Dynamics of Thrombin Generation and Flux from Clots during Whole Human Blood Flow over Collagen/Tissue Factor Surfaces*

Received for publication, August 19, 2016 Published, JBC Papers in Press, September 7, 2016, DOI 10.1074/jbc.M116.754671

Shu Zhu, Yichen Lu, Talid Sinno, and Scott L. Diamond†

From the Department of Chemical and Biomolecular Engineering, Institute of Medicine and Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Coagulation kinetics are well established for purified blood proteases or human plasma clotting isotropically. However, less is known about thrombin generation kinetics and transport within blood clots formed under hemodynamic flow. Using microfluidic perfusion (wall shear rate, 200 s⁻¹) of corn trypsin inhibitor-treated whole blood over a 250-μm long patch of type I fibrillar collagen/lipidated tissue factor (TF; ~1 TF molecule/μm²), we measured thrombin released from clots using thrombin-antithrombin immunoassay. The majority (>85%) of generated thrombin was captured by intrathrombus fibrin as thrombin-antithrombin was largely undetectable in the effluent unless Gly-Pro-Arg-Pro (GPRP) was added to block fibrin polymerization. With GPRP present, the flux of thrombin increased to ~0.5 × 10⁻¹² nmol/μm²·s over the first 500 s of perfusion and then further increased by ~2–3-fold over the next 300 s. The increased thrombin flux after 500 s was blocked by anti-FXa antibody (O1A6), consistent with thrombin-feedback activation of FXI. Over the first 500 s, ~92,000 molecules of thrombin were generated per surface TF molecule for the 250-μm-long coating. A single layer of platelets (obtained with α1bβ3 antagonism preventing continued platelet deposition) was largely sufficient for thrombin production. Also, the overall thrombin-generating potential of a 1000-μm long activation are less well understood in this hierarchical structure involving platelet adhesion and activation on a surface as well as rapid build-up of a dense platelet core surrounded by less activated platelets in surrounding shell of the clot. The core of the clot is highly contracted with P-selectin-positive platelets (21, 22), localized thrombin and fibrin (23, 24), and localized disulfide reductase activity (25). The kinetics of thrombin generation are less well understood in this hierarchical structure where intrathrombus transport and binding effects may control reactions (26–30). For example, fibrin is known to inhibit thrombin via its antithrombin-I activity (31–33). Under venous flow, γ'-fibrin has been shown to limit thrombin transport, fibrin production, and clot size (34).

Direct thrombin or Factor Xa (FXa) inhibitors are orally available and clinically approved and do not requiring frequent coagulation monitoring in patients (35). The reduction of thrombotic risk, although still allowing for sufficient hemostasis to prevent undesired bleeding, is central to therapeutic potency and dosing. Calibrated automated thrombinography (CAT) is a calibrated thrombin generation assay that reports thrombin concentration in activated plasma samples as a function of time (36). CAT measurement determines the coagulability of plasma, potentially helpful for clinical diagnosis and drug monitoring. However, the CAT assay provides limited information on the actual dynamics of thrombin generation in platelet-rich clots formed under flow. By recreating hemodynamic flow over procoagulant surfaces, the dynamics and pharmacology of thrombin generation can be studied with human blood ex vivo. In microfluidic clotting assays, fibrin generation is often used as an indirect indicator for thrombin activity (5, 6). The extrinsic coagulation pathway is triggered by tissue factor/Factor VIIa (TF/FVIIa), which generates Factor Xa (FXa) and Factor IXa (FIXa) and is essential for hemostasis. In contrast, deficiencies in the contact pathway (Factor XII [FXII] and FXI) are not linked to strong bleeding phenotypes.

The kinetics of the extrinsic tenase (TF/FVIIa), the intrinsic tenase (FIXa/FVIIIa), and prothrombinase (FXa/FVa) have been extensively measured (7–9) and kinetically modeled for plasma (10–13) or purified enzymes with added lipid (14–16) and platelet-rich plasma (17–20). These prior kinetic studies explore rate processes in a closed and isotropic context (i.e. a tube). However, clotting under hemodynamic flow is an open system involving platelet adhesion and activation on a surface.
Thrombin Flux from Clots Formed under Flow

Our laboratory previously developed a peptide-based platelet targeting biosensor to report platelet associated thrombin activity in both microfluidic and animal thrombosis models (23, 24). To our knowledge there has been no direct quantitative measurement of thrombin flux from a growing thrombus under hemodynamic flow. This lack of measurement may be due to the limited sensitivity of immunoassays to detect released thrombin in the stable complex of thrombin-antithrombin (TAT) as released thrombin would be severely diluted in macroscopic flow systems with ml/min-scale perfusion. In contrast, microfluidics allows detection of clot-released species by limiting their dilution through use of μl/min-scale perfusion.

Theoretical and experimental studies have suggested thrombin generation is sensitive to prevailing shear rate and surface TF levels (41–43). At a venous shear rate condition, thrombin generation is sensitive to prevailing shear rate and surface whole blood microfluidic thrombosis assay by collecting effluent by heparin. TAT complex measurement has been routinely than a minute in the presence of AT (46), a reaction accelerated and rapid thrombin inhibitor (45). Thrombin half-life is less venous shear rates (5). In blood, antithrombin (AT) is a potent blood thrombi formed on the surface with collagen and TF at which is the fibrin structure that is usually observed in whole blood when compared with the contribution of the TF-surface, very little TAT was detected over the 800-s experiment unless Gly-Pro-Arg-Pro (GPRP, 5 mM) was used to prevent fibrin polymerization (Fig. 2B). Collection of effluent into benzamidine (instead of EDTA) to inhibit thrombin prevented the immunodetection of TAT (not shown), indicating that free thrombin was eluted off the clot in the presence of 5 mM GPRP, which then complexed with antithrombin to form TAT in the EDTA-treated sample. Fibrin captured >85% of locally generated thrombin (Fig. 2B). Thus, the fibrin polymerization inhibitor GPRP was added to all subsequent experiments to eliminate fibrin capture of thrombin and allow detection of TAT in the effluent. Prior studies have shown that GPRP has a minor effect on platelet deposition at the low forces of venous flow conditions (40). Also, the contribution of the contact pathway in this microfluidic assay has been shown to be minimal for 40 μg/ml cronyptinin inhibitor (CTI)-treated whole blood when compared with the contribution of the TF-driven extrinsic pathway (50).

Results

Device Response Time—Release of a diffusible species from a surface into a flow stream is a classic concentration boundary layer phenomenon that can influence the dynamics of measurement of the species at the system outlet. Additionally, for sampling parabolic flows at the system outlet, the faster streamlines at and near the center of the flow contribute more volume per unit time than the slower streamlines at the wall boundary. The discrete sampling of volume at the system exit flow where the species concentration may be nonuniform is defined as the “mixing-cup” concentration and involves weighting the concentration profile by the velocity profile (49). To calculate the lag time of the microfluidic device and exit line and thus relate surface events to remotely sampled volumes at the exit, a computational model with a rectangular domain was created, and the transport of thrombin was calculated by standard finite element method in COMSOL (Inc., Burlington, MA) (Fig. 1). A flux with constant amplitude of 10^-11 nmol/μm^2-s and duration of 1 s was applied at the bottom of the channel near the entrance. Within 1 s, the thrombin concentration was high over the entrance (where collagen/TF resides in the experiment) due to the release of soluble thrombin from the bottom boundary; there was no significant thrombin yet reaching the outlet. After 1.6 s, the outlet (mixing-cup average) thrombin concentration began to rise as thrombin was transported advectively by the flow and also by molecular diffusion. By 5 s, there was essentially no thrombin left near the entrance as the imposed thrombin flux only lasted for 1 s, indicating complete and rapid wash-out at a wall shear rate of 200 s^-1. By 7 s the concentration of thrombin collected at the outlet reached a maximum. It took almost 20 s for the concentration of thrombin to decay to zero at the outlet (Fig. 1, B and C). Overall, the simulation demonstrated that the microfluidic assay (Fig. 1A) allows the detection of locally generated thrombin at the collagen site with only a minor lag time of ~10 s. Also, dilution of thrombin (or TAT) in the prevailing microfluidic flow was predicted to allow detection of nanomolar levels of TAT in the effluent.

Clotting under Flow: Fibrin Rapidly Captures Thrombin—Platelets immediately adhered and accumulated on the collagen surface (~1 TF molecule/μm^2) after initiation of whole blood flow, whereas fibrin generation was detected after a 250-s lag followed by a nearly linear increase until the end of the experiment at 800 s (Fig. 2A and supplemental Fig. S1). Under these conditions of platelet deposition and fibrin generation on the collagen/TF surface, very little TAT was detected over the 800-s experiment unless Gly-Pro-Arg-Pro (GPRP, 5 mM) was used to prevent fibrin polymerization (Fig. 2B). Collection of effluent into benzamidine (instead of EDTA) to inhibit thrombin prevented the immunodetection of TAT (not shown), indicating that free thrombin was eluted off the clot in the presence of 5 mM GPRP, which then complexed with antithrombin to form TAT in the EDTA-treated sample. Fibrin captured >85% of locally generated thrombin (Fig. 2B). Thus, the fibrin polymerization inhibitor GPRP was added to all subsequent experiments to eliminate fibrin capture of thrombin and allow detection of TAT in the effluent. Prior studies have shown that GPRP has a minor effect on platelet deposition at the low forces of venous flow conditions (40). Also, the contribution of the contact pathway in this microfluidic assay has been shown to be minimal for 40 μg/ml cronyptinin inhibitor (CTI)-treated whole blood when compared with the contribution of the TF-driven extrinsic pathway (50).

Increased Surface TF Concentration Promotes Thrombin Flux—Because fibrin formation requires a threshold concentration of surface TF (43), we tested if the amount of immunodetected TAT complex in the effluent was dependent on TF concentration on the collagen surface. At ~1 TF mol/μm^2 (collagen/high TF), the TAT concentration displayed a slow increase during the first 500 s and then an accelerated increase during the following 300 s. By 800 s, the thrombin flux reached ~0.8 × 10^-12 nmol/μm^2-s (Fig. 2C). At ~0.1 TF mol/μm^2 (collagen/low TF), there was a brief initial increase of TAT concentration within the first 200 s. Between 200 and 600 s, TAT concentration remained largely constant. After 600 s, accelerated increase of the TAT concentration was observed. By 800 s, thrombin flux from collagen/low TF surface reached ~0.4 × 10^-12 nmol/μm^2-s, which was only half that of the thrombin flux from collagen/high TF surface (Fig. 2C).

The collagen/high TF surface was used as the test surface for all subsequent experiments. A master thrombin generation
Thrombin Flux from Clots Formed under Flow

curve (Fig. 2D) was developed from 13 individual experiments with blood from 13 donors, representing the average dynamics of human thrombin generation from clots formed on collagen/high TF surface (~1 molecule TF/μm², 5 mM GPRP, 40 μg/ml CTI): a slow increase in thrombin flux during the first 500 s to 0.5 × 10⁻¹² nmol/μm²-s followed by an accelerated increase (~4× higher than the rate of increase during the first 500 s), reaching a thrombin flux of ~1.5 × 10⁻¹² nmol/μm²-s by 800 s at the end point of the experiment. The standard error was attributed to interdonor variation in thrombin generation of about ±50%, as has been previously observed with measurements of thrombin generation by the CAT assay (47). Given this observed variation, subsequent experiments were conducted with at least four donors under matched conditions.

Thrombin Amplification via Thrombin Feedback Activation of FXI—The large and late stage increase in thrombin flux detected between 500 and 800 s was consistent with the late stage fibrin and thrombin activity previously detected as a result of platelet polyphosphate enhancement of the thrombin-mediated FXIa feedback pathway (5, 51–53). This late stage participation of thrombin-mediated activation of FXI has also been theoretically predicted (54). The FXI antibody O1A6 inhibits FXIIa activation of FXI and disrupts the FXI-dependent thrombin amplification loop by inhibiting FXIa generation of FIXa and subsequent FXa activation (Fig. 3A). Adding O1A6 (20 μg/ml) into the blood sample abolished the accelerated increase of thrombin flux from the collagen/TF surface after 500 s (Fig. 3B). Thrombin flux increased to 0.2 × 10⁻¹² nmol/μm²-s during the first 400 s and remained relatively constant after that.

The First Layer of Collagen-activated Platelets Generates the Majority of Thrombin—We tested the thrombin generating capacity of a monolayer of collagen-adherent platelets to that of a thick and dense platelet deposit. As previously observed (55),
the glycoprotein IIb/IIIa (\( \alpha_{IIb}\beta_3 \)) antagonist, GR144053 (500 nM), abolished secondary platelet deposition on the collagen/high TF surface. For the first 500 s of blood perfusion, the thrombin production was essentially the same for a platelet monolayer and a thick platelet deposit, indicating that the thickened platelet mass did not diminish thrombin production by limiting FX transport or by additional coverage/hindrance of surface TF. Interestingly, the accelerated increase of thrombin flux from 600 to 800 s was largely prevented by GR144053 (Fig. 4A), indicating a role for the thickened platelet deposit in thrombin-feedback activation of FXIa (Fig. 3B) that was operative in this late-stage time regime (Fig. 4A) and also consistent with platelet-dependent release of polyphosphate (56). Additionally, in the absence of GPRP, a platelet monolayer was sufficient to support localized fibrin formation (Fig. 4B).

Longer Collagen/TF Zones Are Less Efficient in Thrombin Production—The TAT concentration in effluent collected downstream of 1000-\( \mu \text{m} \) long collagen/high TF was ~2× higher than the detected concentration in effluent from 250-\( \mu \text{m} \)-long collagen/high TF (Fig. 5A). However, the 1000-\( \mu \text{m} \)
accumulation was observed on the first 250 m long zone of the overall flux from the 250-m long zone was less efficient in supporting overall thrombin generation on a per unit area basis. The overall thrombin flux from the entire 1000-μm-long zone of collagen/high TF was only half that of the overall flux from the 250-μm-long zone of collagen/high TF (Fig. 5B). On a 1000-μm zone, most of the platelet accumulation was observed on the first 250 μm, with significantly less platelet deposition between 250 and 1000 μm (Fig. 5, C and D). This is consistent with boundary layer depletion of depositing platelets in the red blood cell-free layer of plasma nearest the collagen coating. Roughly half of detected thrombin flux from the 1000-μm collagen/high TF zone originated from the first 250-μm subregion of the 1000-μm-long zone, assuming the first 250 μm had the exact efficiency in supporting thrombin generation as for the 250-μm-long zone (Fig. 5, E and F).

Discussion

In this study ELISA allowed direct measurement of TAT in plasma isolated from whole blood samples collected from the outlet of a microfluidic thrombosis assay. Numerical simulation demonstrated the delay in thrombin detection in this setup was small (~10 s) compared with the time interval (120 s) between sample collections. Thus, TAT measurements can be directly converted to thrombin fluxes within each time interval. Previous theoretical and experimental studies suggested alterations in shear rates and surface TF affect coagulation reaction dynamics and cause substantial changes in thrombin flux (41, 42).

The microfluidic experiments were operated at a constant flow rate mode resulting in an initial wall shear rate of 200 s−1. The thrombin flux released from growing thrombi on collagen/high TF (~1 molecule/μm²) increased over time and reached a level of ~10⁻¹² nmol/μm²·s by 800 s at the end of the experiment. This final thrombin flux falls within the suggested range from previous computational models for similar shear conditions (41, 42, 44). In our flow system, fibrin localized >85% of thrombin within the thrombus and traveled downstream, indicating that free thrombin lasted less than a second or two before its capture by fibrin. The TAT ELISA-based measurement might underestimate the actual thrombin flux due to the following factors: (i) other protease inhibitors such as αt-macro-globulin, even though far less effective than AT, also complex thrombin (45), (ii) thrombin bound to the clot by fibrin-independent mechanisms would not be detected in the plasma TAT-ELISA assay, and (iii) robust thrombin generation at later time points boosted fibrin polymerization, which was not fully quenched by 5 mM GPRP (supplemental Fig. S2).

A thrombin generation curve from 13 individual measurements provides the average dynamic change of thrombin flux from growing thrombi on 250-μm-long collagen/high TF. We calculate that each TF molecule supported generation of ~92,000 thrombin molecule via the extrinsic pathway during the first 500-s time period. This curve displayed an accelerated increase in thrombin flux starting at ~500 s. This accelerated increase was prevented by FXI antibody O1A6. Our previous work indicated that participation of the TF-triggered extrinsic pathway was required to generate enough thrombin to initiate the FXIa-dependent thrombin amplification mechanism, which can be interrupted by O1A6 (5). This FXI function-blocking antibody also reduced platelet aggregation downstream of thrombi formed on collagen/TG, suggesting a role of the FXI-thrombin axis in distal thrombi formation (57). Therefore, thrombin flux after 500 s is most likely augmented by the FXIa-dependent thrombin amplification loop. FXI inhibition did not significantly interfere with thrombin flux within the first 500 s, indicating the extrinsic pathway is the major contributor of initial thrombin generation in CTI-inhibited whole blood clotting. By 500 s, ~70% of collagen/TG had been covered with accumulated platelets (supplemental Fig. S1), potentially reducing access to surface-immobilized TF and enhancing platelet-dependent pathways (such as polyphosphate pathways).

It is known that platelets provide lipid surface for prothrombinase assembly and, therefore, are essential for promoting coagulation (58, 59). Interestingly, a monolayer of platelets provided enough lipid surfaces for robust thrombin generation in the initial phase. The prevention of amplified thrombin generation during later times suggests the importance of aggregated platelets in aiding the FXI-dependent thrombin feedback loop.
It is possible that platelet aggregates promote thrombin generation by limiting flow dilution and active transport of activated coagulation factors. Additionally, the accumulated platelets release polyphosphate to act as a cofactor and promote thrombin activation of FXI (5, 51, 53) at later times.

We found increased collagen/TF patch length reduced the overall efficiency of thrombin generation on a per unit area basis, most likely due to the decay in platelet deposition along the flow direction on 1000-μm long collagen/high TF patch. Furthermore, we have shown in our previous study that a single...
collagen/TF fiber (patch length <1 μm) prompts a clotting response of multiple layers of platelet deposition and thrombin generation at a venous shear condition (60). We suspect that the total thrombin generation might be greater on larger thrombotic patches, but thrombin generation on a per unit area basis decays once the patch size gets too large, which is probably due to boundary layer platelet depletion of the near-wall platelet layer and consequently reduced platelet deposition in downstream regions of larger patches.

In summary, we made direct measurements of TAT complex concentration in effluent from microfluidic thrombosis assay. We estimated thrombin flux from growing thrombi on the collagen/TF surface can reach up to 10^{-12} nmol/μm²-s at a venous shear condition. Our results suggest polymerized fibrin fibers consume most of the free thrombin and likely serve as a mechanism of localizing clotting response near the injury site and potentially promoting a hierarchical structure of self-limiting clots. Furthermore, aggregated platelets were found to play pivotal roles in amplifying thrombin generation via the FXI-dependent feedback loop.

Experimental Procedures

Materials—Anti-human CD61 (BD Biosciences), Alexa Fluor®647 conjugated human fibrinogen (Life Technologies), H-Gly-Pro-Arg-Pro-OH (GPRP, EMD Chemicals, San Diego, CA), CTI (Hematologic Technologies, Essex Junction, VT), GR144053 trihydrochloride (Tocris Bioscience, Minneapolis, MN), EDTA (Sigma), Sigmacote® siliconizing reagent (Sigma), human thrombin-antithrombin complex ELISA kit (Abcam, Cambridge, MA), Dade® Innovin® PT reagent (Siemens, Malvern, PA), collagen Type I Chrono-Par™ aggregation reagent (Chrono-log, Havertown, PA), and Sylgard® 184 Silicone Elastomer kit (Dow Corning, Auburn, MI). O1A6 FXI antibody was a gift from Dr. Andras Gruber (Department of Biomedical Engineering, Oregon Health and Science University).

Polydimethylsiloxane (PDMS) Patterning and Flow Devices—PDMS devices were fabricated as previously described (61, 62). Protein patches with defined dimensions were patterned on glass slides with single channel devices (250 or 1000 μm in width, 60 μm in height). Multichannel flow chambers with 8 evenly spaced flow channels (250 μm in width, 10000 μm in length, and 60 μm in height) diverging from a single inlet and converging into a single outlet were used for microfluidic thrombosis assay. All PDMS devices have features on their bottom allowing them to be reversibly vacuum-bonded onto glass slides.

Analysis of System Response Time—A model microfluidic system was characterized in COMSOL Multiphysics® Modeling Software (COMSOL). A rectangular domain (10,000 μm by 60 μm) was created to represent one lane of the actual microfluidic device. The local wall shear rate was maintained at 200 s⁻¹, consistent with the experiments. The thrombin flux was imposed between 100 μm and 350 μm, and the transport was mainly governed by the convection and diffusion assuming no hindrance from the developing clot. To calibrate the system response time, a rectangular flux signal with amplitude of 10⁻¹¹ nmol/μm²-s was applied for duration of 1 s. The system was propagated with a constant time step of 0.05 s to obtain the average thrombin concentration at the outlet. Similarly, an empirical thrombin flux fitted with the experimental measures was applied to obtain the resultant mixing-cup average concentration at the outlet.

Preparation of Thrombotic Patches—Glass slides were treated with Sigmacote® to retard surface-triggered clotting. To generate collagen patches on glass slides, collagen type I (1 mg/ml, 5 μl) followed by bovine serum albumin (0.5% BSA in Hepes-buffered saline, 20 μl) was perfused through the main channel on a single channel patterning device (62). The length of a collagen patch (250 μm or 1000 μm) is defined by the dimension of the main channel. Tissue factor factor can be added by subsequent perfusion of 10 μl of Dade Innovin® PT reagent through the main channel and incubation over patterned collagen for 30 min before a BSA wash. The PT reagent stock (23 nm recombinant human TF and synthetic phospholipids) was diluted 10-fold and 200-fold to achieve high and low surface TF surface densities of ~1 and ~0.1 TF molecule/μm², respectively. Sorbed TF lipid vesicles were stained with FITC-annexin V. TF surface densities were estimated by fluorescent imaging of stained vesicles, as previously described (5, 39).

Blood Collection and Sample Preparation—Blood was collected via venipuncture from healthy donors who provided consent under approval of University of Pennsylvania Institutional Review Board and were free of any medication or alcohol for at least 72 h before donation. Blood was collected into syringes with a high dosage of CTI (40 μg/ml) and was transferred into Eppendorf tubes where it was labeled by CD61 antibody (2%/w/v) and fluorescent fibrinogen (1.3% v/v) for platelet and fibrin epifluorescence detection, respectively. In some of the microfluidic experiments, additional treatments were added to the blood sample before perfusion to block platelet aggregation or fibrin polymerization. These additional treatments are indicated under “Results.”

Microfluidic Thrombosis Assay—After protein patterning, the single channel patterning device was replaced with the multichannel flow device. The flow channels were placed perpendicularly to patterned TF bearing collagen patches. Labeled whole blood was transferred to syringe patches which were then mounted on a PHD 2000 syringe pump (Harvard Apparatus, Holliston, MA) operating on an infusing mode. Blood was infused into the flow device at a constant flow rate of 16 μl/min, which corresponds to an initial wall shear rate of 200 s⁻¹. Platelet aggregation and fibrin formation were simultaneously monitored with a fluorescence microscope (IX81, Olympus America Inc., Center Valley, PA). Images were captured with a CCD camera (Hamamasu, Bridgewater, NJ) and analyzed with ImageJ (NIH, Bethesda, MD). The outlet of the flow device was blocked with 10 μl of EDTA. Calcium-dependent thrombin generation in blood was immediately quenched by EDTA once blood exits the device. Blood samples were collected from the outlet every other minute. Another 10 μl of EDTA was added to block the outlet immediately after sample collection. Collected blood samples were allowed to sit for at least 10 min for TAT complex formation before subsequent steps.

TAT-ELISA Assay—Collected blood samples were centrifuged at 1300 × g for 15 min. TAT complex concentration in isolated platelet poor plasma was detected in a sandwich TAT-
ELISA assay. Background TAT level was determined by measuring TAT concentration in plasma sample isolated from blood that was quenched with EDTA right after phlebotomy. Because AT reacts with thrombin in a 1:1 stoichiometric ratio, measured average TAT concentration (̄C) within the 2-min time interval between sample collections was converted to an average thrombin flux (J) using the equation,

\[
J = \frac{̄CQ}{A}
\]

where \(Q\) is flow rate (16 \(\mu\)l/min), and \(A\) is total thrombotic area in each device.

Author Contributions—S. Z. and S. L. D. designed the experiments. S. Z. conducted the experiments. Y. L. performed numerical simulation. S. Z., Y. L., T. S., and S. L. D. analyzed the data and wrote the manuscript.

References
1. Coughlin, S. R. (2000) Thrombin signalling and protease-activated receptors. Nature 407, 258–264
2. Wolberg, A. S. (2007) Thrombin generation and fibrin clot structure. Blood Rev. 21, 131–142
3. Hornyak, T. J., Bishop, P. D., and Shafer, J. A. (1989) α-Thrombin-catalyzed activation of human platelet factor XIII: relationship between proteolysis and factor XIIIa activity. Biochemistry 28, 7326–7332
4. Schwartz, M. L., Pizzo, S. V., Hill, R. L., and McKee, P. A. (1973) Human Factor XIII from plasma and platelets: Molecular weights, subunit structures, proteolytic activation, and cross-linking of fibrinogen and fibrin. J. Biol. Chem. 248, 1395–1407
5. Zhu, S., Travers, R. J., Morrisey, J. H., and Diamond, S. L. (2015) FXIa and platelet polyphosphate as therapeutic targets during human blood clotting on collagen/tissue factor surfaces under flow. Blood 126, 1494–1502
6. Kravtsov, D. V., Matafonov, A., Tucker, E. I., Sun, M. F., Walsh, P. N., Gruber, A., and Gailani, D. (2009) Factor XI contributes to thrombin generation in the absence of factor XII. Blood 114, 452–458
7. Krishnaswamy, S., Church, W. R., Neishem, M. E., and Mann, K. G. (1987) Activation of human prothrombin by human prothrombinase: influence of factor Va on the reaction mechanism. J. Biol. Chem. 262, 3291–3299
8. Krishnaswamy, S., Jones, K. C., and Mann, K. G. (1988) Prothrombinase complex assembly: kinetic mechanism of enzyme assembly on phospholipid vesicles. J. Biol. Chem. 263, 3832–3834
9. Walker, R. K., and Krishnaswamy, S. (1994) The activation of prothrombin by the prothrombinase complex: the contribution of the substrate-membrane interaction to catalysis. J. Biol. Chem. 269, 27441–27450
10. Butenas, S., Orfeo, T., Gissel, M. T., Brummel, K. E., and Mann, K. G. (2004) The significance of circulating factor IXa in blood. J. Biol. Chem. 279, 22875–22882
11. Brummel-Ziedins, K., Vossen, C. Y., Rosendaal, F. R., Umezaki, K., and Mann, K. G. (2005) The plasma hemostatic proteome: thrombin generation in healthy individuals. J. Thromb. Haemost. 3, 1472–1481
12. Brummel-Ziedins, K. E., Orfeo, T., Gissel, M., Mann, K. G., and Rosendaal, F. R. (2012) Factor Xa generation by computational modeling: an additional discriminator to thrombin generation evaluation. PLoS ONE 7, e29178
13. Willems, G. M., Lindhout, T., Hermens, W. T., and Hemker, H. C. (1991) Simulation model for thrombin generation in plasma. Haemostasis 21, 197–207
14. Hockin, M. F., Jones, K. C., Everse, S. J., and Mann, K. G. (2002) A model for the stoichiometric regulation of blood coagulation. J. Biol. Chem. 277, 18322–18333
15. Neishem, M. E., Tracy, R. P., and Mann, K. G. (1984) “Clotspeed,” a mathematical simulation of the functional properties of prothrombinase. J. Biol. Chem. 259, 1447–1453
16. Bungay, S. D., Gentry, P. A., and Gentry, R. D. (2003) A mathematical model of lipid-mediated thrombin generation. Math. Med. Biol. 20, 105–129
17. Chatterjee, M. S., Denney, W. S., Jing, H., and Diamond, S. L. (2010) Systems biology of coagulation initiation: kinetics of thrombin generation in resting and activated human blood. PLoS Comput. Biol. 6, e1000950
18. Sorensen, E. N., Burgren, G. W., Wagner, W. R., and Antaki, J. F. (1999) Computational simulation of platelet deposition and activation: I. Model development and properties. Ann. Biomed. Eng. 27, 436–448
19. Sorensen, E. N., Burgren, G. W., Wagner, W. R., and Antaki, J. F. (1999) Computational simulation of platelet deposition and activation: II. Results for Poiseuille flow over collagen. Ann. Biomed. Eng. 27, 449–458
20. Hoffman, M., and Monroe, D. M., 3rd. (2003) The action of high-dose factor VIIa (FVIIa) in a cell-based model of hemostasis. Dis. Mon. 49, 14–21
21. Stalker, T. J., Traxler, E. A., Wu, J., Wannemacher, K. M., Cermignano, S. L., Voronov, R., Diamond, S. L., and Brass, L. F. (2013) Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. Blood 121, 1875–1885
22. Stalker, T. J., Welsh, J. D., Tomaiuolo, M., Wu, J., Colace, T. V., Diamond, S. L., and Brass, L. F. (2014) A systems approach to hemostasis: 3. Thrombus consolidation regulates intrathrombus solute transport and local thrombin activity. Blood 124, 1824–1831
23. Welsh, J. D., Colace, T. V., Muthard, R. W., Stalker, T. J., Brass, L. F., and Diamond, S. L. (2012) Platelet-targeting sensor reveals thrombin gradients within blood clots forming in microfluidic assays and in mouse. J. Thromb. Haemost. 10, 2344–2353
24. Welsh, J. D., Stalker, T. J., Voronov, R., Muthard, R. W., Tomaiuolo, M., Diamond, S. L., and Brass, L. F. (2014) A system approach to hemostasis: 1. The interdependence of thrombus architecture and agonist movements in the gaps between platelets. Blood 124, 1808–1815
25. Zhu, S., Welsh, J. D., Brass, L. F., and Diamond, S. L. (2016) Platelet-targeting thrombin reduction sensor detects thrombi isomerase activity on activated platelets in mouse and human blood under flow. J. Thromb. Haemost. 14, 1070–1081
26. Voronov, R. S., Stalker, T. J., Brass, L. F., and Diamond, S. L. (2013) Simulation of intrathrombus fluid and solute transport using in vivo clot structures with single platelet resolution. Ann. Biomed. Eng. 41, 1297–1307
27. Leiderman, K., and Fogelson, A. L. (2013) The influence of hindered transport on the development of platelet thrombi under flow. Bull. Math. Biol. 75, 1255–1283
28. Kim, O. V., Xu, Z., Rosen, E. D., and Alber, M. S. (2013) Fibrin networks regulate protein transport during thrombus development. PLoS Comput. Biol. 9, e1003095
29. Xu, Z., Lioi, J., Xu, Z., Komacka, M. M., Liu, X., Chen, D. Z., Rosen, E. D., and Alber, M. (2010) A multiscale model of venous thrombus formation with surface-mediated control of blood coagulation cascade. Biophys. J. 98, 1723–1732
30. Tomaiuolo, M., Stalker, T. J., Welsh, J. D., Diamond, S. L., Sinno, T., and Brass, L. F. (2014) A systems approach to hemostasis: 2. Computational analysis of molecular transport in the thrombus microenvironment. Blood 124, 1816–1823
31. Meh, D. A., Siebenlist, K. R., and Mosesson, M. W. (1996) Identification and characterization of the thrombin binding sites on fibrin. J. Biol. Chem. 271, 23121–23125
32. Lovesey, R. S., Meaddel, M., and Farrell, D. H. (2003) Fibrinogen γ chain binds thrombin exosite II. J. Thromb. Haemost. 1, 124–131
33. Frederburgh, J. C., Stafford, A. R., Pospisil, C. H., and Weitz, J. I. (2004) Modes and consequences of thrombin’s interaction with fibrin. Biophys. Chem. 112, 277–284
34. Muthard, R. W., Welsh, J. D., Brass, L. F., and Diamond, S. L. (2015) Fibrin, γ-m-γ-fibrinogen, and translocat pressure gradient control hemostatic clot growth during human blood flow over a collagen/tissue factor wound. Arterioscler. Thromb. Vasc. Biol. 35, 645–654
35. Yeh, C. H., Frederburgh, J. C., and Weitz, J. I. (2012) Oral direct factor Xa inhibitors. Circ. Res. 111, 1069–1078
36. Hemker, H. C., Giesen, P., AlDieri, R., Regnault, Y., de Smed, E., Wagenvoord, R., Lecompte, T., and Béguin, S. (2002) The calibrated automated
thrombogram (CAT): a universal routine test for hyper- and hypocoagu-
ability. *Pathophysiol. Haemost. Thromb.* **32**, 249–253
37. Zhu, S., and Diamond, S. L. (2014) Contact activation of blood coagulation
on a defined kaolin/collagen surface in a microfluidic assay. *Thromb. Res.*
**134**, 1335–1343
38. Colace, T. V., Fogarty, P. F., Panckeri, K. A., Li, R., and Diamond, S. L.
(2014) Microfluidic analysis of hemophilic blood clotting: distinct deficits in
platelet and fibrin deposition at low factor levels. *J. Thromb. Haemost.* **12**, 147–158
39. Colace, T. V., Jobson, J., and Diamond, S. L. (2011) Relipidated tissue
factor linked collagen surfaces potentiates platelet adhesion and fibrin
formation in a microfluidic model of vessel injury. *Bioconj. Chem.* **22**, 2104–2109
40. Colace, T. V., Muthard, R. W., and Diamond, S. L. (2012) Thrombus
growth and embolism on tissue factor-bearing collagen surfaces under
flow: role of thrombin with and without fibrin. *Arterioscl. Thromb. Vasc.
Biol.* **32**, 1466–1476
41. Folie, B. J., and McIntire, L. V. (1989) Mathematical analysis of mural
thrombogenesis: concentration profiles of platelet-activating agents and
effects of viscous shear flow. *Biophys. J.* **56**, 1121–1141
42. Kuharsky, A. L., and Fogelson, A. L. (2001) Surface-mediated control of
blood coagulation: the role of binding site densities and platelet deposi-
tion. *Biophys. J.* **80**, 1050–1074
43. Okorie, U. M., Denney, W. S., Chatterjee, M. S., Neeves, K. B., and Dia-
mond, S. L. (2008) Determination of surface tissue factor thresholds that
trigger coagulation at venous and arterial shear rates: amplification of 100
fM circulating tissue factor requires flow. *Blood* **111**, 3507–3513
44. Neeves, K. B., Illing, D. A., and Diamond, S. L. (2010) Thrombin flux and
wall shear rate regulate fibrin fiber deposition state during polymerization
under flow. *Biophys. J.* **98**, 1344–1352
45. Downing, M. R., Bloom, I. W., and Mann, K. G. (1978) Comparison of the
inhibition of thrombin by three plasma protease inhibitors. *Biochemistry*
**17**, 2649–2653
46. Rußh, H., Müller, J., Harbrecht, U., Fimmers, R., Oldenburg, J., Mayer, G.,
and Pützsch, B. (2012) Thrombin inhibition profiles in healthy individuals
and thrombophilic patients. *Thromb. Haemost.* **107**, 848–853
47. Chandler, W. L., and Velan, T. (2003) Estimating the rate of thrombin and
fibrin generation in vivo during cardiopulmonary bypass. *Blood* **101**, 4355–4362
48. Rivard, G. E., Brummel-Ziedins, K. E., Mann, K. G., Fan, L., Hofer, A., and
Cohen, E. (2005) Evaluation of the profile of thrombin generation during
the process of whole blood clotting as assessed by thrombelastography. *J.
Thromb. Haemost.* **3**, 2039–2043
49. Bird, R. B., Stewart, W. E., and Lightfoot, E. N. (2006) *Transport Pheno-
mena*, 2nd Ed., pp. 543–568, John Wiley & Sons, Inc., New York
50. Li, R., Panckeri, K. A., Fogarty, P. F., and Diamond, S. L. (2015) Recombi-
nant factor VIIa enhances platelet deposition from flowing haemophilic
blood but requires the contact pathway to promote fibrin deposition. *Hae-
nophila* **21**, 266–274
51. Choi, S. H., Smith, S. A., and Morrissey, J. H. (2011) Polyphosphate is a
cofactor for the activation of factor XI by thrombin. *Blood* **118**, 6963–6970
52. Choi, S. H., Smith, S. A., and Morrissey, J. H. (2015) Polyphosphate acceler-
ates factor V activation by factor Xla. *Thromb. Haemost.* **113**, 599–604
53. Smith, S. A., Choi, S. H., Davis-Harrison, R., Huyck, J., Boettcher, J., Rien-
stra, C. M., Reinstra, C. M., and Morrissey, J. H. (2010) Polyphosphate exerts
differential effects on blood clotting, depending on polymer size. *Blood*
**116**, 4353–4359
54. Fogelson, A. L., Hussain, Y. H., and Leiderman, K. (2012) Blood clot for-
mation under flow: the importance of factor XI depends strongly on plate-
let count. *Biophys. J.* **102**, 10–18
55. Colace, T. V., and Diamond, S. L. (2013) Direct observation of von Will-
ebrand factor elongation and fiber formation on collagen during acute
whole blood exposure to pathological flow. *Arterioscler. Thromb. Vasc.
Biol.* **33**, 105–113
56. Ruiz, F. A., Lea, C. R., Oldfield, E., and Docampo, R. (2004) Human platelet
dense granules contain polyphosphate and are similar to acidocalcisomes
of bacteria and unicellular eukaryotes. *J. Biol. Chem.* **279**, 44250–44257
57. Zilberman-Rudenko, J., Itakura, A., Wiesenekker, C. P., Vetter, R., Maas,
C., Galiani, D., Tucker, E. I., Gruber, A., Gerdes, C., and McCarty, O. J.
(2016) Coagulation factor XI promotes distal platelet activation and single
platelet consumption in the bloodstream under shear flow. *Arterioscler.
Thromb. Vasc. Biol.* **36**, 510–517
58. Swords, N. A., and Mann, K. G. (1993) The assembly of the prothrombi-
nase complex on adherent platelets. *Arterioscler. Thromb.* **13**, 1602–1612
59. Tracy, P. C., and Mann, K. G. (1983) Prothrombinase complex assembly
on the platelet surface is mediated through the 74,000-dalton component
of factor Va. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2380–2384
60. Zhu, S., Tomaíulo, M., and Diamond, S. L. (2016) Minimum wound size
for clotting: flowing blood coagulates on a single collagen fiber presenting
tissue factor and von Willebrand factor. *Arterioscler. Thromb. Vasc.
Biol.* **36**, 2193–2201