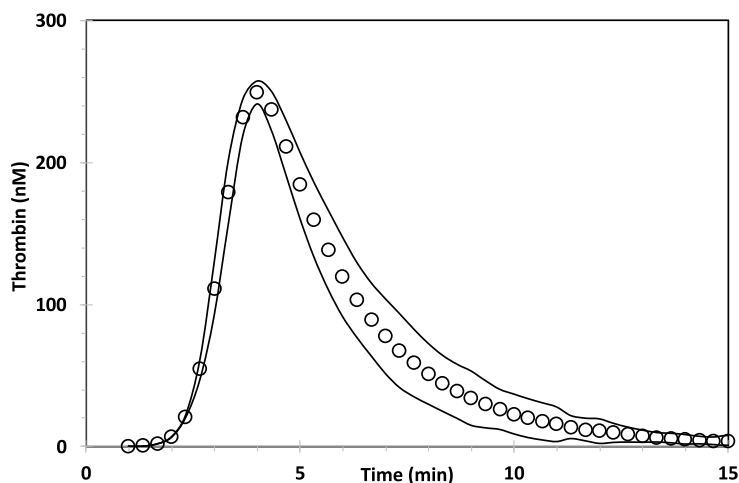
$$z'(t) = -b.z(t)$$
$$y'(t) = y(t).z(t)$$

Fig. 3. Comparison of real TG curves to those simulated with the minimal reaction mechanism. OO: Best fitting curve.
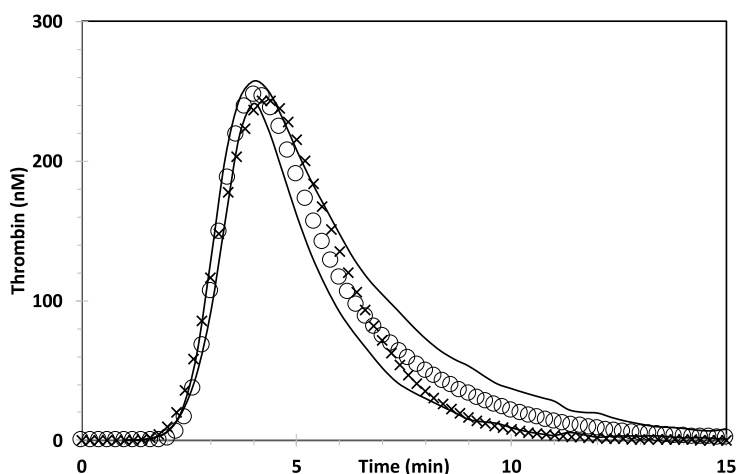

Fig. 4. Comparison of real TG curves to those simulated with formula A. Black lines: Boundaries of the normal range. XX: Best fitting curve using formula A. OO: Best fitting sum of two such curves.

This set has an analytical solution, that, respecting the appropriate boundary conditions, reads:

$$y(t) = a e^{-e^{b(p-t)}}$$

(*formula A*)

This formula has the important benefit that the constants have a well-defined physiological meaning. The constant *a* represents the area under the TG-curve (endogenous thrombin potential – ETP), *b* is the decay constant of thrombin (k_{dec}) in plasma and *p* is the time to peak, while $k_{dec} = 2{,}72 * peak/ETP$.

Fig. 4 shows that there is a fair resemblance between the curve based on formula A (crosses) and the experimental one. For most simulation purposes this might suffice. A perfect similarity can be obtained when two formulas of type A are added (circles). This has the disadvantage, however, that not three but six parameters are required. Moreover, the parameters have lost their physiological meaning, except that the sum of the constants *a* still represents the ETP.

In conclusion, we hope that this contribution will foster the modelling of thrombosis in two ways. In the first place by discouraging attempts to simulate thrombin generation on basis of our knowledge of the biochemistry of the process. In the second place by offering a simple technique for incorporating thrombin generation in larger models of thrombosis.

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