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Modelling of Enzymatic Action in Heterogeneous Phase Assay

Kiril Tenekedjiev

Technical University
1 Studentska Str., 9010 Varna, Bulgaria
E-mail: kiril@dilogos.com

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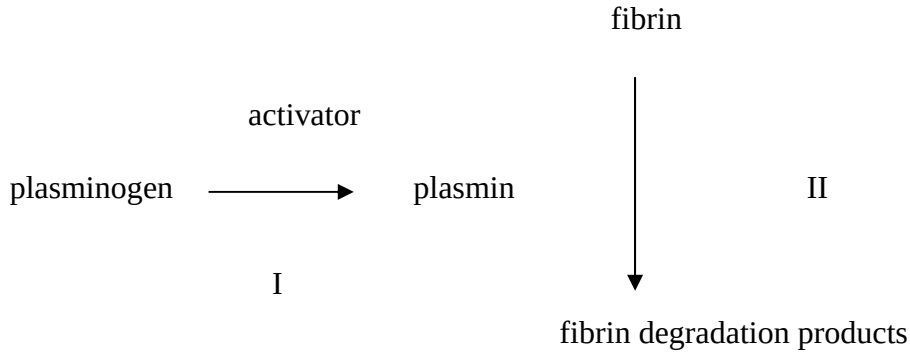
Abstract: *Thrombolysis is based on the action of a fluid-phase enzyme (plasminogen activator) on a gel-phase bound plasminogen with subsequent generation of an active protease (plasmin), which dissolves the gel matrix (fibrin). The design of new therapeutic tools requires adequate in silico models of the process for efficient preliminary testing and characterization of the thrombolytic agents. The present study describes an approach for simulation of the heterogeneous phase enzymatic process and identification of model parameters for the enzymes.*

Keywords: *Thrombolysis, Simulation, Heterogenous phase.*

Introduction

Platelets and fibrin provide a temporary hemostatic plug at sites of blood vessel wall injury, which is removed by the physiological thrombolytic machinery following restoration of wall integrity [3]. The potential of this machinery is exploited by therapeutic strategies designed to dissolve thrombi blocking the arteries in ischemic cardiovascular and cerebrovascular diseases, the leading cause of death in the world. Because the solid matrix of the thrombi consists of fibrin, the classic concept of thrombolysis restricts the dissolution mechanisms to fibrinolysis, which proceeds according to Scheme 1.



Scheme 1

The overall process can conveniently be broken down into two stages: (I) is the activation of plasminogen to plasmin by a plasminogen activator (PA) and (II) is the degradation of the structural protein, fibrin, to fibrin degradation products (FDP). The participants in stage (I) are assembled on the surface of the fibrin fibers and the propagation of plasmin action in blood circulation is limited by the potent plasma inhibitor system. Thus, the fibrin plug is a self-destructive compartment, which provides the transient scaffold for the arrangement of the

fibrinolytic machinery. The surface of this compartment is a fluid-solid interface, through which, under the hemodynamic conditions of blood circulation, diffusion and permeation maintain a continuous supply of fibrinolytic components. The present lecture discusses fibrinolysis from the aspect of the interfacial phenomena related to its initiation and progress and the methodology applicable for adequate evaluation of these phenomena.

Methodology for evaluation of interfacial fibrinolysis

Recently it has proved possible to separate reaction (I) from reaction (II) in Scheme 1 for most PAs by manipulating the ionic strength of the clotting stage, to produce a transparent fibrin. In this way reaction (I) can be studied in isolation in a system containing fibrin. This is achieved by first of all mixing fibrinogen, thrombin and plasminogen to create a transparent clot in a microtitre plate to which can be added PA subsequently. If plasmin-sensitive chromogenic substrate is added with PA, the plasmin generated as the PA enters the clot can be easily monitored. A detailed description of the method has been published elsewhere [4]. Such a method is quite straightforward in design and at its simplest may be viewed as a chromogenic reaction taking place on a pad of (invisible) fibrin. The components are all purified and hence it is possible that the reaction can be standardized to allow comparison of results between laboratories.

A critical feature of this assay is the isolation of the PA step from the fibrin lysis reaction. The intention is to study the fine details of the PA kinetics and better understand the regulation of plasmin generation. From the point of view of developing and improving thrombolytic therapy the behaviour of the PA is inevitably the focus of attention. The composition of the clot target is “a given” and the characteristics of the PA need to be optimized to promote rapid dissolution. The only way of doing this is to maximize the generation of plasmin (while minimizing the side effects) and this is why the characteristics of PAs are of overriding importance.

Modelling of fibrinolysis kinetics

By looking at a range of plasminogen substrate concentrations in this assay system we can begin to determine apparent K_M and k_{cat} values for PAs in this system. This is not new and many such values are available in the literature for different PAs. Unfortunately, it is well known that these published values are widely disparate for a variety of reasons [5], including different methodologies or conditions. However, by using a simple, standardized method some sources of variation can be eliminated and regulation of enzyme activity can be investigated. Moreover, because we have the opportunity to estimate the generation of plasmin in SI units (e.g. pM/s) we can attempt to understand the regulation of PA in terms of binding interactions (K_d) and real K_M and k_{cat} values for the action of PA on plasminogen in association with fibrin. This is crucial as it is well known that the binding of plasminogen and many (though not all) PAs with fibrin is key for the regulation of activity. Thus, it is possible, in theory at least, to quantitate the binding of PA and plasminogen to fibrin in the same format and ultimately take all the thermodynamic and kinetic parameters and build a computer model of PA activity in the presence of fibrin.

Based on the simplicity of Scheme 1 and the apparent simplicity of the clear clot assay method, modeling fibrinolysis may appear to be a trivial problem. However, there are many complications. First of all there is the difficulty associated with the inherent heterogeneity of the system: fibrin is a solid matrix and reactions take place on this protein by adsorbing reactants from the surrounding solution, as discussed above. Enzyme kinetics studies have

been developed, and traditionally take place in, homogeneous solutions. However, even when model systems are used to study PAs that include soluble fibrin analogs as models for real fibrin, the standard approaches of Michaelis-Menten kinetics cannot be simply applied. For instance, in the case of tPA as activator, and some form of soluble fibrin analog as template (for example CNBr fragments of fibrinogen or DESAFIB) there is an equilibrium between activator and template and plasminogen and template. Hence changes in the concentration of plasminogen (for example when determining apparent K_M and k_{cat} values) will affect the position of this equilibrium. Similarly, variations in the concentration of tPA also affect the equilibrium of the tPA-template complex. In other words, for conventional enzyme substrate reactions, where substrate is not limiting one would expect a linear relationship between enzyme concentration and reaction rate in solution; but if a template is involved and the rate depends on bound enzyme, then the relationship will be hyperbolic. Moreover, variations in template concentration lead to bell shaped responses in activity. This is a natural consequence of equilibrium reactions between template and enzyme and substrate to form a ternary complex. The importance of ternary complex formation has been investigated in a number of studies and models proposed to help understand PA activity regulation and observed effects on apparent K_M and k_{cat}.

Optimized kinetic parameters for a PA may be self evidently a good target, but it should be borne in mind that plasminogen concentrations may be reduced during therapy, highlighting the need for a low K_M. This factor also relates to fibrin specificity since a PA that only works in the small confines of a clot, rather than systemically in the circulation, is going to have a lesser impact on the concentration of circulating plasminogen. Fibrin binding may be an important consideration but it is not clear whether very high affinity binding is desirable. tPA binds tightly to fibrin and lysis is believed to proceed in a narrow zone a few microns thick from the surface of the clot. However, reteplase (a recombinant variant of tPA consisting only of its kringle-2 and catalytic domains), which displays weaker fibrin binding is at least no worse than tPA in clinical trials, and may benefit from improved diffusion through the clot. Weaker fibrin binding in conjunction with better catalytic parameters are the considerations behind the engineering of a new thrombolytic agent, amediplase, which is a chimera of kringle-2 of tPA and the protease domain of urokinase [7]. It remains to be seen whether this approach will offer any significant benefits therapeutically.

Resolving kinetic parameters from the clear clot plasminogen activation assay

Legend

Volumes:

- V_d – gel layer
- V_u – fluid layer
- V_R – reactive layer inside V_d

Concentrations:

- $PAPlg$ – plasminogen activator-plasminogen complex in the reactive layer
- PA_R – free plasminogen activator in the reactive layer (not in complex with the plasminogen)
- PA_{Rt} – total plasminogen activator in the reactive layer: $PA_{Rt} = PA_R + PAPlg$
- PA_u – free plasminogen activator in the fluid layer:

$PA_u = PA_{Rt}/f$

Plg_R – plasminogen bound to fibrin in the reactive layer (not in complex with the plasminogen activator)

Plg_{Rt} – total plasminogen in the reactive layer:
$Plg_{Rt} = Plg_R + PAPlg$

Plg_d – plasminogen bound to fibrin in the sub-volume of the gel layer below the reactive layer:
$Plg_d = Plg_{Rt}$

Plg_u – free plasminogen in the reactive and in the fluid layers

ES – plasmin-substrate complex in the reactive and in the fluid layers

EFg – plasmin-fibrin complex in the reactive layer

E – free plasmin in the reactive and in the fluid layers (not in complex with the synthetic substrate or with the fibrin)

E_t – fictitious total enzyme concentration in the reactive and in the fluid layers:
$E_t = E + ES + EFg*V_R/(V_R + V_u)$

Fg_R – fibrin in the reactive layer (not in complex with the plasmin)

Fg_{Rt} – total fibrin in the reactive layer:
$Fg_{Rt} = Fg_R + EFg$

Fg_0 – fibrin in the sub-volume of the gel layer below the reactive layer:
$Fg_0 = Fg_{Rt}$

S_u – synthetic substrate in the reactive and the fluid layers

S_d – synthetic substrate in the sub-volume of the gel layer below the reactive layer

FDP – fibrin degradation product in the reactive and in the fluid layers

P – product in the reactive layer and in the gel layers

Subscript 0 indicates the initial volumes and concentrations, without subscript 0 the volumes and concentrations at time t.

Composition of the system

The system contains a lower solid (gel) phase V_d and an upper fluid phase V_u. The reactions proceed mainly in the interface layer with volume V_R, which is a sub-volume of V_d.

The sub-volume V_d below V_R consists of the solid phase Fg_0 and the attached Plg_d as well as the soluble S_d and P. The plasminogen can move through the intrafiber capillaries, that's why its concentration is varies in time.

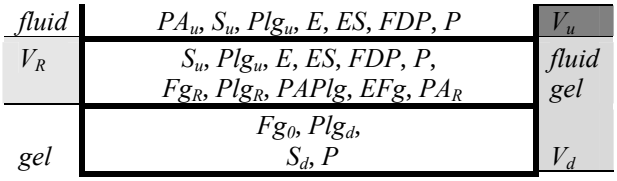
The V_u volume consists of the soluble FDP, PA_u, S_u, ES, E, Plg_u, and P.

The V_R volume contains soluble E, ES, FDP, Plg_u (all distributed at uniform concentrations in V_u+V_R), and soluble P (distributed at uniform concentration in V_d+V_u). The soluble S_{u0} is initially in V_{u0} and in V_{R0}, but it diffuses continuously into V_d. So S_u is present in V_R at varying concentration. The solid phase of V_R contains constant Fg_{Rt} (because of the progressive dissolution of fibrin Fg_{Rt} coincides with Fg_0) and as well as varying EFg, Plg_R, PA_R and $PAPlg$. Here PA_R is the concentration of plasminogen activator bound to the fibrinogen in V_R.

The V_R volume moves down at the rate Fg is converted to FDP, but it increases in depth at a constant rate because of the penetration of the plasminogen activator into V_d (see equation for V_R below). As a result part of the volume V_R is continuously transformating into V_u. The attached plasminogen in that transrforming volume is released to soluble plasminogen, while the plasminogen activator-plasminogen complex is released to solubale plasminogen and to

plasmin (α is the fraction of the complex released as plasmin and (1-α) is the fraction of the complex released as soluble plasminogen).

The V_R volume moves down (because *FDP* formation), but it increases in depth at a constant rate because of the penetration of the plasminogen activator into V_d (see equation for V_R below). As a result part of the sub-volume V_d below V_R is continuously transformating into V_R. The substrade in that transrforming volume is changing to substrade in the fluid phase.



Geometric dependencies

$$V_d = V_{d0} + V_{u0} - V_u \tag{g.1}$$

$$V_{d0} - V_d = V_u - V_{u0} \tag{g.2}$$

$$V_R = V_{R0} + v_{VR}.t \tag{g.3}$$

taking time derivative in (g.1)

$$\frac{dV_d}{dt} = -\frac{dV_u}{dt} \tag{g.4}$$

taking time derivative in (g.3)

$$\frac{dV_R}{dt} = v_{VR} \tag{g.5}$$

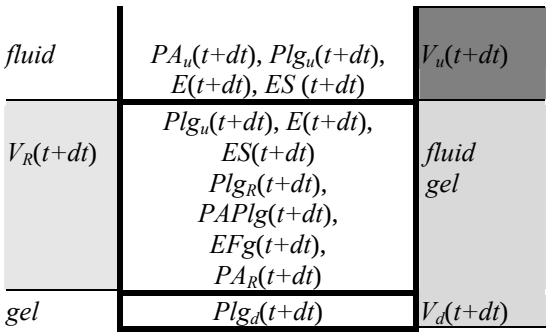
Simultaneous processes in the system

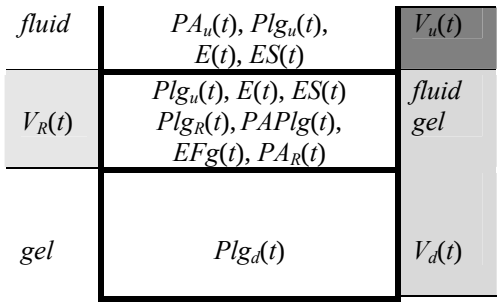
Process I - plasmin concentration's change in V_R and V_u

Plasmin formation in V_R

$$PA_R + Plg_R \underset{k_{-1P}}{\overset{k_{1P}}{\rightleftarrows}} PAPlg \xrightarrow{k_{catP}} PA_R + E_t \text{ , where } K_M^P = \frac{k_{-1P} + k_{catP}}{k_{1P}}$$

In V_u – PA_u, E_t, Plg_u
In V_R – PA_R, PAPlg, E_t, Plg_R, Plg_u
In sub-volume V_d below V_R – Plg_d





The assumption $\frac{d\left(PAPlg.V_R\right)}{dt} \approx 0$ leads to:

$$PAPlg = \frac{PA_R.Plg_R}{K_M^P} \tag{1.1}$$

The differential balance of the total plasminogen is:

$$\begin{aligned} &E_t\left(t+dt\right).\left[V_u\left(t+dt\right)+V_R\left(t+dt\right)\right]-E_t\left(t\right).\left[V_u\left(t\right)+V_R\left(t\right)\right]= \\ &=\int_t^{t+dt} k_{catP}.PAPlg\left(\tau\right).V_R\left(\tau\right)d\tau+\int_t^{t+dt}\alpha.PAPlg\left(\tau\right).dV_u\left(\tau\right) \end{aligned} \tag{I.2}$$

As long as $k_{catP}.PAPlg\left(\tau\right).V_R\left(\tau\right)$ is a continuous function of the time τ, $\exists r \in \left(0;1\right)$ that:

$$\begin{aligned} &\int_t^{t+dt} k_{catP}.PAPlg\left(\tau\right).V_R\left(\tau\right)d\tau= \\ &=k_{catP}.PAPlg\left[t+r.\left(t+dt-t\right)\right].V_R\left[t+r.\left(t+dt-t\right)\right].\left(t+dt-t\right)= \\ &=k_{catP}.PAPlg\left(t+r.dt\right).V_R\left(t+r.dt\right).dt \end{aligned} \tag{I.3}$$

As long as $\alpha.PAPlg\left(\tau\right).dVu\left(\tau\right)$ is continuous function of the time τ, $\exists s \in \left(0;1\right)$ that:

$$\begin{aligned} &\int_t^{t+dt}\alpha.PAPlg\left(\tau\right).dV_u\left(\tau\right)=\int_t^{t+dt}\alpha.PAPlg\left(\tau\right).V_u^{'}\left(\tau\right).d\tau= \\ &=\alpha.PAPlg\left[t+s.\left(t+dt-t\right)\right].V_u^{'}\left[t+s.\left(t+dt-t\right)\right].\left(t+dt-t\right)= \\ &=\alpha.PAPlg\left(t+s.dt\right).V_u^{'}\left(t+s.dt\right).dt \end{aligned} \tag{I.4}$$

The two sides of (I.2) are divided to the differential of time *dt*, and according to (I.3), (I.4):

$$\frac{d\left[E_t.\left(V_u+V_R\right)\right]}{dt}=k_{catP}.PAPlg.V_R+\alpha.PAPlg.\frac{dV_u}{dt} \tag{I.5}$$

According to (mf.4):

$$\left(V_u + V_R\right)\frac{dE_t}{dt} + E_t\left(\frac{dV_u}{dt} + \frac{dV_R}{dt}\right) = k_{catP}.PAPlg.V_R + \alpha.PAPlg.\frac{dV_u^{'}}{dt} \qquad \text{(I.6)}$$

Dividing by V_u+V_R and according to (g.5):

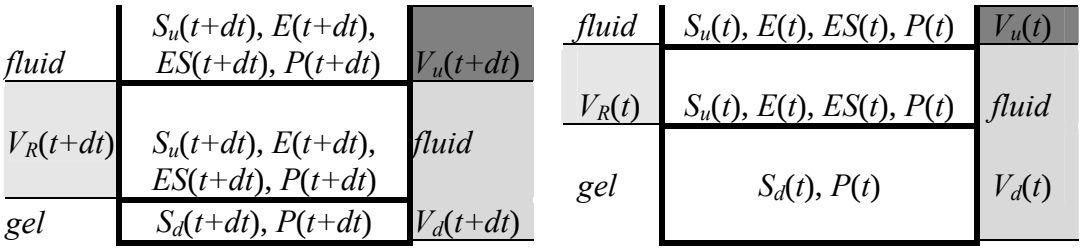
$$\frac{dE_t}{dt} = \left(k_{catP} + \alpha\frac{k_{catF}.EFg}{Fg_0}\right).PAPlg\frac{V_R}{V_u+V_R} - E_t\frac{\frac{dV_u}{dt} + v_{VR}}{V_u+V_R} \qquad \text{(I.7)}$$

Process II – product concentration's change in V_d and V_u

Product formation in V_u and V_R

$$E + S_u \underset{k_{-1S}}{\overset{k_{1S}}{\rightleftarrows}} ES \xrightarrow{k_{catS}} E + P \text{ , where } K_M^S = \frac{k_{-1S} + k_{catS}}{k_{1S}}$$

In V_u – E, S_u, ES, P
In V_R – E, S_u, ES, P
In sub-volume V_d below V_R – S_d, P



The assumption $\frac{d\left[ES.\left(V_R + V_u\right)\right]}{dt} \approx 0$ leads to:

$$ES = \frac{E.S_u}{K_M^S} \qquad \text{(II.1)}$$

The differential balance of the product is:

$$P_t\left(t+dt\right).\left(V_{u0}+V_{d0}\right) - P_t\left(t\right).\left(V_{u0}+V_{d0}\right) = \int_t^{t+dt} k_{catS}.ES\left(\tau\right).\left[V_u\left(\tau\right)+V_R\left(\tau\right)\right]d\tau \qquad \text{(II.2)}$$

As long as $k_{catS}.ES\left(\tau\right).\left[V_u\left(\tau\right)+V_R\left(\tau\right)\right]$ is continuous function of the time τ, $\exists r \in \left(0;1\right)$ that:

$$\int_{t}^{t+dt} k_{catS}.ES(\tau).\left[V_u(\tau)+V_R(\tau)\right]d\tau =$$
$$= k_{catS}.ES\left[t+r\left(t+dt-t\right)\right].\left\{V_u\left[t+r\left(t+dt-t\right)\right]+V_R\left[t+r\left(t+dt-t\right)\right]\right\}.\left(t+dt-t\right) = \quad \text{(II.3)}$$
$$= k_{catS}.ES\left(t+r.dt\right).\left[V_u\left(t+r.dt\right)+V_R\left(t+r.dt\right)\right].dt$$

The two sides of (II.2) are divided to the differential of time *dt*, and according to (II.3):

$$\frac{d\left[P.\left(V_{u0}+V_{d0}\right)\right]}{dt} = k_{catS}.ES.\left(V_u+V_R\right) \quad \text{(II.4)}$$

$$\left(V_{u0}+V_{d0}\right)\frac{dP}{dt} = k_{catS}.ES.\left(V_u+V_R\right) \quad \text{(II.5)}$$

Dividing (II.5) to $V_{u0}+V_{d0}$:

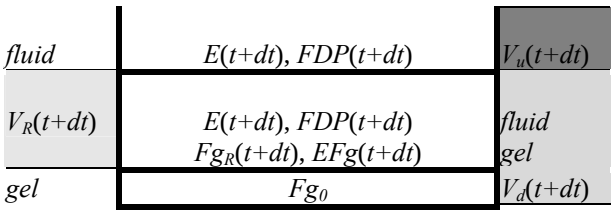
$$\frac{dP}{dt} = k_{catS}.ES\frac{V_u+V_R}{V_{d0}+V_{u0}} \quad \text{(II.6)}$$

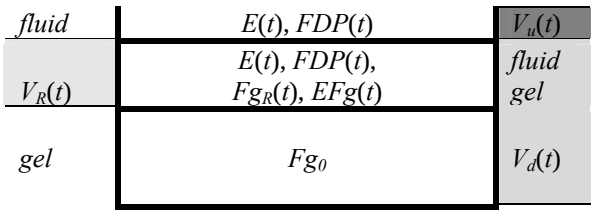
Process III – fibrin degradation product concentration's change in V_R and V_u

Fibrin degradation in V_R

$$E+Fg_R \underset{k_{-1F}}{\overset{k_{1F}}{\rightleftarrows}} EFg \xrightarrow{k_{catS}} E+FDP \text{ , where } K_M^F = \frac{k_{-1F}+k_{catF}}{k_{1F}}$$

In V_u – *E*, *FDP*
In V_R – Fg_R, *E*, *EFg*, *FDP*
In sub-volume V_d below V_R – Fg_0





The assumption $\frac{d\left(EFg.V_R\right)}{dt} \approx 0$ leads to:

$$EFg = \frac{E.Fg_R}{K_M^F} \quad \text{(III.1)}$$

The differential balance of the fibrin degradation product is:

$$FDP(t+dt).\left[V_u(t+dt)+V_R(t+dt)\right]-FDP.\left[V_u(t)+V_R(t)\right]= \\ =\int_t^{t+dt} k_{catF}.EFg(\tau).V_R(\tau)\,d\tau \qquad \text{(III.2)}$$

As long as $k_{catF}.EFg(\tau).V_R(\tau)$ is continuous function of the time τ, $\exists r \in (0;1)$ that:

$$\int_t^{t+dt} k_{catF}.EFg(\tau).V_R(\tau)\,d\tau = \\ =k_{catF}.EFg\left[t+r.(t+dt-t)\right].V_R\left[t+r.(t+dt-t)\right].(t+dt-t)= \\ =k_{catF}.EFg(t+r.dt).V_R(t+r.dt).dt \qquad \text{(III.3)}$$

The two sides of (III.2) are divided to the differential of time *dt*, and according to (III.3):

$$\frac{d\left[FDP.(V_u+V_R)\right]}{dt}=k_{catF}.EFg(t+r.dt).V_R(t+r.dt)=k_{catF}.EFg(t).V_R(t)= \\ =k_{catF}.EFg.V_R \qquad \text{(III.4)}$$

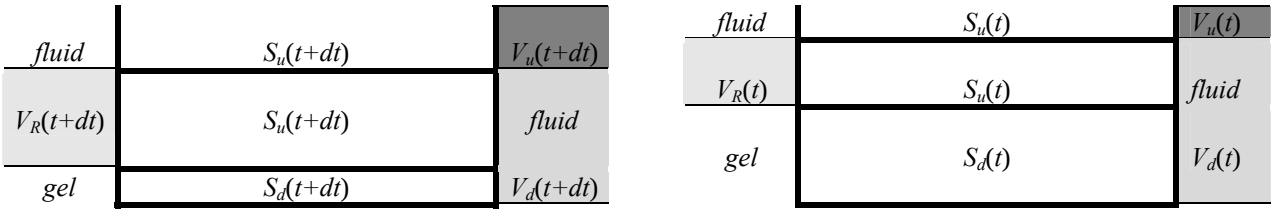
$$(V_u+V_R)\frac{dFDP}{dt}+FDP\left(\frac{dV_u}{dt}+\frac{dV_R}{dt}\right)=k_{catF}.EFg.V_R \qquad \text{(III.5)}$$

Dividing to V_u+V_R and according to (g.5):

$$\frac{dFDP}{dt}=k_{catF}.EFg\frac{V_R}{V_u+V_R}-FDP\frac{\frac{dV_u}{dt}+v_{VR}}{V_u+V_R} \qquad \text{(III.6)}$$

Process IV – substrate diffusion through the inner boundary between V_R and V_d

In $V_u - S_u$
In $V_R - S_u$
In sub-volume V_d below $V_R - S_d$



The differential balance of the substrate in the gel layer under the reactive layer is:

$$S_d(t+dt).\left[V_d(t+dt)-V_R(t+dt)\right]-S_d(t).\left[V_d(t)-V_R(t)\right]= \\ =\int_t^{t+dt} k.B.\left[S_u(\tau)-S_d(\tau)\right]d\tau-\int_t^{t+dt} S_d(\tau)\,d\left[V_u(\tau)+V_R(\tau)\right] \qquad \text{(IV.1)}$$

As long as $k.B.\left[S_u(\tau)-S_d(\tau)\right]$ is continuous function of the time τ, $\exists r\in(0;1)$ that:

$$\int_t^{t+dt} k.B.\left[S_u(\tau)-S_d(\tau)\right]d\tau= \\ =k.B.\left\{S_u\left[t+r.(t+dt-t)\right]-S_d\left[t+r.(t+dt-t)\right]\right\}.(t+dt-t)= \\ =k.B.\left[S_u(t+r.dt)-S_d(t+r.dt)\right].dt \qquad \text{(IV.2)}$$

As long as $S_d(\tau)\left[V_u'(\tau)+V_R'(\tau)\right]$ is continuous function of the time τ, $\exists q\in(0;1)$ that:

$$\int_t^{t+dt} S_d(\tau)\,d\left[V_u(\tau)+V_R(\tau)\right]=\int_t^{t+dt} S_d(\tau)\left[V_u'(\tau)+V_R'(\tau)\right]dt= \\ =S_d\left[t+q.(t+dt-t)\right].\left\{V_u'\left[t+q.(t+dt-t)\right]+V_R'\left[t+q.(t+dt-t)\right]\right\}.(t+dt-t)= \\ =S_d(t+q.dt).\left[V_u'(t+q.dt)+V_R'(t+q.dt)\right].dt \qquad \text{(IV.3)}$$

The two sides of (IV.1) are divided to the differential of time *dt*, according to (IV.2) and to (IV.3):

$$\frac{d\left[S_d.(V_d-V_R)\right]}{dt}=k.B.(S_u-S_d)-S_d\frac{d(V_u+V_R)}{dt} \qquad \text{(IV.4)}$$

Taking the derivatives in (IV.4):

$$(V_d-V_R).\frac{dS_d}{dt}-S_d.\frac{dV_d}{dt}-S_d\frac{dV_R}{dt}=k.B.(S_u-S_d)-S_d.\frac{dV_u}{dt}-S_d\frac{dV_R}{dt} \qquad \text{(IV.5)}$$

Dividing to V_d-V_R and taking into account (g.4):

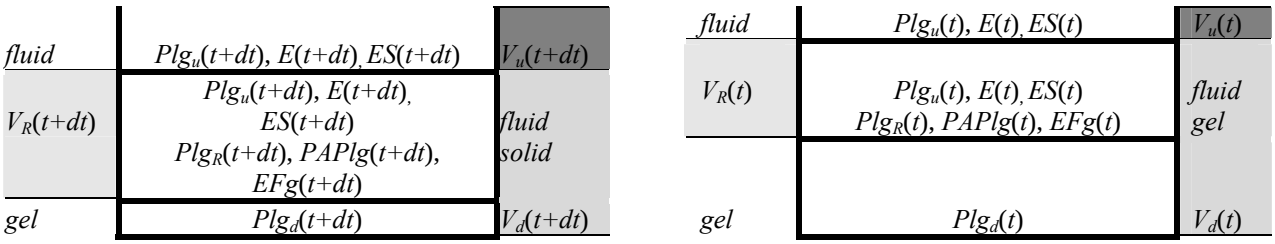
$$\frac{dS_d}{dt}=\frac{k.B.(S_u-S_d)}{V_d-V_R} \qquad \text{(IV.6)}$$

As long as the substrate diffusion from fluid to gel is different from that in the opposite direction, then:

$$k = \begin{cases} k_1 & S_u \geq S_d \\ k_2 & S_u < S_d \end{cases} \qquad \text{(IV.7)}$$

Process V – plasminogen concentration's change in the gel layer

In V_u – Plg_u, E_t
In V_R – Plg_u, Plg_R, $PAPlg$, E_t
In sub-volume V_d below V_R – Plg_d



The differential balance for the plasminogen attached to the fibrin in the gel layer is:

$$\begin{aligned} &\left[Plg_R(t+dt)+PAPlg(t+dt)\right].V_R(t+dt)-\left[Plg_R(t)+PAPlg(t)\right].V_R(t)+ \\ &+Plg_d(t+dt).\left[V_d(t+dt)-V_R(t+dt)\right]-Plg_d(t).\left[V_d(t)-V_R(t)\right]= \\ &=-\left\{E_t(t+dt).\left[V_u(t+dt)+V_R(t+dt)\right]-E_t(t).\left[V_u(t)+V_R(t)\right]\right\}- \\ &-\int_t^{t+dt}\left[Plg_R(\tau)+(1-\alpha)PAPlg(\tau)\right]dV_u(\tau) \end{aligned} \qquad \text{(V.1)}$$

The integrand of (III.1) is simplified according to (plt.1) and (plt.2).

$$\begin{aligned} &Plg_R(\tau)+(1-\alpha).PAPlg(\tau)=Plg_R(\tau)+PAPlg(\tau)-\alpha.PAPlg(\tau)= \\ &=Plg_d(\tau)-\alpha.PAPlg(\tau) \end{aligned} \qquad \text{(V.2)}$$

As long as $\left[Plg_R(\tau)-\alpha.PAPlg(\tau)\right].V_u'(\tau)$ $\alpha.PAPlg(\tau).dVu(\tau)$ is continuous function of the time τ, $\exists q \in (0;1)$ that:

$$\begin{aligned} &\int_t^{t+dt}\left[Plg_R(\tau)+(1-\alpha)PAPlg(\tau)\right]dV_u(\tau)=\int_t^{t+dt}\left[Plg_d(\tau)-\alpha.PAPlg(\tau)\right].V_u'(\tau)dt= \\ &=\left\{Plg_d\left[t+q.(t+dt-t)\right]-\alpha.PAPlg\left[t+q.(t+dt-t)\right]\right\}.V_u'\left[t+q.(t+dt-t)\right].(t+dt-t)= \\ &=Plg_d(t+q.dt).V_u'(t+q.dt).dt-\alpha.PAPlg(t+q.dt).V_u'(t+q.dt).dt \end{aligned} \qquad \text{(V.3)}$$

The two sides of (III.1) are divided to the differential of time *dt*. Then according to (III.2):

$$\frac{d\left(Plg_{Rt}.V_R\right)}{dt}+\frac{d\left[Plg_d.\left(V_d-V_R\right)\right]}{dt}=-\frac{d\left[E_t.\left(V_u+V_R\right)\right]}{dt}-Plg_d\frac{dV_u}{dt}+\alpha.PAPlg\frac{dV_u}{dt} \qquad (V.4)$$

Taking the derivatives in LHS of (II.4) and using (I.5):

$$\begin{aligned} &Plg_d\frac{dV_R}{dt}+V_R\frac{dPlg_d}{dt}+Plg_d\frac{dV_d}{dt}-Plg_d\frac{dV_R}{dt}+V_d\frac{dPlg_d}{dt}-V_R\frac{dPlg_d}{dt}= \\ &=-k_{catP}.PAPlg.V_R-\alpha.PAPlg\frac{dV_u}{dt}-Plg_d\frac{dV_u}{dt}+\alpha.PAPlg\frac{dV_u}{dt} \end{aligned} \qquad (V.5)$$

After canceling out of the opposite terms, dividing by V_d, and according to (g.4):

$$\frac{dPlg_d}{dt}=-k_{catP}.PAPlg\frac{V_R}{V_d} \qquad (V.6)$$

Concentration dependencies for the system

Fictitious total plasmin concentation in the reactive and in the fluid layers

In V_u – *E, ES*
In V_R –*E, ES, EFg*
In sub-volume V_d below V_R – *nothing*

fluid	*E, ES*	V_u
V_R	*E, ES, EFg*	*fluid gel*
gel		V_d

$E_t.(V_u + V_R) = E.(V_u + V_R)+ ES.(V_u + V_R)+ EFg.V_R$ (et.1)

from (II.1) and (III.1) follows:

$$E=\frac{E_t}{1+\frac{S_u}{K_M^S}+\frac{Fg_R.V_R}{K_M^F(V_u+V_R)}} \qquad (et.2)$$

Fibrin concentrations in the gel layer

In V_u – *nothing*
In V_R –Fg_R, *EFg*
In sub-volume V_d below V_R – Fg_0

fluid		V_u
V_R	Fg_R, *EFg*	*fluid*
gel	Fg_0	V_d

$Fg_{Rt} = Fg_R + EFg$ (fgt.1)

but:

$Fg_{Rt} = Fg_0$ (fgt.2)

then:

$Fg_R = Fg_0 - EFg$ (fgt.3)

from (III.1) follows:

$$Fg_R = \frac{Fg_0}{1 + \frac{E}{K_M^F}}$$ (fgt.4)

Plasminogen concentrations in the gel layer

In V_u – *nothing*
In V_R –Plg_R, *PAPlg*
In sub-volume V_d below V_R – Plg_d

fluid		V_u
V_R	Plg_R, *PAPlg*	*fluid*
gel	Plg_d	V_d

$Plg_{Rt} = Plg_R + PAPlg$ (plt.1)

but

$Plg_{Rt} = Plg_d$ (plt.2)

then:

$Plg_R = Plg_d - PAPlg$ (plt.3)

Plasminogen activator concentrations in the reactive and in the fluid layers

In V_u – PA_u
In V_R –PA_R, *PAPlg*
In sub-volume V_d below V_R – *nothing*

fluid	PA_u	V_u
V_R	PA_R, *PAPlg*	*fluid*
gel		V_d

$PA_R = PA_{Rt} - PAPlg$ (pat.1)

from (I.1) follows:

$$PA_R = \frac{PA_{Rt}}{1 + \frac{Plg_R}{K_M^P}} \quad \text{(pat.2)}$$

$$PA_u = PA_{Rt}/f \quad \text{(pat.3)}$$

where

$$f = \begin{cases} C & if \quad C > f_0 \\ f_0 & if \quad C \le f_0 \end{cases} \quad \text{(pat.4)}$$

for

$$C = f_0 + \kappa.EFg - \lambda.FDP \quad \text{(pat.5)}$$

or

$$C = f_0 + \kappa.E_t - \lambda.FDP \quad \text{(pat.6)}$$

or

$$C = f_0 + \kappa.E - \lambda.FDP \quad \text{(pat.7)}$$

Mass dependencies in the system

Mass preservation law for the substrate

In V_u – S_u, ES, P
In V_R – S_u, ES, P
In sub-volume V_d below V_R – S_d, P

fluid	S_u, ES, P	V_u
V_R	S_u, ES, P	*fluid*
gel	S_d, P	V_d

$$S_{u0}^0.V_{u0} = S_{u0}.(V_{u0} + V_{R0}) \quad \text{(ms.1)}$$

$$S_{u0}.(V_{u0} + V_{R0}) = S_u.(V_u + V_R) + P.(V_{u0} + V_{d0}) + ES.(V_u + V_R) + S_d.(V_d - V_R) \quad \text{(ms.2)}$$

From (ms.1) and (II.1) follows:

$$S_{u0}^0.V_{u0} = S_u.(V_u + V_R) + P.(V_{d0} + V_{u0}) + \frac{E.S_u}{K_M^S.}(V_u + V_R) + S_d.(V_d - V_R) \quad \text{(ms.3)}$$

Rearranging for S_u:

$$S_u = \frac{S_{u0}^0.V_{u0} - P.(V_{d0} + V_{u0}) - S_d.(V_d - V_R)}{\left(1 + \frac{E}{K_M^S}\right).(V_u + V_R)} \quad \text{(ms.4)}$$

Mass preservation law for the fibrin

In V_u – FDP
In V_R – FDP, Fg_R, EFg
In sub-volume V_d below V_R – Fg_0

$$Fg_0.V_{d0} = Fg_0.(V_d - V_R) + FDP.(V_u + V_R) + EFg.\ V_R + Fg_R.V_R \qquad \text{(mf.1)}$$

from (g.2) and (fgt.3) follows:

$$V_u = \frac{Fg_0.V_{u0} + FDP.V_R}{Fg_0 - FDP} \qquad \text{(mf.2)}$$

taking time derivative in (mf.1):

$$0 = Fg_0 \frac{dV_d}{dt} - Fg_0 \frac{dV_R}{dt} + \frac{d\left[FDP.(V_u + V_R)\right]}{dt} + Fg_0 \frac{dV_R}{dt} \qquad \text{(mf.3)}$$

After dividing to Fg_0 with respect to (g.4) and (III.4) it follows that:

$$\frac{dV_u}{dt} = \frac{k_{catF}.EFg.V_R}{Fg_0} \qquad \text{(mf.4)}$$

Mass preservation law for the plasminogen

In V_u – E_t, Plg_u
In V_R – E_t, Plg_R, Plg_d, $PAPlg$
In sub-volume V_d below V_R – Plg_d

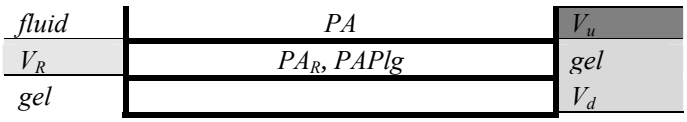
$$Plg_{d0}.V_{d0} = Plg_d.(V_d - V_R) + Plg_R.\ V_R + E_t.(V_u + V_R) + Plg_u.(V_u + V_R) + PAPlg.\ V_R \qquad \text{(mpl.1)}$$

According to (plt.3) and after dividing to $(V_u + V_R)$:

$$Plg_u = \frac{Plg_{d0}.V_{d0} - Plg_d.V_d}{V_u + V_R} - E_t \qquad \text{(mpl.2)}$$

Mass preservation law for the plasminogen activator

In V_u – PA_u
In V_R – PA_{Rt}
In sub-volume V_d below V_R – *nothing*



$$PA_{u0}^{0}.V_{u0} = PA_{u0}.V_{u0} + (PA_{R0} + PAPlg_0).V_{R0} \quad \text{(mpa.1)}$$

$$PA_{u0}.V_{u0} + (PA_{R0} + PAPlg_0).V_{R0} = PA_{Rt}.V_R + PA_u.V_u \quad \text{(mpa.2)}$$

from (mpa.1) and (pat.3) follows:

$$PA_{Rt} = \frac{f.PA_{u0}^{0}.V_{u0}}{f.V_R + V_u} \quad \text{(mpa.3)}$$

Differential equations

$$\left|\begin{array}{l} \dfrac{dE_t}{dt} = \left(k_{catP} + \alpha\dfrac{k_{catF}.EFg}{Fg_0}\right).PAPlg\dfrac{V_R}{V_u+V_R} - E_t\dfrac{\dfrac{dV_u}{dt}+v_{VR}}{V_u+V_R} \\ \\ \dfrac{dP}{dt} = k_{catS}.ES\dfrac{V_u+V_R}{V_{d0}+V_{u0}} \\ \\ \dfrac{dFDP}{dt} = k_{catF}.EFg\dfrac{V_R}{V_u+V_R} - FDP\dfrac{\dfrac{dV_u}{dt}+v_{VR}}{V_u+V_R} \\ \\ \dfrac{dS_d}{dt} = \dfrac{k.B.(S_u-S_d)}{V_d-V_R} \\ \\ \dfrac{dPlg_d}{dt} = -k_{catP}.PAPlg\dfrac{V_R}{V_d} \end{array}\right. \quad \text{(ode.1)}$$

The first one comes from (I.7).
The second one comes from (II.5).
The third one comes from (III.6).
The fourth one comes from (IV.6).
The fifth one comes from (V.6).

Initial conditions:

$$\begin{array}{l} E_t(0) = 0 \\ P(0) = 0 \\ FDP(0) = 0 \\ S_d(0) = 0 \\ Plg_d(0) = Plg_{d0} \end{array} \quad \text{(ode.2)}$$

Algorithm for calculating the right-hand side of (ode.1) as a function of t, E_t, P, FDP, S_d and Plg_d

1) Calculate V_R as function of t according to (g.3)
2) Calculate V_u as function of FDP and V_R according to (mf.2)
3) Calculate V_d as function of V_u according to (g.1)
4) Form a cubic equation for E by plugging S_u from (ms.4) and Fg_R from (fgt.4) in (et.2)

$$E = \frac{E_t}{1 + \frac{S_{u0}^0.V_{u0} - P.(V_{d0} + V_{u0}) - S_d.(V_d - V_R)}{(K_M^S + E).(V_u + V_R)} + \frac{Fg_0.V_R}{(K_M^F + E).(V_u + V_R)}} \quad \text{(equ.1)}$$

5) Find the solution E of (equ.1) as a function of E_t, P, S_d, V_R, V_d and V_u in the interval ($Emin$, $Emax$) where

$$E_{min} = \frac{E_t}{1 + \frac{S_{u0}^0.V_{u0} - P.(V_{d0} + V_{u0}) - S_d.(V_d - V_R)}{K_M^S.(V_u + V_R)} + \frac{Fg_0.V_R}{K_M^F.(V_u + V_R)}}$$

$$E_{max} = \frac{E_t}{1 + \frac{S_{u0}^0.V_{u0} - P.(V_{d0} + V_{u0}) - S_d.(V_d - V_R)}{(K_M^S + E_t).(V_u + V_R)} + \frac{Fg_0.V_R}{(K_M^F + E_t).(V_u + V_R)}} \quad \text{(equ.2)}$$

6) Calculate S_u as function of E, P, S_d, V_R, V_d and V_u according to (ms.4)
7) Calculate Fg_R as function of E according to (fgt.4)
8) Calculate ES as function of E and S_u according to (II.1)
9) Calculate EFg as function of E and Fg_R according to (III.1)
10) Select a formula for additional activation of PA_{Rt}:
   a) Calculate C according to (pat.5) as a function of EF and FDP
   b) Calculate C according to (pat.6) as a function of E_t and FDP
   c) Calculate C according to (pat.7) as a function of E and FDP
11) Calculate f as function of C according to (pat.4)
12) Calculate PA_{Rt} as function of f, V_R, and V_u according to (mpa.3)
13) Form a quadratic equation for $PAPlg$ by plugging PA_R from (pat.1) and Plg_R from (plt.3) in (I.1)

$$PAPlg^2 - (PA_{Rt} + Plg_d + K_M^P).PAPlg + PA_{Rt}.Plg_d = 0 \quad \text{(equ.3)}$$

14) Find the solution $PAPlg$ of (equ.3) as a function of PA_{Rt} and Plg_d in the interval (0, min{PA_{Rt},Plg_d})

$$PAPlg = \frac{PA_{Rt} + Plg_d + K_M^P - \sqrt{(PA_{Rt} + Plg_d + K_M^P)^2 - 4 * PA_{Rt}.Plg_d}}{2} \quad \text{(equ.4)}$$

15) Calculate $\frac{dV_u}{dt}$ as function of EFg, and V_R, according to (mf.4)
16) Calculate k as function of S_d and S_u according to (IV.7)

17) Calculate the right-hand side of (ode.1) as a function of E_t, FDP, S_d, V_R, V_u, V_d, $PAPlg$, ES, EFg, S_u, $\frac{dV_u}{dt}$, and k.
18) **Optional:** Calculate PA_R as function of PA_{Rt} and $PAPlg$ according to (pat.1)
19) **Optional:** Calculate PA_u as function of PA_{Rt} and f according to (pat.3)
20) **Optional:** Calculate Plg_u as function of E_t, Plg_d, V_R, V_d and V_u according to (mpl.2)
21) **Optional:** Calculate Plg_R as function of $PAPlg$ and Plg_d according to (plt.3)

Experiment

Measurements

The $A=\varepsilon.P.l$ is measured in the course of time t for various known concentrations of PA_{u0}^0, Plg_{d0}, Fg_0, and S_{u0}^0.

Mathematical tasks:

1. Integrate A (i.e. P) over time t.
2. Fit the measured data A to the model equations to determine the optimal parameters K_M^P, k_{catP}, κ, λ, α and their confidence intervals.

Known e.g.:

l = 0.31 cm
V_{u0} = 40 µl
V_{d0} = 80 µl
$B = 7^2.\pi/4$ mm^2
ε = 0.00885 µM^{-1}·cm^{-1}

K_M^S = 10 µM
k_{catS} = 810 min^{-1}
K_M^F = 0.14 µM
k_{catF} = 8.4 min^{-1}

V_{R0} = 0.77 µl
v_{VR} = 0.013 µl·min^{-1}
$k_1 = 6/(49.\pi)$ mm·sec^{-1}
$k_2 = 4/(49.\pi)$ mm·sec^{-1}

f_0 is determined by table-look-up using a specific table,
e.g $\left(f_{0,i}^{table} - PA_{u0,i}^{0,table}\right)$ for i = 1, 2, ..., n

f_0 = 6.2 (-) for PA_0 = 0.00048529 µM
f_0 = 4.8 (-) for PA_0 = 0.00073529 µM
f_0 = 3.7 (-) for PA_0 = 0.0012941 µM
f_0 = 2.3 (-) for PA_0 = 0.0024559 µM
f_0 = 1.4 (-) for PA_0 = 0.0036765 µM
f_0 = 1 (-) for PA_0 = 0.0048971 µM
f_0 = 0.58 (-) for PA_0 = 0.0073529 µM

What can a model of fibrinolysis achieve?

Based on the quantitative characterization of the two stages in Scheme 1 a computer model of fibrinolysis was attempted. Clearly, any such model requires some simplifications. These will need to be applied with care so as not to overlook vital aspects of the mechanisms that regulate plasmin generation in a fibrin clot. If this is possible, such a model could be very useful in optimizing current thrombolytic treatment in acute myocardial infarction and stroke and designing new thrombolytics in addition to the simple aim of understanding physiological fibrinolysis under normal or pathological conditions. A good computer model of the system may help us understand how the available concentrations of PA and their fibrin binding characteristics, their “zymogenicity”, and their kinetic parameters all interact and regulate plasmin generation in a clot. Attention could then be focused on improving the important

characteristics of PAs (e.g. optimum fibrin binding, better pharmacokinetics and optimum circulating concentrations, better K_M and k_{cat}) to rationally design better molecules. Approaches to date have been based on simple assumptions that do not appear to be valid [9].

Furthermore, understanding of the regulation of PAs might lead to better treatment using mixtures of PAs. There may be optimized mixtures of fibrin specific and non-fibrin specific PA that could be identified using computer models. Mixtures of PA with different properties seem to have evolved in the case of the vampire bat for example, which produces a mix of PAs to prevent blood clotting during feeding. One form is currently under development for the treatment of ischaemic stroke (desmoteplase), but the mixture present naturally in the saliva of the bat may be an example of an "experiment of nature" that has evolved to optimize fibrinolysis rates. In a similar way it may be possible to improve properties of a single molecule by combining domains from different proteins to promote clot penetration, as is the rationale behind amediplase [7]. Additional advantages may be expected from combining members of the plasmin system with fibrinolytic proteinases from leukocytes (e.g. reduced embolization risk as discussed later in relation to the effects of flow rate on clot dissolution).

Exploration of fibrinolysis *in silico* may also reveal some possible explanations that account for resistance to thrombolytics of aged clots in complex environment [2, 6] and thus extend the therapeutic window. Or it may be possible to tailor treatment according to risk factors in a patient's history, their sex or age (since older patients are at much greater risk of cerebral bleeding) [9]. Finally, it may also be possible to improve current thrombolytic treatment for ischaemic stroke. Currently only tPA (alteplase) is licensed to treat stroke in the USA and only within 6 hours of onset of symptoms [1]. Bearing in mind ischaemic stroke accounts for 80% of all strokes, incidence increases in ageing populations, and there are so few treatments currently available, improvements here would have a major impact on mortality and morbidity [8].

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