Contact activation of the plasma coagulation cascade. III. Biophysical aspects of thrombin-binding anticoagulants

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Abstract: A biophysical model linking fibrin polymerization kinetics (following release from a thrombin–fibrinogen complex), coagulation time, and competitive inhibition of thrombin illustrates the utility of thrombin-binding ligands as anticoagulants in blood collection applications. The resulting mathematical relationship connecting fibrinogen, ligand, and thrombin concentrations was tested against experimentally observed anticoagulation of whole, platelet-poor porcine plasma induced by short, single-stranded DNA oligonucleotides originally found to bind thrombin by screening combinatorial libraries. The thrombin–fibrinogen dissociation constant $K_d$ served as the single adjustable parameter in a least-squares fitting of the model to experimental anticoagulation data. Best-fit $K_d$ values corroborated $\mu$M values measured in plasma-free systems, and application of the model to a ligand challenge to the intrinsic pathway of plasma coagulation corroborated nM endogenous thrombin concentrations measured in porcine blood activated by endotoxin insult in vivo. The model fit to data suggests that only about 20% conversion of blood fibrinogen to fibrin is required to coagulate (gel) porcine plasma. This prediction is consistent with the common clinical laboratory observation of latent fibrin formation in “serum” separated from blood before fibrinogen is fully converted to fibrin. It was concluded that the thrombin-binding anticoagulation model was a reasonable simulation of the situation in which an initial bolus of either exogenous or endogenous thrombin is rapidly partitioned between fibrinogen-bound and ligand-bound forms with little or no additional free thrombin created over time. © 1998 John Wiley & Sons, Inc. J Biomed Mater Res, 40, 92–103, 1998.

Key words: blood; plasma; coagulation; mathematical model; anticoagulation; thrombin; thrombin ligand; DNA ligands; aptamers

INTRODUCTION

Disposable biomedical devices, such as syringes, catheters, and blood collection tubes, constitute very high-volume applications of biomaterials in the health care industry. For example, more than 100 million evacuated blood collection tubes, both plastic and glass, are used annually in domestic blood collection for down-stream chemical and hematological testing.1 Venous blood is drawn either into evacuated tubes or into syringes, and peripheral blood is collected into microvials from finger or heel “sticks,” as is typically practiced in pediatric and geriatric medicine. In either event, collected blood eventually will coagulate even if no procoagulant is contained within the collection device because the intrinsic pathway of blood coagulation is triggered during blood collection by activation of Factor XII through contact with the interior surfaces of stainless steel needles and collection tubes.2 The presence of tissue thromboplastin derived from the puncture wound also can lead to blood coagulation, especially in peripheral blood collection. Thus an anticoagulant must be used in all collection devices used for long-term preservation of whole blood or plasma.

Calcium chelators, such as citrate and EDTA, or biochemicals, such as heparin, are the anticoagulants of choice for most traditional blood testing. The mechanism of anticoagulation by calcium-chelator anticoagulants is a straightforward titration of available blood calcium, a necessary divalent ion for certain enzymes of the coagulation cascade. Long-term anticoagulation is guaranteed by addition of an excess of chelator. The mechanistic aspects of heparin anticoagulation are more complicated, involving antithrombin III (ATIII),3 but again the basic strategy is addition of an overwhelming excess that prevents cleavage of the zymogen prothrombin into thrombin and inhibition of thrombin proteolytic activity.4 Thus in the use of these traditional anticoagulants, the coagulation cascade is forced into an indefinite state of suspended
animation until and unless anticoagulated blood or plasma is recalcified (in the case of chelator-type anticoagulants) or heparin is cleared by exogenous agents, such as protamine, polylysine, or heparinase.\textsuperscript{5,6}

With the advent of widespread blood cell separation/culture, DNA amplification, and ultrasensitive immunoassays, however, certain clinical interferences caused by the above-mentioned anticoagulants have arisen, sparking interest in alternative anticoagulants. Thrombin-binding anticoagulants (also termed thrombin ligands), such as hirudin and hirudin-based peptides,\textsuperscript{7} peptidomimetic inhibitors,\textsuperscript{8} or oligonucleotide ligands,\textsuperscript{9} have potential in blood collection applications (and as therapeutics)\textsuperscript{9,10} because these do not radically change blood ion chemistry or introduce into blood broad molecular-weight polysaccharides that can interfere with DNA amplification or blood cell culture. The role of a thrombin-binding anticoagulant is to block production of fibrin by inhibiting hydrolysis of fibrinogen by thrombin and, in so doing, postponing coagulation for some period of time. That is to say, a state of anticoagulation can be achieved by using sufficient ligand to bind up all thrombin as it is produced from prothrombin in the penultimate steps of the plasma coagulation. Duration of the anticoagulated state so obtained is determined not only by the absolute blood concentration, as in the case of the traditional anticoagulants above, but also by the dissociation constant characteristic of the thrombin ligands ($K_d$ in molar units). Clearly, an excess addition of an ineffective thrombin ligand with $K_d \to \infty$ (no affinity for thrombin), however large the excess, cannot neutralize thrombin. The minimum ligand concentration required to insure permanent anticoagulation occurs at the other thrombin-binding extreme, $K_d \to 0$ (no dissociation from a thrombin-ligand complex), at a one-to-one molar ratio with thrombin. In between these extremes ($0 < K_d < \infty$) there is a relationship among thrombin, ligand, and fibrinogen that ultimately determines the utility of thrombin-binding anticoagulants.

The purpose of this work was to develop a biophysical model of thrombin-binding anticoagulation that quantifies relationships between anticoagulation efficiency, ligand-to-thrombin concentration ratio, and thrombin-binding affinity that provides broad guidelines in the design of thrombin-binding anticoagulants for blood collection applications. Although the model was written explicitly for DNA ligands, propositions of the model are sufficiently general to embrace all kinds of thrombin-binding ligands, including those that reversibly unfold/renature into an inactive, non-binding form. Anticoagulation of whole, platelet-poor porcine plasma induced by single-stranded DNA oligonucleotides was used as an experimental system against which the model was tested. Interesting relationships between anticoagulation efficiency and blood fibrinogen concentrations were illustrated.

**MATERIALS AND METHODS**

### Preparation of platelet-poor porcine plasma

Whole citrated porcine blood was used as received under refrigeration (Environmental Diagnostics Inc., Burlington, NC). The preparation of platelet-poor plasma and its utility as a coagulation test system has been described in detail previously.\textsuperscript{2} The coagulation time of 0.5 mL straw-yellow or light-pink plasma without excessive hemolysis in glass test tubes (Fisher, 13 x 100 mm, approximately $4.1 \times 10^{-3}$ m$^2$ interior surface area or $8.2 \times 10^{-3}$ m$^2$/mL of plasma) was $3.64 \pm 0.98$ min after recalcification by addition of $100 \mu$L of 0.2M CaCl$_2$ while rotating on an inverting hematology mixer ($N = 9$; 3 tests of plasma preparations from three different blood lots).

### Thrombin and oligonucleotide solutions

Bovine thrombin was used in all coagulation experiments (Sigma, 50% protein by weight). Stock solutions were freshly prepared from lyophilized powder in phosphate-buffered saline (PBS, Gibco) to $3.2 \mu$M (0.25 mg/mL lyophilized powder) for subsequent dilution into porcine plasma. Thrombin-binding oligonucleotides\textsuperscript{11} used in this work (Table I) were constructed on an automated synthesizer (ABI Model 380B) employing standard B-cyanoethyl phosphoramidite chemistry. Resulting oligonucleotides were purified by denaturing gel electrophoresis, electroelution, and ethanol precipitation. Concentrations of the final purified oligonucleotide solutions were assayed by spectrophotometry at 298 nm.

### Thrombin titration and anticoagulation assay

Frozen plasma was thawed and brought to room temperature (~20°C) in a water bath. Approximately 0.5 mL of plasma was aliquoted into polystyrene test tubes (Falcon Brand, Becton Dickinson). Thrombin titrations were carried out by addition of 25 $\mu$L of 1:1 diluted thrombin stock solutions into 0.5 mL citrated porcine plasma to a final thrombin concentration $[T^+]$, with the superscript denoting the initial concentration. A tube without thrombin served as a negative control that measured coagulation due to "background" activation.\textsuperscript{2} The tubes were held upright in a standard inverting hematology mixer (Fisher). Plasma was recalcified by addition of 100 $\mu$L of 0.2M of CaCl$_2$ and a stop-watch was started. The tubes quickly were capped with parafilm and the mixer started. The coagulation time was noted by a distinct change in a fluid-like rheology to gel formation. This end point time was very sharp and reproducible within 30 s. Coagulation times at various [T$^+$] in the
TABLE I
Thrombin-Binding Oligonucleotide Anticoagulants\(^{(a)}\)

<table>
<thead>
<tr>
<th>Compound Reference #</th>
<th>DNA Sequence(^{(b)})</th>
<th>(K_d) (nM)</th>
<th>(K_c)</th>
<th>(\alpha @ \text{Min. Std. Error} )</th>
<th>(K_c @ \text{Min. Std. Error} )</th>
<th>Calculated (K_c) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (consensus)</td>
<td>5(^{\prime})-GGTTGGTGTGGTGGTGGTGG(^{3\prime})</td>
<td>5.35 ± 0.90</td>
<td>0.11 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>28.82 ± 3.54</td>
<td>0.15</td>
</tr>
<tr>
<td>5</td>
<td>5(^{\prime})-GGTTGGTGTG(^{3\prime})p(^{\prime})-p(^{\prime})-GGTTGGTGGTGG(^{3\prime})</td>
<td>6.20 ± 1.91</td>
<td>0.46 ± 0.15</td>
<td>0.24 ± 0.01</td>
<td>40.28 ± 6.82</td>
<td>0.25</td>
</tr>
<tr>
<td>8</td>
<td>5(^{\prime})-GGTTGGTGTG(^{3\prime})p(^{\prime})-p(^{\prime})-GGTTGGTGGTGG(^{3\prime})</td>
<td>26.14 ± 5.85</td>
<td>0.50 ± 0.06</td>
<td>0.11 ± 0.01</td>
<td>21.31 ± 3.15</td>
<td>0.56</td>
</tr>
<tr>
<td>1-tail(^{(c)})</td>
<td>5(^{\prime})-ATGGTTGGTGTGGTGGTGG-tail(^{3\prime})</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Mean ± SD (\%CV)

| —           | 0.19 ± 0.07 | —                | 0.32 ± 0.21 | (35.7) |

(a) Values for \(K_d\) and \(K_c\) were determined by SPR and DNA melting curves, respectively.\(^{11}\) Fixed parameters of the fit were \([F^\prime] = 14.1 \mu M, [T^\prime] = 128 nM, S = 182.1 nM \cdot \text{min, } k_r = 0.7 \text{ min}^{-1}, \text{ and } C_T = 3.1 \text{ min} \) (see Appendix I). (b) \(L_3\) = hexaethylene glycol chain; \(L_2\) = glycerol chain; \(p\) = phosphodiester linkage. (c) tail = TCGAGCTAGATCTTCAGTACGT.

The absence of thrombin ligand were termed “thrombin times” and given the symbol \(CT^\circ\). Anticoagulation assays were carried out in the same manner as thrombin titrations except that \([T^\circ]\) was fixed at 128 nM and the oligonucleotide ligand concentration \([L^\circ]\) was varied to obtain different ligand/thrombin ratios \(R = [L^\circ]/[T^\circ]\). Final concentrations were adjusted with PBS as required, and the plasma was recalcified by addition of 0.50 \(\mu\)L of 0.4M of CaCl\(_2\), yielding a 625 \(\mu\)L final test volume. Scatter in the experimental data, particularly at longer coagulation times, is not inherent to the coagulation time assay itself but rather thought to be related to the variable production of thrombin, over-and-above the exogenous concentration \([T^\circ]\), induced by progressively longer contact with polystyrene test-tube surfaces that exhibit weak procoagulant properties.\(^{2}\) Anticoagulation efficiency (\(\alpha\)) was defined as the ratio of the coagulation time \(CT\) noted at variable \(R^\circ\) to the coagulation time with \(R^\circ = 0\) (\(\alpha \equiv CT/CT^\circ\)).

Likewise, an initial ligand bolus concentration \([L^\circ]\)\(_q\), comprised of an active form \(L^\circ_q\) that binds thrombin and an inactive form \((L^\circ_r)\) that does not bind thrombin, is distributed between free \(L^\circ_q\) or \(L^\circ_r\) and a thrombin-bound form TL. The subscripts “q” and “r” herein refer to quadruplex and coil forms of DNA but generally can be interpreted as active and inactive forms, respectively; the q subscript is not carried in TL for notational simplicity. Concentration of the inactive form \([L^\circ_r]_q\) is connected to \([L^\circ_q]_q\) through a separate competing equilibrium. Equilibrium dissociation constants \(K_{q}, K_{q}, K_{c},\) and \(K_{c}\) in Scheme 1 control steady-state partitioning, with \(K = K_{q}/K_{q}\) serving as a dimensionless variable comparing the affinity of thrombin binding to F (numerator) and L (denominator).

Unlike thrombin and ligand concentrations, \([F]\) does not achieve steady state. Instead, an initial concentration of fibrinogen \([F^\prime]\) decreases to a time-variable concentration \([F]\) up to and including coagulation time \(CT\) (0 ≤ \(t\) ≤ \(CT\)), as a polymerizable fibrin fragment \(F^\prime\) accumulates after rapid cleavage from TF and rate-limiting polymerization into a fibrin polymer network denoted F-F.\(^{13-18}\) The model assumed that irreversible

Computational methods

Mathematical statements of biophysical models used to deduce \(K_c\) from experimental \(\alpha\) measurements (see Appendix I) were coded into research programming language (RPL) programs and statistically fit to experimental data through the agency of the FITFUNCTION facility of RS/1 (a scientific information handling software package, Bolt Beranek and Newman, Cambridge, MA) residing on a VAX (Digital Equipment Corp.) computer system.

RESULTS

Biophysical model of thrombin-binding anticoagulation

The model illustrated in Scheme I links previously published kinetic constructions for thrombin-catalyzed hydrolysis of fibrinogen and fibrin polymerization leading to blood coagulation.\(^{12-15}\) The underlying premise is an instantaneously established
polymerization of F$^*$ into F-F was pseudo-first order in F$^*$ as a means of simplifying a more complex set of reactions leading to fibrin oligomerization that ultimately displays consecutive first-order reaction kinetics. This view of coagulation effectively combines a manifold of enzyme reactions with interconnected feedback loops into a net forward reaction leading to F-F with a characteristic polymerization rate constant ($k_p = 0.7 \pm 0.1 \text{ min}^{-1}$) deduced from previous investigations of the coagulation cascade. Notably, these feedback loops include the fibrinolytic pathways the model does not explicitly consider.

First-order production and polymerization of F$^*$ means that [F$^*$] results from simple source-sink kinetics that depend on the mole fraction of thrombin bound to fibrinogen ($X_{TF}$) according to Equation (A2) of Appendix I. Figure 1 illustrates [F$^*$] kinetics at different $X_{TF}$ (in the form of [F$^*$]/$k_3$). For example, a steady state value [F$^*$]/$k_3 = 182.9 \text{ nM} \times \text{min}$ was computed for $X_{TF} = 1$ at [T$^0$] = 128 nM from Equation (A2). Incidentally, the [F$^*$] kinetics curve of Figure 1 strongly resembles turbometric and spectrophotometric measurement of fibrin aggregation as well as kinetics of thrombin-mediated release of fibrinopeptides A and B.

Coagulation time (CT) is related to [TF] through the model assertion that blood plasma coagulates only after some specific fraction ($\alpha$) of [F$^0$] is converted into fibrin polymer (see Appendix I for details). Anticoagulation efficiency ($\epsilon$) is defined as a dimensionless multiple expressing the extension of coagulation time (CT) noted at variable $R^0 = [L^0]/[T^0] > 0$, over-and-above the coagulation time observed at $R^0 = [L^0] = 0$ (termed thrombin time, CT$^0$) measured at thrombin concentration [T$^0$]; $\epsilon = CT/CT^0$; $\epsilon = 1 \Rightarrow$ no anticoagulation; $\epsilon > 1 \Rightarrow$ temporary anticoagulation; $\epsilon = \infty \Rightarrow$ permanent anticoagulation. Detailed arguments and assumptions disclosed in Appendix I lead to an approximate analytical relationship between $\epsilon$ and dependent variables $R^0$, $K$, [T$^0$], and [F$^0$]. Figure 2 illustrates $\epsilon = \mathcal{F}(R^0,K)$ at different [F$^0$] computed from the quadratic solution of Equation (A13). Parameter values used in the computations and listed in the figure annotations were obtained either from the literature or from experimental determinations, as will be discussed subsequently.

**Thrombin titration**

Figure 3 is a typical thrombin titration curve obtained by plotting thrombin time (CT) at different

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**Figure 1.** Kinetics of accumulation of a polymerizable fibrin monomer (F$^*$) in the coagulation of platelet-poor porcine plasma at different mole fractions of thrombin-bound fibrinogen ($X_{TF}$) [see Eq. (A2) of Appendix I; assumes [T$^0$] = 128 nM and irreversible pseudo-first-order polymerization with a characteristic net forward rate constant of $k_p = 0.7 \text{ min}^{-1}$]. The vertical line marks the experimental thrombin time (CT$^0$) observed at [T$^0$] = 128 nM, suggesting that [F$^*$]/$k_3$ approaches a steady-state value for all $X_{TF}$ before plasma coagulates.

**Figure 2.** Variation in anticoagulation efficiency ($\epsilon$) with increasing ligand-to-thrombin concentration ratio $R^0 = [L^0]/[T^0]$ and dissociation constant ratio $K = K_c/K_a$. When ligand affinity for thrombin exceeds that of fibrinogen ($K_d < K_c; K > 1; \text{log} K > 0$), increasing $R^0$ leads to increasing anticoagulation efficiency $\epsilon$. When fibrinogen affinity for thrombin exceeds that of ligand ($K_d > K_c; K < 1; \text{log} K < 0$), increasing $R^0$ has no anticoagulation effect and $\epsilon \rightarrow 1$ (see Results section). Annotations list parameter values used in the computation [see Eq. (A13) of Appendix I]. The strong dependence of $\epsilon$ on blood fibrinogen concentration [F$^0$] illustrates that attainment of long-term anticoagulation with thrombin-binding ligands requires either small $K_d$ or large $R^0$ or a judicious combination of both.
fixed \([T^+]\). Thrombin titration curves were linear in 
\([T^+]^{-1}\) over the concentration range of interest, as 
anticipated by Equation (A7) of Appendix I, yielding a 
slope \( [F^+] / k_3 = 182.1 \pm 17.8 \text{ nM} \cdot \text{min} \). The intercept of 
\(1.6 \pm 0.3 \text{ min} \) at \([T^+]^{-1} = 0 \) \((T^+ = \infty)\) has no physical 
meaning for finite \([F^+]\). Thrombin times correspond to 
\([L^+] = 0\), for which \(X_{TF} \rightarrow 1\) because \([F] \gg [T]\). (See 
Discussion section and Appendix II for more details.)

### Oligonucleotide anticoagulants

Table I provides sequences for the thrombin-
binding oligonucleotides employed in this work along 
with \(K_f\) and \(K_c\) values determined by surface plasmon 
resonance (SPR) and DNA melting curves, respectively.\(^{11}\) For the current purposes, it is important only 
that these palindromic GG-TT-GG-TGT-GG-TT-GG 
sequences bind thrombin with variable affinities, 
which are related to the folded DNA quadruplex 
structure that is, in turn, related to the sequence and 
linking chemistry given in Table I.

### Exogenous thrombin spike experiments

Figure 4 illustrates the effect of added ligand on \(X_{TF}\) 
for a typical experimental case in which a thrombin 
spike (final \([T^+] = 128 \text{ nM}\)) was added to recalcified 
porcine plasma containing variable amounts of 
compound 5 (expressed as \(R^0\), see Materials and Methods 
and Table I). Similar results were obtained for 
compounds 1 and 8 (not shown). Focusing first on the 
experimental data points, it will be noted that \(X_{TF} \rightarrow 1\) 
as \(R^0 \rightarrow 0\), in general agreement with the model, and 
increasing \(R^0\) leads to the progressive reduction \(X_{TF}\) 
anticipated by Equation (A11) of Appendix I. Smooth 
curves through the data correspond to the best fit of 
the quadratic solution to Equation (A13) to the data 
with \(K\) as the single adjustable parameter at different 
fixed values of \(\alpha\) (see Appendix II for a brief statistical 
discussion). A reasonable fit of the model to the data 
could be obtained only with \(0 \leq \alpha < 0.3\), implying that 
less than 30% of \([F^+]\) was required to coagulate (gel) 
porcine plasma, consistent with the common clinical 
laboratory observation of latent fibrinogen formation 
in "serum" separated from blood before fibrinogen is 
fully converted to fibrin. The standard error of the best 
fit minimized between \(0.1 < \alpha < 0.25\) (column 5 of 
Table I) and, as a consequence, it is concluded that \(\alpha = 
0.2\) (20% conversion of \([F^+]\) at \(CT\)) is a reasonable value 
for the porcine plasma system. Therefore, \(\alpha = 0.2\) has 
been used in all computations reported herein.

Column 6 of Table I summarizes best fit values of \(K = 
K_f / K_d\) for the DNA ligands in rows 1–3. Column 7 
lists \(K_f\) values calculated from \(K\) using \(K_d\) determined 
from SPR (column 3; see Materials and Methods). It 
will be noted that the variation in \(K_f\) is quite large and
about twice that in the standard error of the fit for \( K \) (as a % of the mean of three trials, row 6). As it turns out, about half of this uncertainty is directly attributable to error in \( K \) and \( K_d \) propagating into \( K_s \) (see Appendix II under “Model Fit to Experimental Anti-coagulation Data”).

### DISCUSSION

**Predictive utility of the biophysical model of thrombin-binding anticoagulation**

Predictive utility of biophysical models sometimes can be assessed by examining statistical fit to experimental data and comparing the magnitude of adjustable parameters to similar quantities determined by independent means. In the current case, such a test is not straightforward because the model has a multiplicity of dependent variables (see Eq. (A13A) of Appendix I that are unknown or at least uncharacterized in the porcine plasma used in this work. Specifically, these variables include thrombin-binding constants (\( K_s \) and \( K_d \)), enzyme (thrombin) activity, and porcine plasma fibrinogen concentrations [F*]. More specifically, ligand \( K_d \) values were determined by SPR in

**TABLE AII**

<table>
<thead>
<tr>
<th>Physical Limits</th>
<th>Solutions to ( X_{TF} ) Quadric</th>
<th>Quadratic Coefficients</th>
<th>Quadratic Solution Implied root</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K ) \rightarrow 0 ( (K_d \rightarrow \infty \text{ or } K_s \rightarrow 0) )</td>
<td>( X_{TF} = (\gamma + \beta)/\beta )</td>
<td>( a = 1 ) ( b = -\gamma/\beta ) ( c = 1 )</td>
<td>( + ) root</td>
</tr>
<tr>
<td>( K ) \rightarrow \infty ( (K_d \rightarrow 0 \text{ or } K_s \rightarrow \infty) )</td>
<td>( 0 ) ( 1 ) ( (R^0 - 1) )</td>
<td>( a = 0 ) ( b = 0 ) ( c = 1 - R^0 )</td>
<td>( + ) root</td>
</tr>
<tr>
<td>( K = \beta (1 + K_s) )</td>
<td>Single valued</td>
<td>0</td>
<td>Not quadratic</td>
</tr>
</tbody>
</table>

\[ X_{TF} = \gamma[\beta(K_s(1 + K_s)/[T^0] + R^0) + \gamma]^{-1} \]
buffer solutions of human α-thrombin that are not necessarily relevant to either exogenous bovine thrombin added to platelet-poor porcine plasma or endogenous porcine thrombin released directly from the porcine plasma coagulation cascade. Likewise, thrombin activities derived from bovine-thrombin titration curves in porcine plasma (Fig. 3) do not necessarily reflect activity of endogenous thrombin released from the intrinsic cascade. And fibrinogen concentrations in porcine plasma were not measured to verify literature values. Nevertheless, in spite of these inherent difficulties, the quadratic solution to Equation (A13A) of Appendix I was used to fit experimental data for compounds 1, 5, and 8 with \( K = K_s/K_d \) as the single adjustable parameter. Fixed values for all other parameters are listed in the notes of Table I.

Figure 4 illustrates the model fit to experimental data at different fixed values of α for compound 5. Similar results (not shown) were obtained for the other thrombin-binding ligands listed in Table I. It is apparent that the model adequately can simulate experimental results with 0.01 < α < 0.3, but not for α > 0.3. Column 5 of Table I lists α values leading to a minimum in the standard error of the statistical fit for all of the compounds listed in Table I. Column 6 lists fitted values of \( K_s \) and column 7 gives \( K_d \) values calculated from \( K_s \) using \( K_d \) determined from SPR (column 3; see Materials and Methods). The mean value \( (K_d) \) is about sixfold smaller than the 1.8 μM reported for fibrinogen-thrombin binding in plasma-free systems. About half of the uncertainty observed in the mean \( K_d \) value is attributable to error inherent in \( K_s \) and \( K_d \) (see Appendix II). In consideration of statistical confidence in this fitted value and all of the above issues associated with testing the biophysical model against the experiment, the best-case conclusion is that the model adequately simulates experimental conditions obtained in thrombin-spike experiments. The worst-case interpretation is that the test of the model against thrombin-spike experiments was inconclusive.

Whereas the thrombin spike experiments discussed above were a convenient means of measuring and controlling (bovine) thrombin activity in whole (porcine) plasma, end use application of thrombin-binding anticoagulants in blood collection is more like circumstances established in the ligand-challenge experiments because a known added concentration of ligand binds to an unknown concentration of endogenous thrombin released from prothrombin in the final steps of the plasma coagulation cascade initiated by the blood drawing process. Smooth curves through the data points in Figure 5 result from fitting the model with \([T^0] \) as the single adjustable parameter (instead of \( R^0 \)) under the premise that the concentration of endogenous thrombin was an unknown constant throughout the time course of the experiment. Setting α = 0.2 and \( K_d = 5.35 \) nM for compound 1 (Table I) led to a best-fit value for \([T^0] = 13.4 ± 1.2 \) nM (smooth curve through the data corresponding to 1-tail was obtained in the same way although \( K_s \) was not measured for this compound). In consideration of the aforementioned complications associated with testing the biophysical model, this value is in surprising agreement with the 10–15 nM endogenous thrombin concentrations measured in porcine blood after endotoxin insult in vivo.25

Quantitative arguments aside, it is apparent from Figure 5 that \( X_{TF} \) decreased monotonically with [L] even though integrated contact time with the activating glass test tube surface increased with duration of temporary anticoagulation. Thus, according to this interpretation of ligand-challenge experimental results, decreasing \( X_{TF} \) results from a titration effect of added ligand in a manner similar to the exogenous thrombin-spike experiments in which thrombin activity is controlled by added concentration. Taken together with the saturating surface area effect on activation of the plasma coagulation cascade reported previously,2 all of the above suggests that the total concentration of endogenous thrombin was, in fact, more-or-less constant throughout the time course of the ligand-challenge experiments. Production of thrombin from the plasma cascade therefore must result from rapid and complete conversion of a fixed (but unknown) fraction of prothrombin to thrombin within the first few minutes of plasma contact with the glass test tube surface.

It therefore is apparent that the premise of an instantaneously established steady-state partitioning of an initial thrombin concentration \([T^0] \) into the various
bound and free forms shown in Scheme I can be applied to circumstances encountered in blood collection only if the plasma coagulation cascade is rapidly potentiated upon blood draw, producing a bolus of endogenous thrombin, as might arise if the tube interior surface strongly activates the cascade or a procoagulant purposely has been added to the tubes. Otherwise, in the circumstance of partial activation of the plasma coagulation cascade in plastic tubes with no added procoagulant, it might be anticipated that thrombin slowly but continuously is released from prothrombin.² The initial \([T^*]\) bolus concept has no meaning under such circumstances, and the model would not be applicable. Thus a principal conclusion that can be drawn is that predictions based on the biophysical model represent worst-case scenarios for the use of thrombin-binding anticoagulants in blood collection tubes that do not contain procoagulant surfaces.

**Implications for thrombin-binding anticoagulants in blood collection**

It is quite clear from Figure 2 (see Results section) that a substantial excess of ligand over thrombin \((R^o \gg 1)\) is required to achieve modest \(\epsilon\), even with relatively efficient thrombin binding. For example, about a five-fold excess ligand over thrombin \((R^o = 5)\) is required to achieve \(\epsilon \approx 100\) (at \([F^o] = 20 \mu M\) and log \(K = 3\) obtained with ligand \(K_d \approx nM\)). Also, the strong dependence of anticoagulation on \([F^o]\) illustrated in Figure 2 is somewhat surprising given that fibrinogen already is in at least a ten-fold excess over thrombin in most practical clinical situations. For example, fibrinogen concentrations vary from about 5 \(\mu M\) in preterm human infants,²⁶ 10 \(\mu M\) in normal adult humans,²⁷ 15 \(\mu M\) in geriatric humans²⁸ and adult swine,²⁹ to 44 \(\mu M\) for porcine infants (piglets).²⁹ Thus, it can be anticipated that practical use of thrombin-binding anticoagulants may require some level of customization for use in human and veterinarian blood applications.

**CONCLUSIONS**

A biophysical model of anticoagulation induced by thrombin-binding ligands was developed that linked previously published kinetic constructions for thrombin-catalyzed hydrolysis of fibrinogen and polymerization of fibrin leading to the coagulation of blood. The model was based on the premise of an instantaneous steady-state partitioning of thrombin into fibrinogen-bound, ligand-bound, and free thrombin forms. Equilibrium constants \(K_d\) and \(K_d\) controlled steady-state partitioning with \(K = K_d/K_d\) serving as a dimensionless variable comparing thrombin binding affinity to fibrinogen and ligand. An approximate, closed-form mathematical relationship between anticoagulation efficiency \((\epsilon)\), \(K\), and ligand concentration was derived based on this biophysical model, revealing that \(\epsilon\) was surprisingly sensitive to blood fibrinogen concentrations even though fibrinogen always was in substantial concentration excess over thrombin or ligand.

The model was tested against experimental anticoagulation of platelet-poor porcine plasma obtained with short single-stranded oligonucleotides, originally found to bind thrombin by screening combinatorial libraries. The equilibrium dissociation constant measuring binding affinity of thrombin to fibrinogen \(K_d\) served as the single adjustable parameter in least-squares fitting of the model to experimental anticoagulation data. Best-fit \(K_d\) values corroborated \(\mu M\) values measured in plasma-free systems, and application of the model to a ligand challenge to the intrinsic pathway of plasma coagulation corroborates nM endogenous thrombin concentrations measured in porcine blood activated by endotoxin insult in vivo. The model further predicted that only about 20% conversion of blood fibrinogen was required to coagulate (gel) porcine plasma. It was concluded that the thrombin-binding anticoagulation model was a reasonable simulation of the situation in which an initial bolus of either exogenous or endogenous thrombin is rapidly partitioned between fibrinogen-bound and ligand-bound forms with little or no free thrombin created over time.

**APPENDIX I—MATHEMATICAL MODEL OF THROMBIN-BINDING ANTICOAGULATION**

**Thrombin and ligand inventory**

Reacting species in the various forms listed in columns 1 and 2 of Table A1 are deduced by inspection of Scheme I (see Results section). Important mole fractions and mass balances utilized in development of the mathematical model are given in columns 3 and 4 and are based on the premise of an instantaneously established steady-state partitioning of initial thrombin and ligand concentrations (column 1) into the various bound and free forms (column 2).

Thrombin mass balance is straightforward and it is assumed that the bound forms \([TF]\) and \([TL]\) predominate because \([F] + [L] \gg [T]\) (see Appendix II for more discussion). Ligand mass balance is somewhat more complicated due to the steady-state partition between a quadruplex form \((L_q)\) that binds thrombin and an inactive random-coil form \((L_c)\). Distribution between
ligand isomers is controlled by $K_r$. Thus there are three mole fractions representing two free forms, in either quadruplex or coil form, and a bound form involving only the quadruplex form (the “q” subscript designation is not applied to TL for the sake of notational simplicity). The ligand fraction in coil form can be eliminated as an unknown using $K_r$ as in row 6 of column 1 under “Ligand.” Mole fractions of fibrinogen are not explicitly utilized in the mathematical model. Instead, the absolute concentrations of the various forms listed in rows 7–10 of column 2 are deduced as described in the following section.

Fibrinogen inventory at the time of coagulation

The model proposes that $F^*$ accumulates as a result of rapid cleavage of the fibrinogen-bound form $[TF]$ and rate-limiting, irreversible polymerization into F-F (see Results section for more discussion). Fibrin polymerization is characterized by a pseudo-first-order rate constant $k_p = 0.7 \pm 0.1 \text{ min}^{-1}$ deduced from previous investigations of the coagulation cascade. Accumulation of $F^*$ is thus given by a simple source-sink expression (Eq. A1) with the solution (Eq. A2) obtained by application of the boundary condition that $[F^*] = 0$ at time $t = 0$.

$$\frac{d[F^*]}{dt} = k_s[TF] - k_p[F^*] \quad (A1)$$

$$\frac{[F^*]}{k_3} = \frac{[TF]}{k_p} \left(1 - e^{-k_p t}\right) \quad (A2)$$

Equation (A2) describes an exponential increase in $[F^*]/k_3$ to a steady-state value attained after a nearly linear accumulation phase, as shown in Figure 1, for various values of $X_{TF}$. Initial rates and steady-state values readily are obtained by limits on Equation (A2):

$$\lim_{t \to 0} \frac{[F^*]}{k_3} = X_{TF}[T^*] \quad (A3)$$

$$\lim_{t \to \infty} \frac{[F^*]}{k_3} \approx X_{TF}[T^*]/k_p \quad (A4)$$

It is very unlikely that coagulation can occur in the initial rate phase measured by Equation (A3) because insufficient $[F^*]$ has accumulated to support polymerization into $[F-F]$ (that is, $k_p[F^*] = 0$). Coagulation is much more likely to occur at or near the steady-state limit [Eq. (A4)] when sufficient $F^*$ has been converted to F-F to gel plasma. As an illustration, the thrombin time $CT^*$ observed at $[T^*] = 128 \text{ nM}$ is noted by the horizontal line on Figure 1 showing that $[F^*]/k_3$ approaches the steady-state limit even in the case of thrombin-catalyzed hydrolysis with no added anticoagulant ($R^*=0$). It follows that (A4) is an even better estimate of $[F^*]$ at longer coagulation times ($CT$) occurring at either lower $[T^*]$ or $[L^0] > 0$ ($X_{TF} < 1$). This reasoning leads to the following approximate identities (see Appendix II for discussion of error):

$$[F^*]_{CT^*} \approx k_3[T^*]/k_p \quad (A5)$$

$$[F^*]_{CT} \approx k_3X_{TF}[T^*]/k_p \quad (A6)$$

The model further proposes that coagulation occurs after some specific fraction ($\alpha$) of the original blood fibrinogen concentration $[F^*]$ is converted to polymer $F-F$. Stated more precisely, coagulation occurs only after sufficient time has passed that the integrated rate of $[F-F]$ production (loss of accumulated $[F^*]$) equals the product $\alpha[F^*]$: $\int_{t=0}^{t=CT^*} k_p[F^*]dt = k_pCT[F^*]_{CT^*} = \alpha[F^*]$

$$= \int_{t=0}^{t=CT^*} k_p[F^*]dt = k_pCT[F^*]_{CT^*} \quad (A7)$$

where the limits of integration extend from $t = 0$ to coagulation time corresponding to either CT at $[L^0] > 0$ and $X_{TF} < 1$, or thrombin time $CT^*$ at $[L^0] = 0$ and $X_{TF} \equiv 1$. Equation (A7) precipitates three main conclusions regarding the fibrinogen inventory at or near plasma coagulation:

1. The anticoagulation efficiency $\epsilon \equiv CT/CT^* = [F^*]_{CT}/[F^*]_{CT^*}$. That is, the extension of coagulation time induced by the thrombin ligand, over and above thrombin time at a given thrombin concentration, is a direct measure of the relative concentration of activated fibrin levels at the time of coagulation.

2. A relationship that permits computation of $X_{TF}$ directly from measured coagulation time data can be derived from (A7) using (A2) to evaluate $[F^*]$ at CT and CT*, respectively:

$$X_{TF}[T^*] k_p^{-1} \left(1 - e^{-k_p CT^*}\right) CT = \frac{[T^*]}{k_p} \left(1 - e^{-k_p CT}\right) CT \Rightarrow X_{TF} \approx \frac{CT^*}{CT} \left(1 - e^{-k_p CT^*}\right) \quad (A8)$$

A useful simplification of (A8) with somewhat improved intuitive appeal relates $X_{TF}$ directly to $\epsilon$ using the identities (A5) and (A6) in (A7):

$$\epsilon = \frac{1}{X_{TF}} \quad (A9)$$

from which it is evident that anticoagulation efficiency is inversely proportional to the mole fraction of thrombin bound to fibrinogen.

3. Insertion of (A5) into the right-hand side of (A7) leads to the conclusion that $CT^* \approx (\alpha[F^*]/k_p)(1/[T^*])$, anticipating that a plot of thrombin times observed at various $[T^*]$ (a thrombin titration curve) should be linear in $[T^*]^{-1}$ with slope $S = (\alpha[F^*]/k_p)$. 
At this juncture, concentrations of the various fibrinogen forms have been deduced or approximated, allowing the fibrinogen mass balance at the time of coagulation to be written:

\[
[F]_{CT} = [F^*] - [TF] - [F^*] - \alpha [F^*] = 
\begin{cases} 
[F^*](1 - \alpha) - X_{TF}[T^o] 
\end{cases} 
\left(1 + \frac{k_i}{k_p}\right) 
\]

\(= \left(1 + \frac{\alpha[F^*]}{Sk_p}\right) \]  \hspace{1cm} (A10)

where \(S\) has been used to eliminate the unknown quantity \(k_s\).

**Simple intersecting linear competitive inhibition**

The biophysical model of Scheme I is not fundamentally different from standard competitive inhibition enzyme kinetics. \(^{30}\) Using the definitions of \(K_d\) and \(K_c\) from Table A1 to eliminate \([TF]\) and \([T]\) from \(X_{TF} \equiv [TF]/[T^o]\) leads immediately to the standard equation relating velocity of an enzyme-catalyzed reaction in the presence of a binding inhibitor \(\nu\) to the maximal initial velocity \(V_{MAX}\) observed in the absence of an inhibitor:

\[
X_{TF} = \frac{\nu}{K_s + \frac{[L]_q}{K_i}} = \frac{\nu}{1 + \frac{[F]}{K_i} + \frac{[L]_q}{K_i}} = V_{MAX} 
\]

\hspace{1cm} (A11)

where \([L]_q\) and \(K_i\) are equivalent to the inhibitor \(I\) and \(K_i\) terminology commonly employed in enzyme kinetics.

The number of unknowns in (A11) can be reduced using two key substitutions. First, \(K_s\) and \(K_i\) explicitly can be eliminated by using the identity \(K \equiv \frac{K_s}{K_i}\). The second substitution involves estimation of \([L]_q\) using the mass balance equations listed in Table A1:

\[
[L]_q = [L^o] - [TL] - [L]_q = [L^o](1 - Y_{TL}) - K_s[L]_q = \frac{[L^o](1 - Y_{TL})}{1 + K_c} = \frac{R'[T^o] - [T^o](1 - X_{TF})}{1 + K_c} \]  \hspace{1cm} (A12)

where it has been recognized that \(Y_{TL} = X_{TL}/R^o\) and the simplification \(X_{TL} \equiv (1 - X_{TF})\) has been employed in deference to the approximation \(X_r = 0\) (see discussion of error in Appendix II). Insertion of (A12) into (A11) leads to a second order polynomial in \(X_{TF}\), which maybe written in two equivalent forms:

\[
X_{TF}^2 \left(\frac{K}{1 + K_c} - \beta\right) + X_{TF} \left(\frac{K[K(R^o - 1) + K_i(1 + K_c)]}{[T^o]^2} + (\gamma + \beta)\right) - \gamma = 0 
\]

\[
X_{TF}^2 \left(\frac{K}{1 + K_c} - \beta\right) + X_{TF} \left(\frac{K[K(R^o - 1) + K_i(1 + K_c)]}{[T^o]^2} + (\gamma + \beta)\right) - \gamma = 0 
\]

where \(\beta\) and \(\gamma\) have been defined as above for the sake of notational compactness. Note that \(\beta\) effectively measures relative rates of \([F^*]\) production and consumption into \(F-F\); \(\beta \rightarrow 1\) when \(k_p \gg k_3\) (little or no net accumulation of \([F^*]\)) and \(\beta > 1\) when \(k_3 > k_p\) (\([F^*]\) accumulates due to rate-limiting polymerization). The latter case is pertinent for these studies with porcine plasma with \(k_3/k_p = 27.1\) calculated from \(\alpha = 0.20, [F^o] = 14 \mu M, k_p = 0.7\) min\(^{-1}\), and \(S = 182.1\) nM. \(\gamma\) is the molar excess of blood fibrinogen over thrombin; \(41 < \gamma < 292\) over the range \(34 < [T^o] < 267\) nM used herein.

Equation (A13(b)) is less compact than (A13(a)) but is useful in deducing limits on the coefficients \(a, b, c\) of the quadratic solution \(- b \pm \sqrt{(b^2 - 4ac)}/2a\) and, through this exercise, the appropriate selection between addition or subtraction of the square root in solution of the quadratic equation for \(X_{TF}\). Column 1 of Table AII lists three limits on \(K\) corresponding to the circumstances in which (1) the ligand has little or no binding capacity for thrombin \((K_i \rightarrow \infty)\), (2) ligand affinity for thrombin greatly exceeds affinity for fibrinogen \((K_i \rightarrow 0)\), and (3) \(K\) has a finite value determined by \(\beta\) and \(K_i\). Under these conditions, \(X_{TF}\) must vary between an upper bound in which all thrombin is bound to fibrinogen \((X_{TF} = 1\), assuming molar excess fibrinogen\) and a lower bound in which no thrombin is fibrinogen bound \((X_{TF} = 0)\), through a finite value determined by the linear equation given in row 5. Examination of the imposed quadratic solutions within these limits (column 7) leads to the conclusion that the sign changes from \((-\) to \((+) as \(K\) passes through these limits. The solution to Equation (A13) is illustrated in Figure 2 for different \([F^o]\). In this work, thrombin ligands were selected such that \(K_s \gg K_i\) and the solution for \(X_{TF}\) is found by addition of the square root.

**APPENDIX II—APPLICATION OF THE MATHEMATICAL MODEL AND EVALUATION OF INHERENT ERROR**

**Thrombin titration**

Figure 3 is a thrombin titration curve for porcine plasma confirming the inverse relationship in throm-
bin concentration anticipated by the model over the range $34 \leq [T^\circ] \leq 267$ nM, from which the slope ($\alpha [F^\circ]/k_3 = 182.1 \pm 17.8$ nM $\cdot$ min is estimated. Linearity of the thrombin titration curve ($R^2 = 91.3\%$) suggests that any deviation from the approximation (A5) must be essentially inconsequential for $X_{TF} = 1$ over the [$T^\circ$] interval shown in Figure 3. Close examination of Figure 1, in which the vertical line marks $CT^\circ$ at $[T^\circ] = 128$ nM, suggests that (A5) underestimates $[F^\circ]/k_3$ by about 14%.

**Model fit to experimental anticoagulation data**

Experimental anticoagulation data for oligonucleotide compounds 1, 5, and 8 listed in column 1 of Table I obtained at $[T^\circ] = 128$ nM (see Materials and Methods) was converted to $X_{TF}$ using Equation (A8) with $k_p = 0.7$ min$^{-1}$ (determined from previous investigations of porcine plasma coagulation) and $CT^\circ = 3.1 \pm 0.3$ min (as calculated from the best-fit line through the thrombin titration curve of Fig. 3; error estimate by simple propagation of error of fitted coefficients into $CT^\circ$). Examination of the sources of error in calculated $X_{TF}$ leads to an estimated uncertainty of ±10% associated with the errors in measured coagulation times. Resulting data was fit to the quadratic solution of Equation (A13) using $K$ as the single adjustable parameter with $K_d$ for each compound listed in column 3 of Table I (i.e., $K_d = K/\alpha$, $\alpha$ = 3.6 (1 to 60% conversion of $[F^\circ]$), $[F^\circ] = 14.1$ $\mu$M, and $S = 182.1$ nM $\cdot$ min (determined from the thrombin titration curve of Fig. 3). The standard error of the fit minimized between 0.1 $< \alpha < 0.25$ for the three compounds, with a relatively flat 96% < $R^2$ < 99% yielding 0.1 $\mu$M < $K_d$ < 1 $\mu$M. A sharply increased standard error of the fit and decreased $R^2$ at $\alpha > 0.3$ was accompanied by obvious lack-of-fit (see Fig. 4). Propagation of the error in $\alpha$ into $K_d$ leads to $\sigma_{K}^2 = K^2 \sigma_{\alpha}^2 + K^{2.5} \sigma_{\alpha}^2$ where the $\sigma$ terms are approximated by the standard errors in $K$ and $K_d$. Substitution of values from Table I leads to the conclusion that simple statistical error in $K_d$ is about 0.1 $\mu$M.

**Mass balance error**

A simplifying assumption used in reducing the biophysical model of Scheme I into a closed-form analytical relationship between dependent and independent variables was that $[T]$ was negligibly small in comparison to all other forms of thrombin ([TF] and [TL]; that is, thrombin mole fraction $X_T = 0$). This assumption is reasonable for the conditions $[F] + [L] \gg [T]$ and $K \gg K_d$, which generally is achieved under the experimentally relevant conditions employed herein because $[F^\circ]$ in porcine plasma is quite large and $K_d$ values were on the order of nM. For example, in the thrombin spike experiments discussed herein, $[T^\circ] = 128$ nM and $[F^\circ] = 14$ $\mu$M virtually guarantees that $[F] \gg [T]$ up to and including $CT$, especially in the event that only 20% of $[F^\circ]$ is consumed at $CT$. However, this simplification is less robust under theoretically relevant conditions when $R^\circ = 0$ or $K_d \gg K$ that might allow a significant fraction of free T unanticipated by the model, even in the presence of excess F. That is to say, the thrombin mass balance deduced from Table AI can be in error at relatively high $K_d$ and/or low $R^\circ$. Mass balance error manifests itself in computational experiments as a positive or negative deviation from the anticipated $X_{TF} = 1$ condition at low $R^\circ$ and $K < 1$. For example, under the extreme conditions of $K = 10^{-4}$ and $R^\circ = 0.5$ ($\alpha = 0.2$, $K_d = 0.5$, $[T^\circ] = 128$ nM) $X_{TF}$ was over estimated by about 10% at $[F^\circ] = 40$ $\mu$M (a physical impossibility) and underestimated by about 25% at $[F^\circ] = 15$ $\mu$M. Thus the model would require appropriate adjustment for accurate application to ligands at low concentration with relatively low affinity for thrombin (low anticoagulation efficiencies).

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**References**


