Glycoprotein Ibα–Mediated Platelet Adhesion and Aggregation to Immobilized Thrombin Under Conditions of Flow

Cees Weeterings, Jelle Adelmeijer, Timothy Myles, Philip G. de Groot, Ton Lisman

Objectives—Thrombin interacts with platelets via the protease-activated receptors (PARs) 1 and 4, and via glycoprotein Ibα (GPIbα). Recently, it was shown that platelets are able to adhere to immobilized thrombin under static conditions via GPIbα.

Methods and Results—Here, we show that platelets are also able to adhere to and form stable aggregates on immobilized thrombin under conditions of flow. Adhesion and aggregation to thrombin was dependent on the interaction with GPIbα, as addition of glycollacitin or an antibody blocking the interaction between thrombin and GPIbα inhibited platelet adhesion. Additionally, platelet adhesion to recombinant thrombin mutants, which are unable to bind GPIbα, was severely suppressed. Furthermore, platelet adhesion to thrombin was dependent on activation of PARs, and partly on granule secretion and thromboxane-A2 synthesis. Immobilization of thrombin on a fibrin network resulted in substantially increased adhesion compared with fibrin alone. The adhesion to fibrin alone was completely abolished by addition of dRGDW, whereas fibrin-bound thrombin still showed substantial platelet adhesion in the presence of dRGDW, indicating that fibrin-bound thrombin is able to directly capture platelets under flow.

Conclusion—These results indicate that platelets are able to adhere to thrombin under flow conditions, which is dependent on the interaction with GPIbα. (Arterioscler Thromb Vasc Biol. 2006;26:670-675.)

Key Words: thrombin • platelet • fibrin • GPIbα • flow conditions

Thrombin has a central role in hemostasis. It activates platelets, cleaves fibrinogen into fibrin, and activates factor XIII. Furthermore, thrombin enhances coagulation by activating factors V, VIII, and XI, but it also inhibits coagulation by activating protein C, and attenuates fibrinolysis by activating thrombin activatable fibrinolysis inhibitor (TAI). On clot formation, thrombin is immobilized to the fibrin clot, and this binding to fibrin could be important in localizing thrombin to the site of vascular injury. Fibrin-bound thrombin is protected against inactivation by the heparin-antithrombin complex, but the active site still remains accessible, as fibrin-bound thrombin is still capable of cleaving fibrinogen and activating factor XI.

Thrombin can activate platelets via the protease-activated receptors (PARs) PAR1 and PAR4, which are generally assumed to account for the moderate- and low-affinity binding sites for thrombin, respectively. GPIbα is described to be the high-affinity receptor for thrombin. GPIbα consists of 2 subunits, GPIbα and GPIbβ, and is expressed in platelets as a complex with GPIX and GPV in a 2:2:2:1 stoichiometry. However, there are ~25 000 copies of GPIbα on the platelet surface, but only a small number (~100 to 1000) appear to be involved in the high-affinity binding of thrombin. The localization of the GPIb–IX–V complex in rafts has proved to be important in platelet activation by von Willebrand Factor (vWF), and it has been postulated that raft association may also account for the difference in high-affinity binding sites for thrombin and GPIbα copies on the platelet.

Thrombin contains 2 anion binding sites or exosites referred to as exosite I and exosite II, a catalytic pocket and a Na+ binding site. Exosite I is important in the binding of multiple substrates, including fibrin and fibrinogen, whereas exosite II is referred to as the heparin binding site. The catalytic pocket is responsible for the actual cleavage of the substrates, and the amount of Na+ bound to the Na+ binding site regulates the affinity of thrombin for its substrates (reviewed by Di Cera). Recently, site-directed mutagenesis has indicated the involvement of many basic exosite II residues in GPIbα binding. In addition, the crystal structures of thrombin bound to GPIbα reported by Celikel et al and Dumas et al revealed the importance of both exosites of GPIbα in the binding of thrombin. Although there were many discrepancies between the 2 structures, which resulted in fundamentally different functional interpretations, both structures showed that 2 thrombin molecules can interact with a single GPIbα mole-

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It was previously shown that platelets are able to adhere to immobilized thrombin under static conditions. However, it is unclear whether this interaction is sufficiently strong to resist shear forces. In this study, we investigated whether platelets could interact with immobilized thrombin under flow conditions. Furthermore, the role of fibrin-bound thrombin is not yet completely understood, and in this study we investigated whether fibrin-bound thrombin contributes to platelet adhesion. We show that thrombin immobilized either directly on a glass coverslip or on fibrin induces platelet adhesion and aggregate formation under flow conditions, which is dependent on its interaction with GPIbα.

Methods
For Methods, please see the data supplement, available online at http://atvb.ahajournals.org.

Results
Platelet Adhesion and Aggregate Formation to Immobilized Thrombin Under Conditions of Flow
To investigate whether thrombin immobilized on a surface is able to interact with platelets under conditions of flow, reconstituted blood was perfused over immobilized thrombin for 5 minutes at a shear rate of 300 \( \text{s}^{-1} \). Real time perfusion experiments showed rapid adhesion of single platelets to the surface, followed by the formation of large aggregates at sites of primary platelet adhesion (for movie capture, please see http://atvb.ahajournals.org). The aggregates were stable, and embolization only occurred sporadically. Figure 1A shows a microscopic picture of platelet adhesion and aggregate formation to immobilized thrombin after 5 minutes of perfusion at a shear rate of 300 \( \text{s}^{-1} \). Scanning Electron Microscope analysis supports our observations that large stable aggregates were formed on top of initially spread platelets (Figure 1B). Platelet adhesion to coverslips that were coated only with blocking buffer (BSA) was virtually absent (Figure 2). In contrast to platelet adhesion to vWF, platelets did not roll on immobilized thrombin before firm adhesion, but rather attached instantly. Perfusion experiments using a range of shear rates (100 \( \text{s}^{-1} \) to 4000 \( \text{s}^{-1} \)) showed platelet adhesion at all shear rates tested, with optimal adhesion at a shear rate of 300 \( \text{s}^{-1} \) (data not shown). For further perfusion experiments, a shear rate of 300 \( \text{s}^{-1} \) was used, which is comparable to venous shear rates.

Platelet Adhesion to Immobilized Thrombin Is Mediated by GPIbα
To investigate whether GPIbα is involved in platelet adhesion to immobilized thrombin, we perfused reconstituted blood over immobilized thrombin in the presence of an antibody directed against the thrombin-binding site of GPIbα (LJIb-10, 100 \( \mu \text{g/mL} \)). As shown in Figure 2A, surface coverage is substantially and significantly reduced on addition of LJIb-10. Also, addition of glycoprotein IIB (GC, 50 \( \mu \text{g/mL} \)), a proteolytic fragment of the extracellular domain of GPIbα, inhibited platelet adhesion to immobilized thrombin (Figure 2A).

Platelet Adhesion to Immobilized Thrombin Requires Activation of PARs, Secretion of ADP and Thromboxane A2 Synthesis
Next, we investigated the role of PAR1 and PAR4 in platelet adhesion to immobilized thrombin. Platelet adhesion to immobilized thrombin could be blocked by addition of an
Inhibitory antibody against PAR1 (Figure 2A). Platelets desensitized for either PAR1 or PAR4 with the PAR1 activating peptide SFLLRN (15 μmol/L, 30 minutes, 37°C) or the PAR4 activating peptide GYPGQV (1 mmol/L, 30 minutes, 37°C) had a reduced capacity to adhere to immobilized thrombin (Figure 2A). Furthermore, platelet adhesion to thrombin was almost completely abolished when thrombin was preincubated for 30 minutes at 37°C with 50 μg/mL wild-type (WT) thrombin or 25 μg/mL thrombin mutant. After perfusion, coverslips were stained with May-Grünwald/ Giemsa and examined by light microscopy. Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. **P<0.01. Error bars indicate standard deviation.

Platelet Adhesion to Immobilized Thrombin Is Dependent on Exosite II. Reconstituted blood was perfused for 5 minutes at a shear rate of 300 s⁻¹ over a coverslip coated with 25 μg/mL wild-type (WT) thrombin or 25 μg/mL thrombin mutant. After perfusion, coverslips were stained with May-Grünwald/Giemsa and examined by light microscopy. Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. **P<0.01. Error bars indicate standard deviation.

Platelet adhesion to immobilized thrombin is dependent on exosite II. Reconstituted blood was perfused for 5 minutes at a shear rate of 300 s⁻¹ over a coverslip coated with 25 μg/mL wild-type (WT) thrombin or 25 μg/mL thrombin mutant. After perfusion, coverslips were stained with May-Grünwald/Giemsa and examined by light microscopy. Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. **P<0.01. Error bars indicate standard deviation.

To study whether platelet adhesion to fibrin with thrombin is also dependent on GPIbα, platelets were pretreated with the snake venom Nk (5 μg/mL), which sheds GPIbα from the platelet surface. Platelet adhesion to fibrin alone is partially inhibited by Nk, indicating the involvement of GPIbα in adhesion to fibrin (Figure 5), which is in agreement with experiments performed by Hantgan et al, who showed that platelet adhesion to fibrin is in part dependent on GPIbα. Recombinant thrombin mutants could also increase adhesion to fibrin as compared with wild-type thrombin.

Fibrin-Bound Thrombin Contributes to Platelet Adhesion and Aggregate Formation

Subsequently, we investigated platelet adhesion to immobilized fibrin and fibrin-bound thrombin. Reconstituted blood was perfused for 5 minutes at a shear rate of 300 s⁻¹ over fibrin-coated coverslips, which were incubated with thrombin (25 μg/mL) or vehicle. Platelets readily adhered to fibrin as shown in Figure 4A. Platelet adhesion and aggregate formation substantially increased on fibrin with bound thrombin compared with fibrin alone as shown in Figure 4B and increased with increasing thrombin concentrations with half-maximum effect obtained at 15 μg/mL thrombin and maximum effect reached at 50 μg/mL thrombin (data not shown). Platelet adhesion to fibrin is fully dependent on α₃β₃, and therefore after addition of dRGDW (200 μmol/L) adhesion was abolished (Figure 4C). However, Figure 4D shows that in the presence of dRGDW platelets did adhere to fibrin-bound thrombin, indicating that fibrin-bound thrombin is able to directly bind platelets. Figure 4E shows the surface coverage results of Figure 4A through 4D.
Platelet adhesion to fibrin-bound thrombin is dependent on GPIbα and PAR1. Reconstituted blood was perfused over fibrin-bound thrombin (Fb+IIa) or fibrin alone (Fb) after pre-treatment with Nk (left section), PPACK (mid section), or an inhibitory antibody against PAR1 (right section). After perfusion, coverslips were stained with May–Grünewald/Giemsa and examined by light microscopy. Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. *P<0.05; **P<0.01. Error bars indicate standard deviation.

Discussion

This study shows that thrombin immobilized on a coverslip or on fibrin is able to capture platelets under conditions of flow. The capacity of thrombin to function as a platelet adhesive protein has not been recognized previously. Perfusion of reconstituted blood over immobilized thrombin resulted in rapid platelet adhesion and the formation of large stable aggregates. Platelet adhesion was shown to be dependent on GPIbα and the proteolytic activity of thrombin. We propose the following sequence of events leading to the formation of a stable aggregate when thrombin is immobilized on a surface. Immobilized thrombin is able to capture platelets from flowing blood via GPIbα. Subsequently, intracellular signaling occurs in response to thrombin binding to GPIbα and activation of PARs, resulting in the formation of thromboxane A2 and secretion of ADP and the activation of α<sub>IIb</sub>β<sub>3</sub>. These processes are responsible for the stable adhesion to thrombin and the formation of aggregates.

Activation of α<sub>IIb</sub>β<sub>3</sub> is not mandatory for primary platelet adhesion to thrombin, as platelets readily adhere in the presence of dRGDW, whereas signal transduction via PARs, thromboxane A2, and ADP is required for primary adhesion. These processes result in inside-out signaling to GPIbα or relocation of GPIbα into lipid rafts, which we hypothesize to be required for a firm GPIbα–thrombin interaction. However, we cannot exclude that other (unknown) receptors contribute to stable platelet adhesion to thrombin.

Platelet adhesion to immobilized thrombin is dependent on the interaction between thrombin exosite II and GPIbα on the platelet surface. This is demonstrated by the fact that antibodies against the thrombin binding site on GPIbα inhibit platelet adhesion to immobilized thrombin. Furthermore, recombinant thrombins with mutations in exosite II, which virtually abolish the interaction with GPIbα, did not induce platelet adhesion and aggregate formation when immobilized directly on a coverslip, whereas the exosite I mutant does support adhesion. Taken together, these results provide strong evidence that exosite II is essential for platelet adhesion to immobilized thrombin mediated by GPIbα, and that the interaction of GPIbα with exosite I apparently is not required or capable of inducing platelet
adhesion under flow conditions. This is in correspondence with the observations of Celikel et al., who reported that thrombin first binds to GPIbα via exosite II, after which a second molecule can bind via exosite I.

Although we show that thrombin can act as a platelet adhesive protein, thrombin is usually not present as a surface-bound protein but functions in hemostasis primarily as a soluble protein. Nevertheless, on clot formation thrombin is immobilized to the fibrin clot and this binding to fibrin may be important in localizing thrombin to the site of vascular injury. Our results show that when thrombin is bound to fibrin, platelet adhesion and aggregate formation are substantially enhanced. Although relatively high concentrations of thrombin are required for this process, it would make sense to believe that local thrombin concentrations bound to fibrin could rise to high levels and thereby contribute in the post-recruitment of platelets to the fibrin-clot. Also, in the presence of dRGDW, which completely blocks adhesion to fibrin, platelets readily adhere to fibrin-bound thrombin. This suggests that fibrin-bound thrombin not only increases platelet adhesion and aggregation by enhancement of platelet activation mediated by PARs, but also is able to directly capture platelets via GPIbα. This is further demonstrated by the experiments shown in Figure 5, which show that the increase in platelet adhesion to fibrin with bound thrombin is abolished when platelets are depleted from GPIbα after pre-treatment with Nk.

When bound to fibrin, exosite II mutants contributed to platelet adhesion in the absence of dRGDW, which most likely reflects enhancement of platelet activation via PARs. As these exosite mutants could not initiate platelet adhesion in the presence of dRGDW, we conclude also that fibrin-bound thrombin is able to capture platelets via GPIbα. Although Y71A has a reduced binding capacity for fibrin, it can still contribute to platelet adhesion to fibrin. However, it is unable to induce platelet adhesion in the presence of dRGDW. It appears that in our experimental setup the amount of Y71A, which has bound to fibrin, still has the potential to activate PARs and contribute to platelet adhesion to fibrin but is present in insufficient amounts to directly capture platelets via GPIbα.

It is important to note that when thrombin is immobilized on a surface, probably thrombin exosomes are not both available for ligand binding. Whether the fibrin-bound thrombin is bound to fibrin via exosite I or exosite II is still a matter of debate. Extensive reviews have been dealing with this controversy (Huntington, Mosesson, and Lane). Although from the results in the present study we cannot confirm the exact mechanism of how thrombin is bound to fibrin, the fact that adhesion of GPIbα-depleted platelets to fibrin-bound thrombin is strongly diminished compared with control platelets, combined with the observation that platelet adhesion to thrombin itself is mediated by thrombin exosite II, suggests that thrombin is bound to fibrin via exosite I and contributes to platelet adhesion via a GPIbα-dependent interaction with exosite II. Although exosite I interacts with fibrin, and thrombin also interacts via exosite I with PAR1, PAR1 can still be hydrolyzed by fibrin-bound thrombin. This seems contradictory, but Mylés et al already described that the ability of thrombin mutants to activate PAR1 or clot fibrinogen differ profoundly, indicating the involvement of different exosite I residues in PAR1 activation and fibrinogen binding.

In conclusion, these experiments show that immobilized thrombin can act as an adhesive surface and is able to directly capture and activate platelets under flow conditions. This platelet adhesion is dependent on the interaction of thrombin with GPIbα, the activation of PARs, and the secretion of ADP and thromboxane A2. Platelet adhesion to fibrin-bound thrombin could be a novel target for new antithrombotic drugs, which could now more specifically interfere with the action of thrombin on platelets on the actual site of thrombosis.

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Data Supplements

Materials

Purified plasma-derived thrombin was kindly provided by Dr. W. Kisiel (University of New Mexico Health Sciences Center, Albuquerque, NM). Recombinant thrombin mutants were generated and purified as described previously. Thrombin and fibrinogen used for generating fibrin-coated coverslips were purchased from Kordia Life Sciences (Leiden, the Netherlands). The RGD-containing peptide D-arginyl-glycyl-L-aspartyl-L-tryptophane (dRGDW) was synthesized at the Department of Membrane Enzymology, Faculty of Chemistry (University of Utrecht, the Netherlands). A rabbit polyclonal inhibitory antibody against the protease-activated receptor 1 (PAR1) was a generous gift of Dr. D. C. Foster (Cytokine Biology, ZymoGenetics, Seattle, WA). Fab fragments of a monoclonal antibody, which specifically inhibits thrombin binding to GPIb (LJIb-10), were a generous gift from Dr Z. M. Ruggeri (The Scripps Research Institute, La Jolla, CA). An antibody against vWF (RAG-35, ascites fluid) that inhibits the binding of vWF to GPIb was a generous gift of Dr J.A. van Mourik (CLB, Amsterdam, the Netherlands). PPACK [D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone] and the PAR1-agonist peptide SFLLRN were from Bachem (Bubendorf, Switzerland). The PAR4-agonist peptide GYPGQV was synthesized by GenScript (Piscataway, NJ). The P2Y1 antagonist adenosine-3’,5’-diphosphate (A3P5P) was purchased from Sigma-Aldrich Chemicals BV (Zwijndrecht, the Netherlands) and the P2Y12 antagonist AR-C69931MX was a generous gift from AstraZeneca (Loughborough, United Kingdom). The thromboxane-receptor antagonist SQ30741 was kindly provided by Bristol-Meyers-Squibb (Maarssen, The Netherlands). Nk, a GPIbα-cleaving metalloproteinase, was purified from cobra (Naja kaouthia) venom (Sigma, St. Louis, MO) using the method previously described for mocarhagin and was a generous gift of Dr R.K. Andrews (Monash...
University, Clayton, Australia). Essentially fatty acid free bovine serum albumin (BSA) and mepacrine were obtained from Sigma-Aldrich (St Louis, MO). ImmunO Human Albumin Fraction V was purchased from MP Biomedicals Inc (Eschwege, Germany). All other chemicals used in the experiments were of analytical grade.

**Purification of glycocalicin**

Glycocalicin was purified from fresh-frozen plasma by immunoaffinity chromatography using an in house mouse monoclonal antibody against human GPIbα coupled to CNBr-activated Sepharose 4B (5 mg antibody/ml column)(Pharmacia, Uppsala, Sweden), followed by affinity chromatography on Wheat-germ agglutinin-agarose (Fluka Chemie, Buchs, Switzerland). The purified glycocalicin appeared as a single band on SDS-PAGE and concentration was measured by using a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

**Platelet preparation**

Blood from healthy volunteers, who claimed not to have used aspirin or other nonsteroidal anti-inflammatory drugs for the preceding 10 days, was drawn into one-tenth volume of 3.4% sodium citrate. Washed platelets were prepared as described previously \(^3\). The blood was centrifuged at 200g for 15 min at room temperature. The platelet-rich plasma (PRP) was removed and acidified by addition of one-tenth volume of ACD (2.5% trisodium citrate, 1.5% citric acid, and 2% D-glucose). Platelets were centrifuged (500g, 15 min) and the platelet pellet was resuspended in Hapes-Tyrode buffer at pH 6.5 (10 mM HEPES [\(N\text{-2-hydroxyethylpiperazine-\(N\text{'-2-ethanesulfonic acid}\)]}, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH\(_2\)PO\(_4\), 1.7 mM MgCl\(_2\), 5 mM D-glucose). Prostacyclin (PGI\(_2\), 10 ng/ml) was added to prevent platelet activation during the subsequent washing step. Platelets were centrifuged
(500g, 15 min) and resuspended in a small volume of HEPES-Tyrode buffer. The platelets were diluted in human albumin solution (HAS; 4% human albumin, 4 mM KCl, 124 mM NaCl, 20 mM NaHCO₃, 2 mM Na₂SO₄, 1.5 mM MgCl₂, 5 mM CaCl₂, 5 mM D-glucose, pH 7.35) to a platelet count of 333 × 10⁹/L (333.000/µl). Red blood cells were obtained by centrifuging blood at 200g for 10 min at room temperature. PRP was removed and used for platelet isolation. The pellet was centrifuged (2000g, 10 min) and resuspended in 0.9% NaCl containing 5 mM D-glucose. The obtained red blood cells were washed twice with 0.9% NaCl containing 5 mM D-glucose, and finally cells were packed (2000g, 15 min). For perfusion experiments, a mixture of 40% red blood cells and 60% washed platelets was prepared. Consequently, the reconstituted blood had a platelet count of 200 x 10⁹/L (200.000/µl) and a hematocrit of 40%.

GPIb-depleted platelets were prepared by using the cobra venom Nk. Like mocarhagin, Nk cleaves GPIbα to release a ~45-kDa N-terminal fragment, and inhibits von Willebrand factor binding to washed platelets (R. Andrews, personal communication). Washed platelets (diluted in HEPES-Tyrode buffer, pH 7.4) were incubated for 30 min at 37 °C with 5 µg/ml Nk or vehicle in the presence of 1 mM Ca²⁺. Nk-activity was stopped by adding 5 mM EDTA. After incubation, platelets were centrifuged (500 g, 15 min) in the presence of prostacyclin (PGI₂, 10 ng/ml) and the platelet pellet was resuspended in a small volume of HEPES-Tyrode at pH 6.5 and diluted in human albumin solution as described above.

Coating of the coverslips

For end-point perfusion experiments, glass coverslips (Menzel Gläser 18x18 mm) were cleaned overnight with 100% alcohol and rinsed with distilled water before coating. Coating was performed by incubating the coverslips with 100 µl thrombin or thrombin mutant (25 µg/ml) in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 8.0) overnight at
4°C, followed by incubation with 2% BSA in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 30 min to block unoccupied sites on the coverslip. For real time perfusion studies, the same coating procedure was used, but larger glass coverslips (Menzel Gläser 24x60 mm) were used. These coverslips were incubated with 300 µl thrombin. To obtain fibrin-coated coverslips, fibrinogen (100 µg/ml) was mixed with 0.15 µg/ml thrombin (Kordia Life Sciences, Leiden, the Netherlands) and immediately sprayed onto glass coverslips with a retouching airbrush. The coverslips were incubated for 30 min at 37°C and afterwards, the coverslips were blocked for at least 30 min at room temperature with 2% BSA in PBS. Possible residual thrombin used to obtain fibrin was shown not to have an effect on platelet adhesion to fibrin under flow conditions as demonstrated by Hantgan et al.\(^4\) and confirmed in Figure 5 of this manuscript. Subsequently, fibrin-coated coverslips were incubated with thrombin or thrombin mutant (25 µg/ml) in TBS (pH 8.0) overnight at 4°C, followed by incubation with 2% BSA in PBS for 30 min.

**Perfusion studies**

Perfusions were carried out in a single-pass perfusion chamber as described previously.\(^5\) Reconstituted blood was perfused over the coated coverslips for 5 min at a constant flow rate. After perfusion, slides were washed with HEPES buffer (10 mM Hepes, 150 mM NaCl, pH 7.35) and fixed in 0.5% glutaraldehyde in PBS or prepared for Scanning Electron Microscopy as described below. Subsequently, slides were dehydrated in methanol and stained with May-Grünwald and Giemsa as described previously.\(^6\) Next, the slides were examined using light microscopy. Platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.0 software (Dutch Vision Systems [DVS], Breda, The Netherlands) and was expressed as the percentage of the surface covered with platelets.
Real time perfusion studies

Real time perfusions were carried out in a single-pass perfusion chamber consisting of a silicon sheet gasket that maintained a flow path height of 0.125 mm and a width of 2 mm. Platelets were labeled prior to perfusion for 15 min with 10 µM mepacrine, a concentration known not to interfere with the functional properties of platelets. Reconstituted blood was perfused over immobilized thrombin for 5 min at a constant flow rate. Platelet interaction with the surface was continuously monitored by a fluorescence microscope (Orthoplan Flu. Leica, Germany) equipped with a LI-low-light-level CCD camera (Lambert Instruments, Leutewolde, the Netherlands) and a Pioneer DVD-recorder.

Scanning Electron Microscopy

For Scanning Electron Microscopy (SEM) examination, slides were washed with HEPES buffer and fixed in 2% glutaraldehyde in PBS. After washing with distilled water, slides were dehydrated with increasing concentrations of ethanol (80% - 100%) and subsequently treated with hexomethyldisylazane (Fluka Chemie, Buchs, Switzerland). Slides were embedded on a stub in carbon glue. Samples were coated with a thin layer of Platinum/Palladium using an Emitech K-575X Sputter Coater and were examined by SEM (XL30 SFEG, Philips, Eindhoven, the Netherlands).

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, CA) software package. Statistical differences in surface coverage were analysed by standard one-way analysis of variance (ANOVA) using Dunnett multiple comparison test. P values less than .05 were considered statistically significant.
References


Supplement figure - Platelet adhesion and aggregation to thrombin mutants bound to fibrin. Fibrin-coated coverslips were incubated with 25 µg/ml wild-type (WT) thrombin or 25 µg/ml thrombin mutant. Reconstituted blood was perfused for 5 min at a shear rate of 300 s⁻¹ in the absence (A) of presence (B) of dRGDW (200 µM). After perfusion, coverslips were stained with May-Grünwald/Giemsa and examined by light microscopy. Graph shows relative mean surface coverage of at least 3 independent experiments performed in triplicate. Statistical significance is compared to fibrin control (A, indicated as (-)) or wild-type thrombin (B, indicated as (WT)). *: P<0.05, **: P<0.01. Error bars indicate standard deviation.
**Movie – Platelet adhesion to immobilized thrombin.** Reconstituted blood was perfused over a coverslip coated with 25 µg/ml thrombin at a shear rate of 300 s⁻¹. Platelet interaction with the surface was continuously monitored by a fluorescence microscope (original magnification (2500x). Movie shows a fast-forwarded capture of a representative perfusion of 5 min.