

Procoagulant Stimulus Processing by the Intrinsic Pathway of Blood Plasma Coagulation

A Contribution from the Hematology at Biomaterial Interfaces Research Group
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By

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Abstract

Potentialiation of the intrinsic pathway of human blood plasma coagulation *in vitro* by contact with a solid procoagulant surface leads to *bolus* release of thrombin (FIIa) in concentration proportion to the intensity of activation as measured by procoagulant surface area or energy (water wettability). This rather remarkable finding is confirmed using two different assays: one triggering coagulation substantially through the intrinsic pathway alone and the second triggering coagulation through the intrinsic pathway in the presence of exogenous FIIa spikes. Similarity of experimental outcomes of these assays strongly suggests that endogenous FIIa production through the intrinsic pathway is independent of the absolute amount of FIIa present in plasma. Furthermore, we corroborate previous work indicating that procoagulant surfaces remain activating after repeated use and are not poisoned or denatured in the process of activating plasma coagulation. It is concluded that the sharp control mechanism that gives rise to bolus-production of FIIa from the intrinsic pathway must occur between surface activation of FXII and the FII → FIIa step, is not related to inhibition by FIIa, and does not involve deactivation of procoagulant surfaces.

Key words: blood coagulation; plasma; procoagulants; Hageman Factor; FXII; mathematical model of coagulation; thrombin; FII

Introduction

Perioperative bleeding and thrombosis are significant barriers to the development and implementation of advanced in-dwelling blood pumps and ventricular assist devices [1, 2]. Indeed, thrombosis remains a risk in the application of ordinary biomedical devices such as catheters, now used in the tens-of-millions annually in various nosocomial procedures [3]. Thrombosis can usually be traced to adverse blood-biomaterial interactions that lead to the formation of emboli. Bleeding, on the other hand, is frequently associated with excessive anticoagulation employed to prevent thrombosis. Thus, blood-contact phenomena persists as one of the more important facets of biomaterials surface science and development of truly hemocompatible materials remains a largely unrealized objective [3, 4]. Future advances in the surface-engineering of blood-contacting biomaterials [4, 5] are critically dependent on a detailed understanding of how blood “processes” presence of an artificial material at a molecular level. Toward this understanding, we have focused on the blood-plasma coagulation cascade *in vitro* as a highly simplified, yet very informative, experimental system that permits us to probe surface-contact events that trigger platelet-poor plasma (PPP) coagulation in a manner that is substantially (but not wholly) independent of cellular interactions [6, 7]. The general expectation is that this information will provide guidance to the design of materials with improved hemocompatibility.

Classical biochemistry of the intrinsic pathway suggests that a “procoagulant stimulus” originating in exposure of plasma to a test surface (herein also referred to as a dose), potentiates a cascade of zymogen-enzyme conversions (a response) that culminate in

the release of the protease thrombin (FIIa). Individual steps of this cascade have been the subject of intense scrutiny over the last few decades, resulting in a quite complete characterization of the biochemistry of separated/purified reactions, especially for the extrinsic cascade that is partly responsible for hemostasis of circulating whole blood *in vivo*. However, much less is known about the holistic operation of the intrinsic cascade that is activated by contact with foreign materials [7] beyond that inferred from the classical tests of coagulation [8]: partial prothrombin time (PTT), and activated prothrombin time (aPTT) (see ref. [9] and citations therein). PTT and aPTT protocols purposely saturate the coagulation cascade with procoagulant dose (thromboplastin or thromboplastin mixed with a high surface area silica or kaolin, respectively) to evoke maximum response (minimum coagulation time) from the so-called extrinsic and intrinsic pathways of plasma coagulation, respectively. These tests effectively side-step the question of “dose processing” that we seek to more fully understand by carefully controlling the kind/intensity of dose as an experimental variable (esp. surface area, chemistry, and energy) and relating this to the observed response (coagulation time or release of enzyme intermediates) using mathematical models of plasma coagulation.

Modeling kinetics of a series of interconnected zymogen-enzyme conversions in which the product of a preceding reaction is the enzyme of a subsequent reaction is a rather complicated affair, especially when self-amplifying loops and feedback inhibition are taken fully into account. Early efforts [10-14] have led to informative computational models of the extrinsic pathway [14-27] but, as already mentioned, much less work has been dedicated to modeling the intrinsic pathway that is activated by contact with

material surfaces [28, 29]. No doubt a limitation has been a dearth of fundamental information relating procoagulant surface properties to the extent that the intrinsic pathway is potentiated [6] and the proportions between dose (surface area, chemistry, energy) and response (release of activated enzyme, coagulation time). It is these structure-property relationships that we seek for the purpose of developing biomaterials with improved hemocompatibility.

As an alternative to developing a manifold of differential equations representing individual enzyme reactions expressed in terms of a number of rate constants and activation efficiencies (related to procoagulant surface properties) that have been derived under conditions that may or may not be entirely relevant to whole-plasma coagulation *in vitro*, we have adopted the “phenomenological” approach used in traditional hematology that abstracts the cascade into functional compartments grouping related multi-step enzyme reactions. This engineering method is especially useful in modeling the holistic behavior of a complex *in vitro* experimental vehicle such as PPP [6, 7] for which all sources and sinks of enzyme or surface activities may not be clearly known or identified. A significant goal is to derive simplified analytical relationships between dose and response written explicitly in terms of rate parameters (typically ratios of rate constants) that measure an effective compartment potentiation. This strategy has the decided advantage over purely computational models in that derived analytical expressions can be statistically fit to experimental data using rate parameters as adjustable factors of the fit. In turn, fitted-rate parameters (with error estimates derived from least-squares statistics) can be scaled against dose characteristics to

induce or infer how dose is processed by the cascade. Although this sort of modeling will not typically yield kinetic information about the *individual* enzyme-mediated reactions comprising the coagulation compartment under investigation and thus cannot supplant/replace detailed computational kinetics, it has been shown to provide useful insights into how the *intact* cascade is activated [6] and deactivated [7] *in whole plasma*. Importantly, this information quantitatively links material properties to the coagulation of whole plasma.

Herein we apply human PPP as an experimental system, coagulation time as the measured response, and phenomenological modeling to demonstrate that procoagulant stimulus originating in contact with surfaces with varying water wettability (surface energy) propagates through the cascade in a manner that releases a bolus of FIIa in remarkable concentration-proportion to procoagulant surface area. This work extends and corroborates previous work that used porcine plasma as a test system, suggesting that the classical mechanism of the intrinsic cascade of blood coagulation requires some revision or upgrading to fully account for the details of surface activation.

Methods and Materials

Procoagulant Surfaces: Test procoagulants applied in this work were 425-600 μm diameter glass beads (Sigma-Aldrich) in either cleaned or silanized form. Clean glass was prepared by 3X serial rinses in 18 M Ω water (obtained from a Millipore Simplicity unit), 2-propanol, and chloroform (reagent grade, VWR) followed by air-plasma

treatment of a single layer of washed beads held in a 15 mm Pyrex glass petri dish (10 min. at 100 watts plasma; Herrick, Whippany NY). Clean glass beads were used within the same day of air-plasma treatment to assure the water-wetted condition. Glass was silanized by either 1.5 hr. reaction with 5% octadecyltrichlorosilane (OTS in chloroform; United Chemical Technologies, Inc.) or by 5 min. reaction with 2% 3-aminopropyltriethoxysilane (APS in acetone; United Chemical Technologies, Inc.). Silanized beads were 3X rinsed in either chloroform or acetone, respectively, and dried in a vacuum oven at 110°C for 24 hr. Contact angles of glass-slide witness samples measured by Wilhelmy balance tensiometry (CDCA-100, Camtel Ltd.) typically yielded advancing/receding contact angles 0°/0°, 70°/40°, and 110°/90° for clean, APS, and OTS procoagulants, respectively, with no more than about 10° variation among batches. Contact angles cannot be read directly on glass beads but optical microscopy of the shape of the liquid meniscus of beads partly immersed in water on a microscope slide qualitatively confirmed that the treated beads were not different from the witness samples.

Plasma and Thrombin: Platelet poor (citrate) plasma (PPP) is a proven experimental vehicle for investigating the coagulation cascade [8, 30], both in traditional hematology (recalcified-citrate plasma is the preferred form of blood for aPTT and PTT testing) [8] and in previous studies of coagulation/anticoagulation [6, 7]. Recalcified PPP is wholly coagulation competent, requiring no additional proteins or phospholipids to recover clotting potential. Human platelet poor plasma (hPPP, citrate) was prepared from outdated (within 2 days of expiration) lots obtained from the M.S. Hershey Medical

Center Blood Bank. This work was performed with a single pooled lot of plasma aliquoted into 15 mL polypropylene tubes (Falcon, Becton Dickinson) and frozen at -20°C until use. We observed consistent results with this plasma over the 1 year time span of experimentation. Experience has shown that different lots of plasma yield quantitatively different but qualitatively similar results. Human α -thrombin (Sigma-Aldrich) solutions were freshly-prepared before each use by dissolution in phosphate buffer saline (PBS, Sigma-Aldrich) to 100 NIH unit/mL.

Coagulation-Time Assays: The basic protocol for coagulation-time assays used in this work has been described in detail elsewhere [6, 7] and applied in this work in three basic variations: surface-area titrations (SAT), thrombin titration (TT), and thrombin-surface area co-titrations (COT). In each variation, 0.5 mL of thawed plasma equilibrated with ambient temperature was transferred into 15×75 mm polystyrene tubes (VWR) and diluted with sufficient PBS to bring the final total liquid volume to 1 mL; including all additives which varied according to the assay. In SAT and COT experiments, tubes containing varying amount of solid procoagulant between 0 – 300 mg (as measured on an electronic balance to within 0.1 mg) were prepared before liquid ingredients were added. In COT and TT experiments, serially-increasing volumes between 0 and 50 μ L of FIIa (100 NIH unit/mL in PBS) were added to each tube in the titration series. In all cases, coagulation was induced by recalcification with 0.1mL of 0.1 M CaCl_2 and contents were mixed on a slowly-turning hematology mixer (Roto-shake Genie, Scientific industries, Inc.). Coagulation time CT after recalcification was noted by a distinct change in fluid-like rheology to gel formation, allowing determination

of the end point of the coagulation process to within 10 s or so [6]. These simple, yet highly sensitive, recalcification-time assays eliminated extraneous contributions to coagulation associated with many modern instrumented tests (e.g. activating surfaces of stirrers and tubing) and yielded smooth dose-response curves that varied with procoagulant properties (surface chemistry and energy) or FIIa concentration.

Multiple Activation of Plasma Coagulation: A single 12 X 75 mm glass tube was cleaned according to the protocol described above for clean glass bead procoagulants, including air-plasma-discharge treatment. 0.1 mL of 0.1 M CaCl_2 and 0.4 mL of PBS buffer was added to 0.5 mL plasma and time to coagulation was measured while the tube was turned on a hematology mixer as described above. After coagulation, clot was removed by draining liquid contents, shaking-out solid contents, and rinsing 3X with ~ 5 mL PBS. An additional 1 mL PBS was added to the tube and the contents turned for 2 hr on a hematology mixer after which the wash was replaced with fresh citrated PPP, PBS, and CaCl_2 . This process was repeated 5X for each of 3 separate trials employing a single glass tube, achieving an average coagulation time of 6.9 ± 0.7 min ($N = 3 \times 5 = 15$).

Mathematical Methods: Models of plasma coagulation applied herein were basically extensions of previously-published work [6, 7]. Very briefly reviewing the core ideas behind these models for current purposes, surface activation of plasma coagulation was proposed to occur in some proportion to procoagulant surface area that led to production of a hypothetical activated fibrin monomer F^* . F^* subsequently entered into rate-limiting oligomerization (measured by a rate constant k_p with min^{-1} units) yielding a

fibrin mesh that ultimately caused plasma to coagulate. A key premise was that coagulation did not occur until a fixed fraction of fibrinogen α had been converted into F^* , independent of procoagulant surface properties, and the rate of F^* production controlled coagulation time CT . Thus, surface-activated coagulation was compared to a negative control (typically time to coagulation in the absence of any activator CT^o), leading to an analytical solution for CT in terms of ratios of unknown rate constants:

$$CT = -\left(\frac{1}{k_p}\right) \ln \left[\frac{K_{act}^{SAT} A + e^{-k_p CT^o}}{(K_{act}^{SAT} A + 1)} \right] \quad (1)$$

where A represented procoagulant surface area and K_{act}^{SAT} a ratio of rate constants that measured the ‘catalytic potential’ of the procoagulant surface under study. An important point to be stressed in application of this model in the current work is that previous work incorrectly identified dimensions of K_{act}^{SAT} . Careful scrutiny of ref. [6] has revealed that correct units of K_{act}^{SAT} were mL/m², not m⁻² procoagulant surface area (note also that k_p was designated k_2 and K_{act}^{SAT} designated K in ref. [6]). Otherwise, Eq. (1) was used exactly as previously described, including assignment of an arbitrary value to the unknown k_p fixed by a two-parameter fit of Eq. (1) to experimental CT data obtained using a positive-control standard. In this case, clean glass beads were positive controls for which $k_p = 0.54 \pm 0.10 \text{ min}^{-1}$ was obtained (average \pm std. dev for N = 3 separate trials; compare to $0.70 \pm 0.09 \text{ min}^{-1}$ for porcine plasma). Thus, K_{act}^{SAT} measured activation potential relative to this internal standard and is not to be interpreted as a quantitative characteristic of procoagulants studied herein. Likewise k_p should not be regarded as representative of human PPP in general (see ref. [6] for more discussion).

The FIIa-titration model used herein was described in ref. [7]. The essential idea underlying this theory was that an exogenous FIIa spike was instantaneously (relative to the timeframe of coagulation) partitioned between free and bound forms. As in the surface-activation model discussed above, it was assumed that FIIa hydrolysis of fibrinogen led to production of F^* and that the fraction of free thrombin was vanishingly small (mole fraction $X_{TF} \rightarrow 1$; see ref. [6] for details). Elaboration of this model led to an analytical relationship between observed CT and FIIa-spike concentration $[T^o]$ in (NIH unit/mL as applied herein):

$$CT = \frac{\alpha[F^o]}{k_3[T^o]} + \frac{(1 - e^{-k_p CT})}{k_p} \quad (2)$$

where the term $\frac{\alpha[F^o]}{k_3}$ was an unknown constant treated as a single variable parameter.

Recognizing that Eq. (2) cannot be explicitly solved for CT without additional simplifying assumptions, a commercial engineering-equation-solver software package (F-Chart Software, Madison, WI) was used to obtain the best-fit statistical solution to experimental FIIa-titration data (observed CT at various $[T^o]$) using $k_p = 0.54 \pm 0.10$ min^{-1} as discussed above in reference to SAT.

The COT model was a simple extension of Eq. (2) wherein it was assumed that activation of the intrinsic cascade produced a bolus concentration of *endogenous* FIIa (measured by the parameter Θ with dimensions of NIH unit/mL) that directly added to the *exogenous* FIIa spike, such that:

$$CT = \frac{\alpha[F^o]}{k_3([T^o] + \Theta)} + \frac{(1 - e^{-k_p CT})}{k_p} \quad (3)$$

Thus, under conditions of this assumption, Eq. (3) permitted extraction of Θ by fitting to COT data, again obtained by use of engineering-equation-solver software mentioned above in reference to TT.

Results

Thrombin Titration of Human Plasma: Sensitivity and reproducibility of human PPP coagulation from outdated blood (see Methods and Materials) was tested by ‘thrombin titration’ (TT) experiments in which coagulation time CT of recalcified plasma induced by increasingly-concentrated exogenous-bolus spikes of human α -thrombin $[T^o]$ was observed. Fig. 1A collects results of a number of FIIa titrations performed on one batch of plasma on different dates spanning approximately 1 year of experimentation. As can be readily appreciated from this plot, CT was approximately sigmoidal on a $\log_{10} [T^o]$ (unit/mL) scale, with a very noisy low-concentration asymptote near 46 min and more consistent high-concentration limit near 0.5 min. Thus, the TT working range appeared to be approximately two decades in $[T^o]$, lying between $1 \times 10^{-2} < [T^o] < 1$ unit/mL.

Fig. 1B redraws data of Fig. 1A falling within this working range on $1/[T^o]$ scale, consistent with Eq. (2) of Methods and Materials (see figure legend for details).

Surface Area Titration (SAT) of Human Plasma: Fig. 2 collects results of human PPP activation by serially-increasing surface area of solid procoagulants with different surface chemistry/energy obtained by silanating clean glass beads with APS or OTS.

Significant effort was invested in the clean-glass SAT curve, especially at low surface area, to verify the trend suggested by Eq. (1) applied to human PPP and to confidently establish a value for k_p (see Methods and Materials). Much less effort was expended in the low surface-area regime for APS and OTS, focusing instead on higher-surface area for the purpose of establishing a confident value for K_{act}^{SAT} as the single adjustable parameter in non-linear least-square fitting to Eq. (1) with $k_p = 0.54 \text{ min}^{-1}$ (see Methods and Materials). Smooth lines drawn through the data of Fig. 2 represent the best fitted curves. Table 1 collects results for the three procoagulants studied in this work where the error estimate given for K_{act}^{SAT} represents standard error of regression through pooled data of three separate SAT experiments for each procoagulant. Evidently, the SAT model represented by Eq. (2) of Methods and Materials adequately simulated experimental data, corroborating previous studies of porcine PPP coagulation [7] using a much broader array of polymer and self-assembled monolayer (SAM) test procoagulant surfaces. K_{act}^{SAT} values in Table 1 will be discussed further in the discussion section.

Thrombin-Surface Area Co-titration (COT) of Human Plasma: Fig. 3 collects representative results from FIIa titration of human PPP in the presence of varying surface areas of water-wettable glass spheres. Smooth lines drawn through the data of Fig. 3 were obtained by non-linear least-square fitting to Eq (3) of Methods and Materials with $k_p = 0.54$ (as discussed above in reference to SAT) and $\frac{\alpha[F^0]}{k_3} = 0.24$ (as discussed above in reference to TT). Evidently, the COT model represented by Eq (3)

adequately simulated experimental data for all cases up-to-and-including 4.7×10^{-4} m^2/mL surface area clean glass procoagulant beads. Co-titration results obtained with more hydrophobic procoagulants (not shown) were similar except that the difference between co-titration (surface activation of FXII in the presence of FIIa) and FIIa titration (with no added solid procoagulant) was much less pronounced. Table 2 collects fitted values for variable Θ for clean and silane-treated glass procoagulants performed in duplicate. Fig. 4 plots Θ as a function of surface area A showing a strong linear relationship ($R^2 \geq 96\%$) for all three test procoagulants with a slope K_{act}^{COT} that measures the amount of endogenous FIIa released per-unit-area procoagulant. Thus, K_{act}^{COT} is a measure of the catalytic power of the procoagulants that is similar to K_{act}^{SAT} . Fig. 5 and Table 1 compare K_{act}^{COT} and K_{act}^{SAT} values for the test procoagulants.

Retention of Procoagulant Catalytic Potential: Fig. 6 collects results of repeated coagulation assays in a single, clean glass tube following the protocol outlined in the Methods and Materials section. Mean and standard deviation of three replicate tubes indicated that no single trial could be confidently distinguished from the average of 6.9 ± 0.7 min. These results corroborate previous studies with porcine PPP [6, 31] and demonstrate that repeated activation of plasma coagulation does not measurably affect the procoagulant potential of a surface exposed to whole plasma.

Discussion

Smooth and reproducible results obtained by thrombin titration (TT), surface-area titration (SAT), and thrombin-surface-area co-titration (COT) coagulation assays applied

to human PPP verify that re-calcified plasma is a reproducible, robust test system for the investigation of enzyme and surface-activated coagulation *in vitro*. Quality of fit of the SAT, TT, and COT models represented by Eqs. (1) to (3) of the Methods and Materials section to experimental data strongly suggests that simplifying assumptions underlying theory were reasonable approximations of reality and the parameters of these models are important controlling factors. When scaled to procoagulant surface properties, these parameters provide insights into the hemocompatibility of materials; at least from the perspective of platelet poor plasma that is substantially depleted of cellular contributions to coagulation.

SAT curves (Fig. 2) obtained using clean glass beads, APS, and OTS silane-modified glass beads formed nested, nearly parallel curves on the same axes, giving visual testimony to the conclusion drawn from studies of porcine PPP that procoagulants have variable, surface-energy-dependent catalytic potential [6, 7, 31]. Fit of the SAT model (Eq. (1) of Materials and Methods) K_{act}^{SAT} quantifies this procoagulant catalytic potential and Fig. 5 suggests that K_{act}^{SAT} scales sharply with procoagulant surface energy (as measured by water adhesion tension) in a manner similar to that observed with porcine PPP; although this work explored a much more limited selection of procoagulant surfaces than previous work.

Co-titration is an interesting extension of SAT experiments in which the intrinsic cascade is activated to produce *endogenous* FIIa in the presence of increasingly-concentrated *exogenous* FIIa spikes. Fig. 3 shows that endogenous FIIa produced by

serially-increasing surface area of clean glass procoagulant adds to the exogenous thrombin spike used in the titration experiment (compare also to Fig. 1B noting differences in $[T^o]^{-1}$ scaling). Interpreted in terms of the COT model of Eq. (3), endogenously-produced FIIa adds to the exogenous spike in a manner dependent on both surface area and surface energy of the solid procoagulant. Smooth lines through the data of Fig. 3 result from the fit of Eq. (3) to the data, yielding an estimate of the amount of endogenous FIIa produced, as measured by the fitted parameter Θ . Fig. 4 shows that Θ scales linearly with procoagulant area with a slope K_{act}^{COT} that is a measure of procoagulant catalytic potential to produce endogenous FIIa. Thus K_{act}^{COT} is analogous to K_{act}^{SAT} obtained from SAT curves in that both measure the propensity of a surface to potentiate the intrinsic pathway. It is important to stress, however, that these two different parameters were obtained from two completely different assays are based on two different models of coagulation, and have different dimensions (unit/m² compared to mL/m², respectively). However, as shown in Fig. 5, these independent measures of procoagulant catalytic potential yield similar trends with procoagulant surface energy, suggesting that there is indeed a sharp dose-response relationship between surface energy and procoagulant catalytic potential, similar to that observed in porcine PPP[6, 31]. Firm functional relationships between procoagulant catalytic potential and procoagulant surface energy for human PPP awaits similar studies embracing a greater selection of surface types.

An important assumption underlying both SAT and COT models was that FIIa is produced by activation of the intrinsic cascade of plasma coagulation as a *bolus*

concentration, as opposed to continuous production of FIIa by the continuous presence of procoagulant surface area. Quality of fit of these models to experimental data suggests this assumption was reasonable and that FIIa was produced in bolus-concentration proportion to the intensity of activation as measured by procoagulant surface area or energy (water wettability). We note that this conclusion is consistent with direct measurement of FIIa generation by chromogenic assays in which it was observed that FIIa concentration rises sharply with time in contact with Ti alloys and remains relatively constant throughout the coagulation reaction [32]. Results also corroborate conclusions based on thrombin-aptamer titration of endogenous thrombin produced by activation of the intrinsic cascade [7]. However, results reported herein seem inconsistent with FIIa-burst kinetics reported to occur in whole blood [24, 33, 34] where FIIa generation occurs predominately on and within cells [34]. Furthermore, burst intensity in proportion to surface activation appears to be inconsistent with a self-amplifying mechanism of FIIa production mediated by cofactors (FVa and FVIIIa) assembled on cell membranes.

It is of interest to speculate how this rather remarkable production of FIIa in bolus-concentration proportion to the intensity of procoagulant activation occurs. The control mechanism must lie between surface activation of FXII and FIIa generation – the two points of cascade activation explored in this work. Apparently, biochemistry of the intrinsic cascade is effectively turned off in the continuous presence of an activating procoagulant surface since experiments summarized in Fig. 6 show that strongly-activating procoagulant surfaces remain activating through multiple applications,

discounting the possibility of procoagulant poisoning or deactivation by fouling with plasma proteins; at least for the clean-glass procoagulants studied herein. Suggestion that FXI is activated by FIIa in plasma [35, 36] seems inconsistent with results reported herein because this would presumably lead to continuous amplification of procoagulant activation rather than punctuated deactivation. Rather, work of Mitropoulos *et al.* [37], showing that FXIIa inhibits the FXII \rightarrow FXIIa appears to be a more likely process that could lead to a self-termination reaction that effectively sends an activating pulse down the coagulation cascade, culminating in discontinuous production of FIIa in proportion to the originating stimulus.

Conclusions

Human plasma has been shown to be a robust experimental system for studies of the surface activation of coagulation *in vitro*. Three different coagulation assays provide a measure of the dynamic range of coagulation time that can be explored using exogenous thrombin (FIIa) spikes (thrombin titration, TT) and/or surface activation of the intrinsic pathway of coagulation (surface area titration, SAT or thrombin-surface area titration, COT). Mathematical models of TT, SAT, and COT assays predicated on the idea that FIIa is available as a bolus concentration (either as an exogenous spike as in TT or an endogenous bolus produced by the intrinsic cascade in SAT) give excellent fit to experimental data, strongly suggesting that simplifying assumptions underlying theory were reasonable approximations of reality and that model parameters are important controlling factors.

Results of SAT and COT assays strongly suggest that endogenous FIIa is produced by potentiation of the intrinsic pathway of plasma coagulation in bolus concentration-proportion to the intensity of procoagulant activation, as measured by surface area and surface energy. The control mechanism leading to bolus FIIa production apparently lies between FXII activation and FIIa generation, is shown not to be inhibited or limited by the absolute concentration of FIIa in plasma, and is not consistent with cell-mediated thrombin-burst kinetics observed in whole blood. Furthermore, experimental results show that procoagulant surfaces remain activating throughout the coagulation process, suggesting that the cascade must somehow be deactivated or 'turned off' soon after being activated or 'turned on', as opposed to continuous FIIa generation by a continuously-activated intrinsic cascade. The FXII→FXIIa reaction itself seems a likely point of control, as has been suggested previously, but the exact surface-mediated steps involved are not apparent from this work. In any event, the traditional biochemistry of surface activation to the plasma coagulation cascade appears to require elaboration/modification to account for bolus production of thrombin in a strict concentration-proportion to the activating procoagulant dose.

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List of figure legends

Figure 1:

(A) Thrombin titrations (TT) plotted on a $\log_{10}[T^o]$ axis. Coagulation time varied sigmoidally with thrombin concentration $[T^o]$, suggesting that the working range lies between $0.5 < CT < 46$ min and $1 \times 10^{-2} < [T^o] < 1$ unit/mL (see annotations).

(B) TT on $1/[T^o]$ coordinates consistent with Eq (2) of Method and Materials.

Line through the data is best fit of Eq (2), with $R^2=89.3\%$ using $k_p=0.54$

and yielding $\frac{\alpha[F^o]}{k_3} = 0.24 \pm 0.09$.

Figure 2:

Surface area titration with glass beads bearing different surface chemistries plotting observed coagulation time CT as a function of procoagulant surface area per mL test solution ($\bullet, \blacktriangle, \blacksquare$ collect results of three independent trials and annotations identify procoagulant types; see Methods and Materials). Smooth curves drawn through the data result from least square fitting to Eq. (1) of Method and Materials yielding K_{act}^{SAT} values collected in Table 1. Low surface area regime was explored only for clean glass procoagulants to secure k_p value by two-parameter fitting to Eq. 1 of Methods and Materials. Dashed lines for OTS and APS is the trend suggested by the fitted curve.

Figure 3:

Representative thrombin titration curves in the presence of varying surface area of fully-water-wettable glass procoagulants. Smooth lines through the data result from non-linear least-squares fitting to Eq. (3) of Method and Materials, extracting Θ values listed in Table 2. Smooth curve corresponding to 0 m²/mL was derived from the data of Fig. 1B.

Figure 4:

Bolus thrombin concentration Θ produced by varying surface area of glass-sphere procoagulants with different surface chemistries (● clean glass, ▲ APS treated glass, ■ OTS treated glass). Range on the mean of duplicate trials (N=2) collected in Table 2 is approximated by symbol size. Note that the Θ axis is scaled by 10⁻ⁿ with n values listed in figure annotations on the left side of the figure.

Figure 5:

Procoagulant catalytic potential derived from SAT (K_{act}^{SAT} , left axis, circles) and COT (K_{act}^{COT} , right axis, triangles) coagulation assays scaled as a function of procoagulant surface energy expressed as water adhesion tension $\tau^o = \gamma_{lv}^o \cos \theta$, where the 'o' superscript denotes pure PBS buffer with interfacial tension $\gamma_{lv}^o = 72$ dyne/cm at 25 °C, exhibiting contact angles θ on test surfaces measured in both advancing (filled symbols) and receding modes (open symbols). Uncertainty

indicated by error bars represent standard deviation of mean for K_{act}^{SAT} (N=3) and range for K_{act}^{COT} (N=2) . Note that K_{act}^{SAT} and K_{act}^{COT} are two different measures of procoagulant catalytic activity with two different dimensions. Each suggests an exponential-like dependence on procoagulant surface energy as measured by τ^o .

Figure 6:

Coagulation time (CT , min) of plasma in a single clean glass tube used in multiple coagulation assays does not vary significantly, implying that procoagulant surfaces remain activating throughout the course of plasma-coagulation experiment (see Methods and Materials for protocol). Uncertainty indicated by error bars represent standard deviation of the mean of three separate trials (N=3).

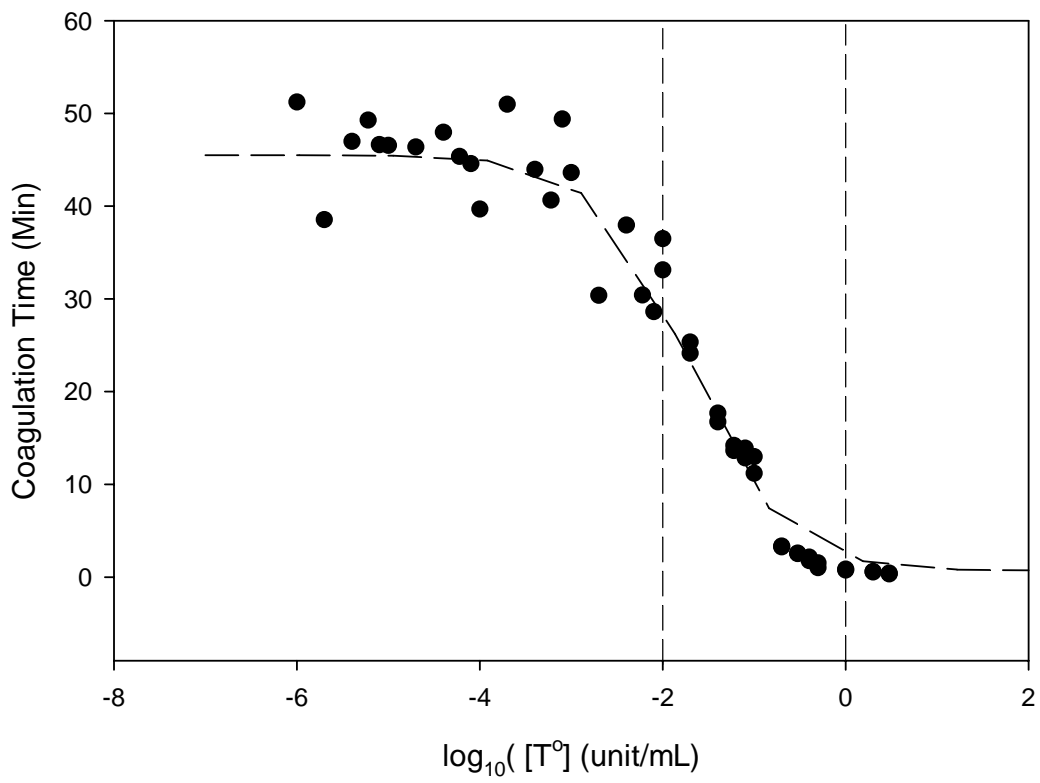
Table 1: Procoagulant Catalytic Activities

| Procoagulant | Norminal Contact Angle (adv. / rec.) | K_{act}^{SAT} from SAT (mL/m ²) N=3 | K_{act}^{COT} from COT (unit/m ²) N=2 |
|-------------------|---|---|---|
| Clean Glass | 0°/0° | 21.1±2.1 | 35.7±3.7 |
| APS Treated Glass | 70°/40° | $(9.0±1.7)×10^{-3}$ | 4.0±0.8 |
| OTS Treated Glass | 110°/90° | $(4.0±1.7)×10^{-4}$ | 2.8±0.5 |

Table 2: Endogenous FIIa Production

| Surface Area of Procoagulants ($10^{-4} \times \text{m}^2/\text{mL}$) | Trial Number | Clean Glass Θ ($10^{-2} \times \text{unit}/\text{mL}$) | APS Treated Glass Θ ($10^{-3} \times \text{unit}/\text{mL}$) | OTS Treated Glass Θ ($10^{-3} \times \text{unit}/\text{mL}$) |
|--|--------------|--|--|--|
| 0.47 | 1 | 2.10 | 9.37 | 7.67 |
| | 2 | 2.31 | 9.56 | 7.80 |
| 1.5 | 1 | 2.55 | 10.2 | 8.20 |
| | 2 | 2.43 | 10.0 | 8.02 |
| 2.4 | 1 | 3.06 | 10.5 | 8.46 |
| | 2 | 2.87 | 10.6 | 8.37 |
| 3.9 | 1 | 3.40 | 10.9 | 8.81 |
| | 2 | 3.27 | 11.0 | 8.66 |
| 4.7 | 1 | 3.61 | 11.3 | 8.94 |
| | 2 | 3.98 | 11.5 | 9.10 |

Figure 1 (A)



(B)

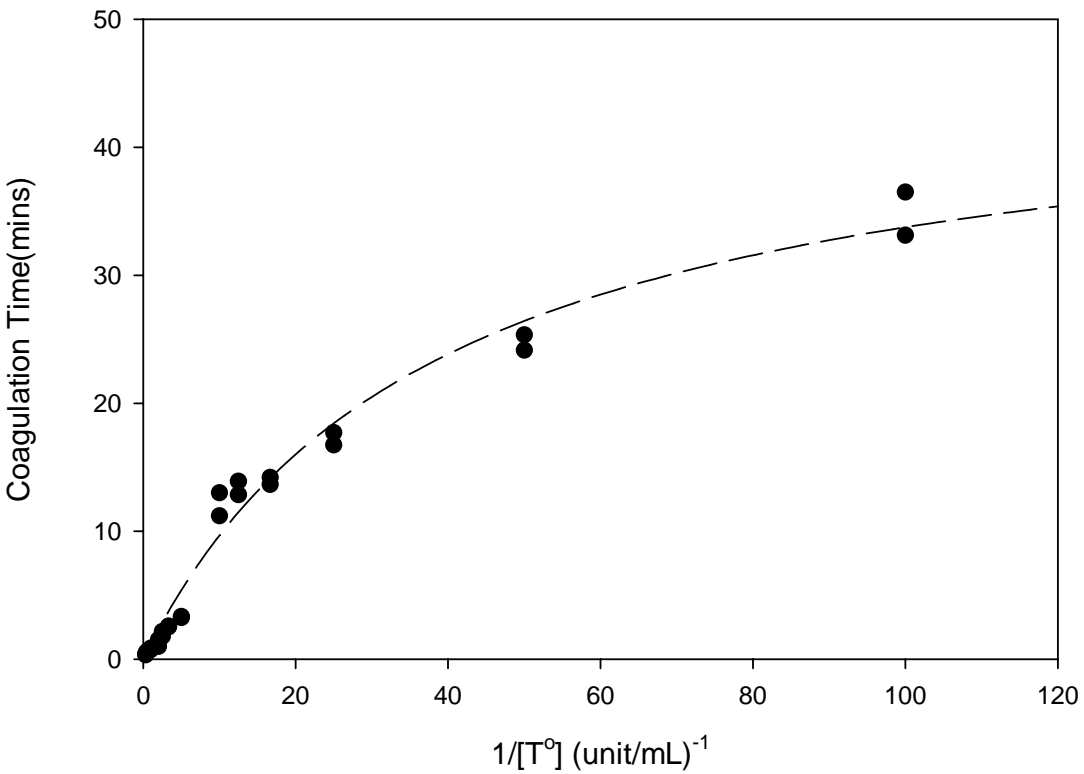


Figure 2

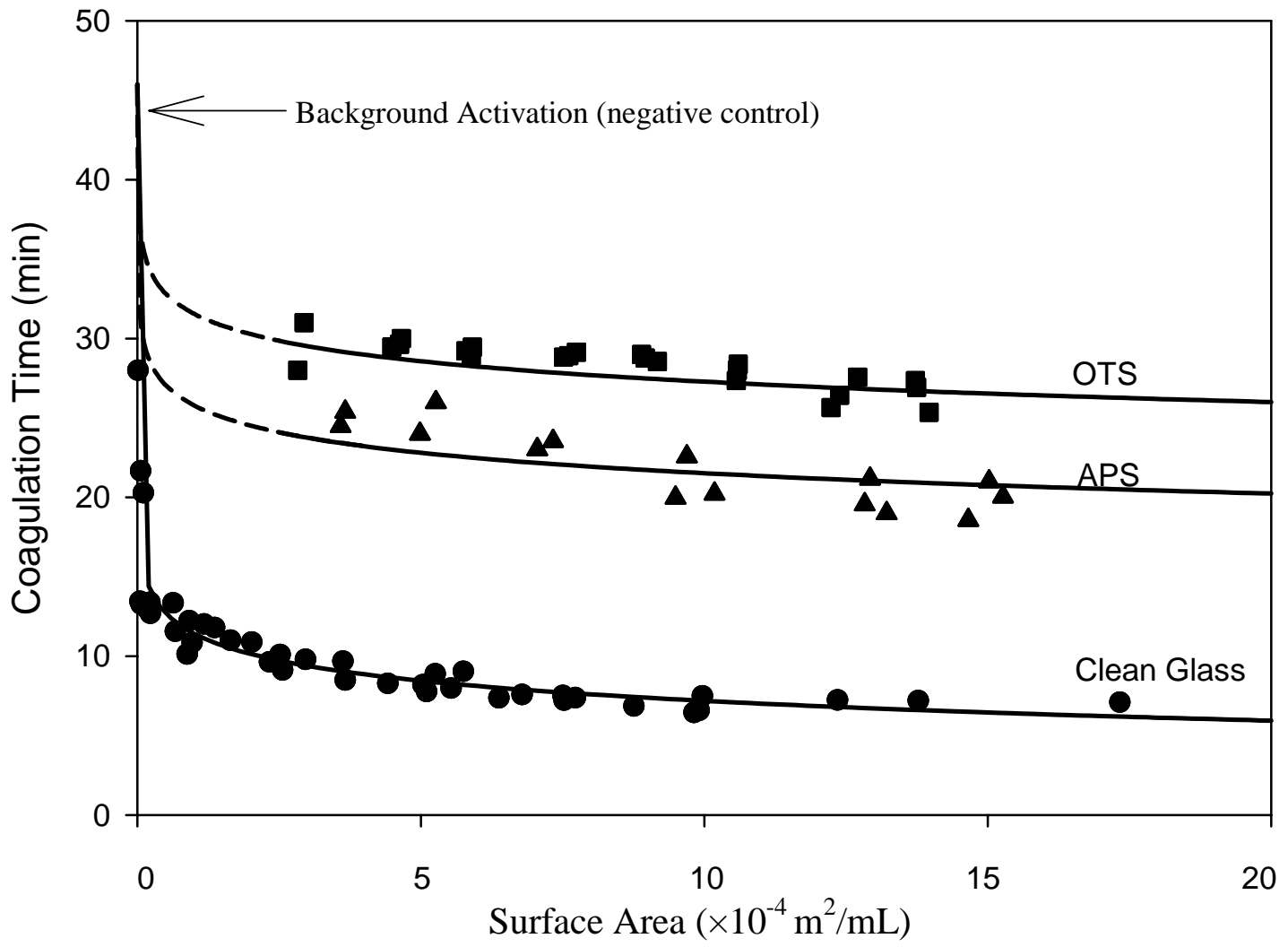


Figure 3

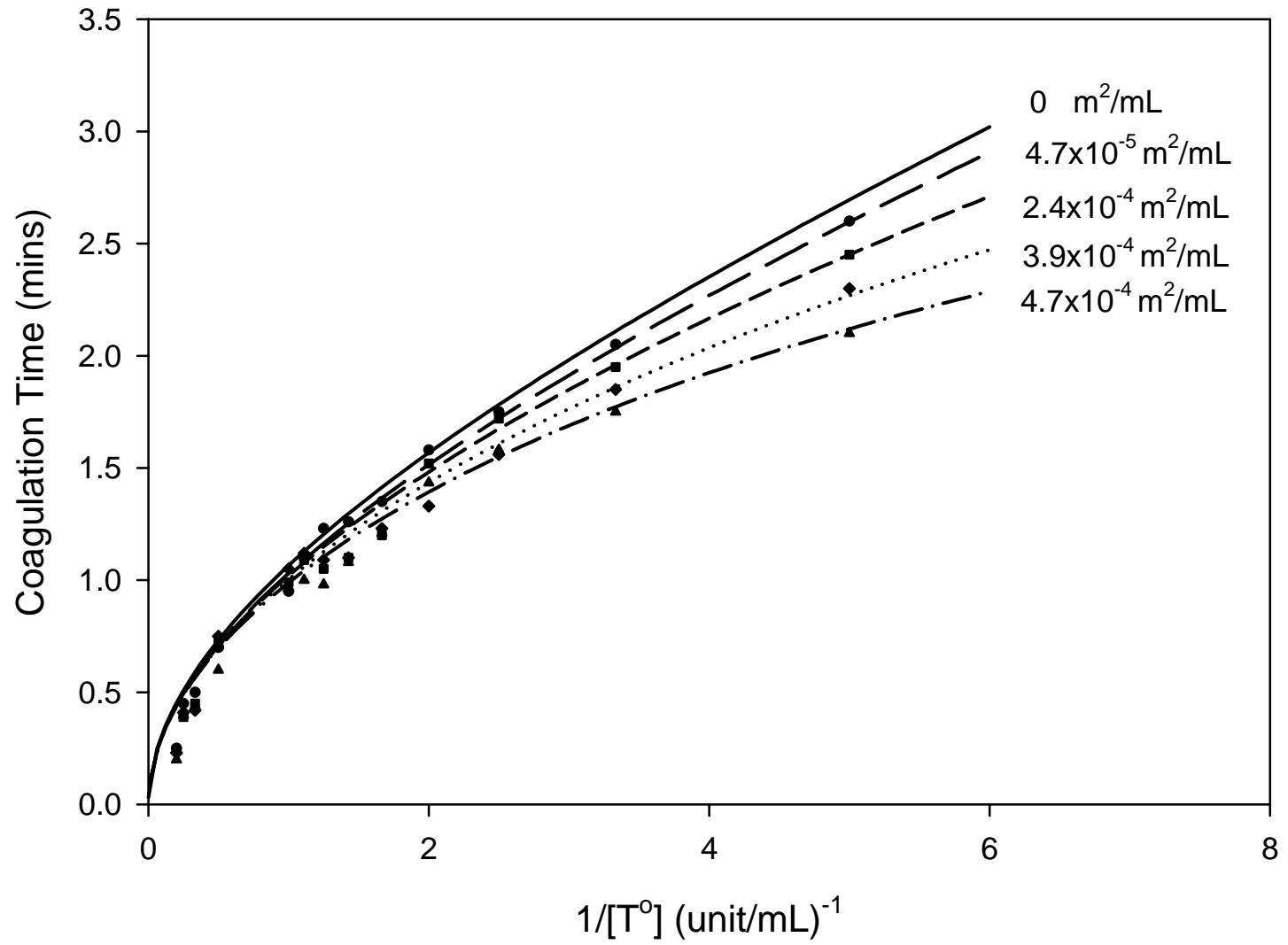


Figure 4

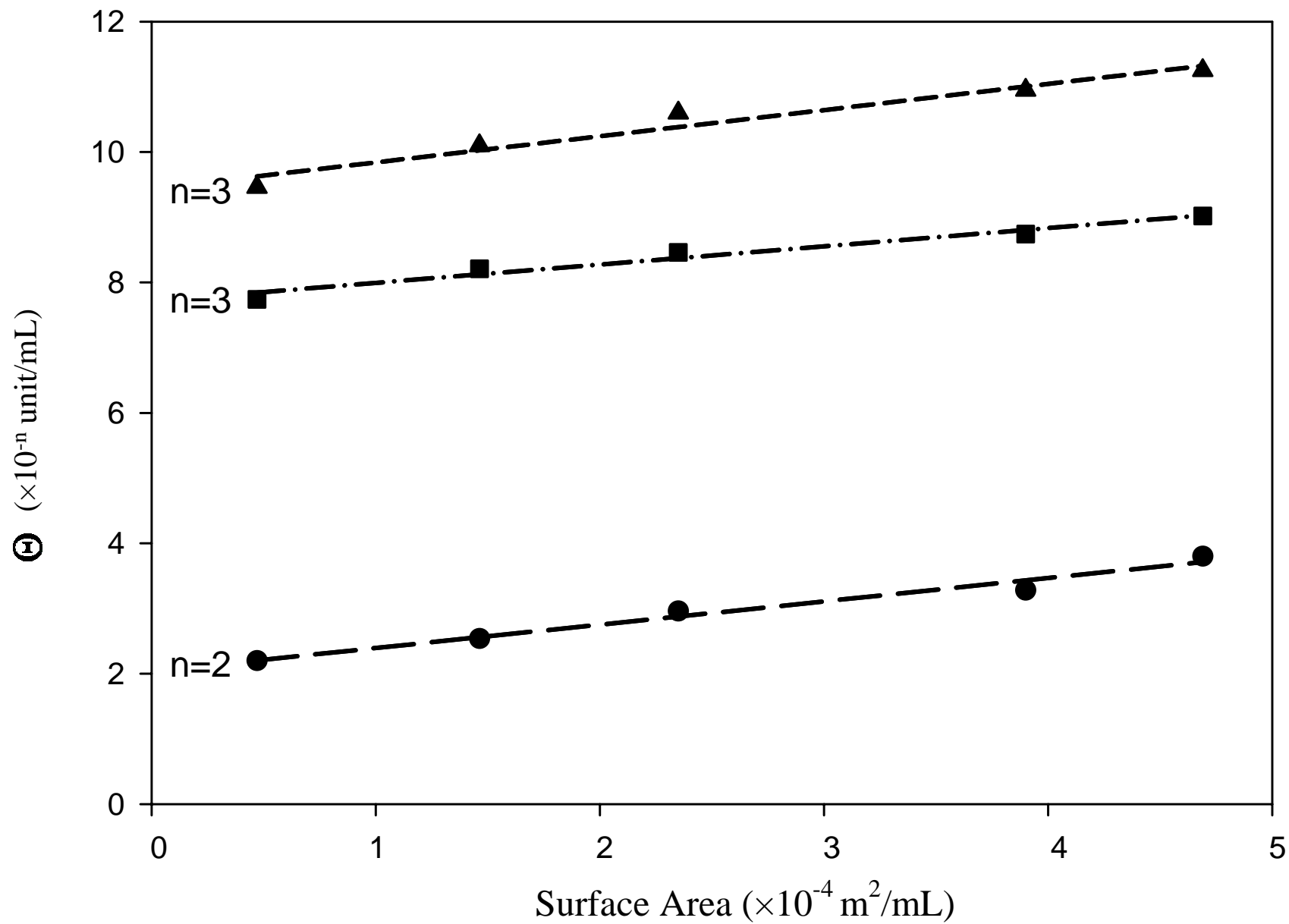


Figure 5

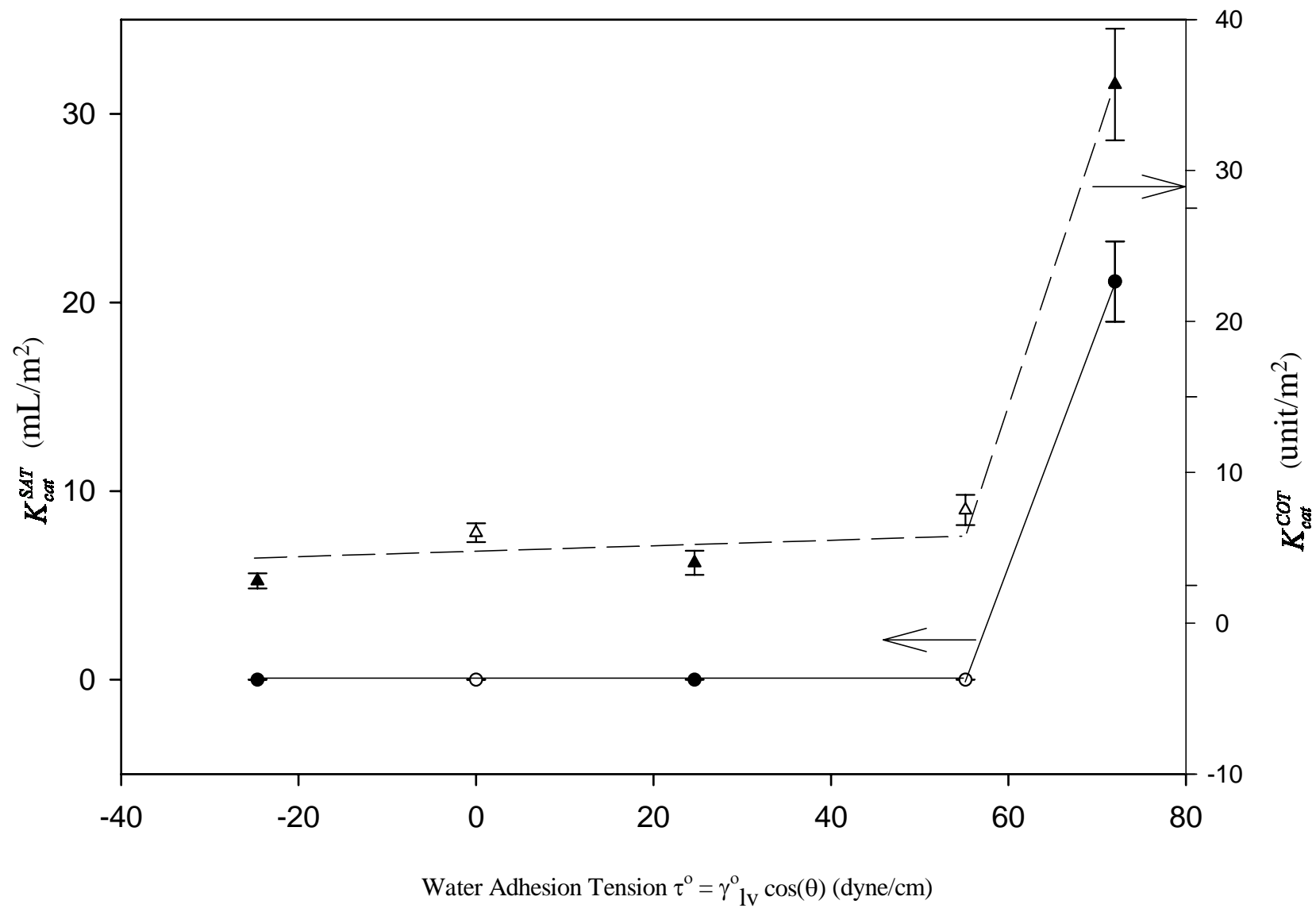


Figure 6

