Supplemental Information for

Threshold Response of Initiation of Blood Coagulation by Tissue Factor in Patterned Microfluidic Capillaries is Controlled by Shear Rate

Feng Shen, Christian J. Kastrup, Ying Liu, Rustem F. Ismagilov*

Department of Chemistry and Institute for Biophysical Dynamics,
The University of Chicago, Chicago, Illinois

Methods

Materials

All solvents and inorganic chemicals used in buffers were purchased from commercial sources and used as received unless otherwise stated. Fibrinogen labeled with Alexa Fluor 488 and Texas Red®, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE) were purchased from Molecular Probes/Invitrogen (Eugene, OR). 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC) and L-α-phosphatidylserine from porcine brain (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). T-butyloxycarbonyl-β-benzyl-L-aspartyl-L-prolyl-L-arginine-4-methyl-coumaryl-7-amide (Boc-Asp(OBzl)-Pro-Arg-MCA) was purchased from Peptides International (Louisville, KY). Human recombinant tissue factor (TF) was purchased from Calbiochem/EMB Biosciences (La Jolla, CA). Corn trypsin inhibitor (CTI) was purchased from Haematologic Technologies (Essex Junction, VT). Silica capillaries (inner diameter, I.D., of 150 μm, 200 μm, 450 μm, and 700 μm) were purchased from Polymicro Technologies (Phoenix, AZ). Teflon tubing (I.D. of 305 μm) was purchased from Weico Wire & Cable, Inc. (Edgewood, NY). Albumin from bovine serum (BSA), anhydrous hexadecane, and butyltricholorosilane (BTS) were purchased from Sigma Aldrich (St. Louis, MO). Sodium Chloride and anhydrous methyl sulfoxide (DMSO, 99.7% purity) were purchased from Fisher Scientific (Pittsburgh, PA). Human normal, platelet poor, pooled plasma (NPP) was purchased from George King Bio-Medical, Inc. (Overland Park, KS). Human whole blood was obtained from individual healthy donors

in accordance with the guidelines set by the Institutional Review Board (protocol # 12502A) at the University of Chicago.

Generating patches of tissue factor (TF) surrounded by an inert phospholipid monolayer

Lipid vesicles were prepared as previously described.[1] Detailed procedures for forming layers of phospholipids containing TF on glass surfaces and for patterning phospholipids patches have been previously described by others.[2, 3] Our procedure is briefly described here. A monolayer of inert neutral lipids of DLPC was initially formed in silanized silica capillaries. Then, this monolayer and silane were selectively removed by deep-UV photopatterning, and a bilayer of clot-inducing lipids was formed in the irradiated region. Silica capillaries were first made hydrophilic by flowing a solution of "piranha" (3:1 sulfuric acid:hydrogen peroxide, WARNING: piranha reacts violently with organic substances, including all human tissues) for 20 min, followed by rinsing with deionized water (18.2 MΩ.cm) and drying with nitrogen gas. A solution of BTS (4 μL) in anhydrous hexadecane (1 mL) was flowed through the capillary at a flow rate of 0.01 mL min^{-1} for 45 min. Additional hexadecane was flowed through the capillary to remove excess BTS. The silanized capillary was then rinsed with ethanol (2 mL) and dried with nitrogen gas. A monolayer of DLPC was formed on the silanized surface by filling the capillary with a solution containing DLPC vesicles (1.25 mg mL^{-1} vesicles in phosphate buffered saline (PBS, pH = 7.4)) and incubating at room temperature for 40 min. Excess vesicles were removed by rinsing with a 150 mM NaCl solution (saline). These monolayers were photopatterned within 24 hr.

A photomask patterned with rectangular windows was placed over the capillary substrate and irradiated with deep UV light.[1, 3] The patterned capillary was backfilled with vesicles containing 79.5 mol % of DLPC, 20 mol % of PS, 0.5 mol % of Texas Red DHPE, and TF. The concentration of TF in the vesicle solution was 0.4 nM^{1} in all the experiments except two: 1) the control experiments comparing the volumetric flow rate, flow velocity, and shear rate (as in Figure 4), in which the concentration of TF was 1.6 nM and 2) the control experiments on whole blood without sodium citrate, in which the concentration of

TF was 0.8 nM. The capillary was incubated at room temperature for 2 min and then extensively rinsed with saline at a shear rate of 2000 s^{-1} to remove excess lipid vesicles.

Preparing blood samples (whole blood, PRP, NPP)

Whole blood, PRP, and NPP were prepared as previously described.[4] Whole blood obtained from individual healthy donors was collected in Vacutainer® tubes (Franklin Lakes, NJ) containing 3.2 % sodium citrate (9:1 by volume). PRP with approximately 3 $\times 10^5$ platelets μL^{-1} was obtained by centrifuging whole blood at 300 g for 10 min.[5] Human normal, platelet poor, pooled plasma (NPP) was purchased from George King Bio-Medical, Inc. (Overland Park, KS). According to the manufacturer, platelet counts in NPP samples were less than 10,000 per μL before freezing. Samples were stored at -80 ˚C and thawed immediately before the experiments. All blood and plasma were incubated with CTI (100 μg mL^{-1}) to inhibit the Factor XII pathway of initiation of coagulation.[6] CTI was added to NPP during the thawing of the frozen plasma. CTI was added to whole blood approximately 30 minutes after collection, although adding CTI during the collection may help extend the background clot time even further in this experimental setup.[7]

For the control experiment on whole blood without sodium citrate, blood was drawn into 10mL BD syringes. The first 10mL blood was discarded, and CTI was added during collection to reach a final concentration of 100 μg mL^{-1}.

Measuring clot time of human blood and plasma on patches of TF

Citrated NPP and PRP (600 μL) were recalcified by adding 200 μL of a solution of $CaCl_2$ (40 mM), NaCl (90 mM), and a thrombin sensitive fluorescent substrate, Boc-Asp(OBzl)-Pro-Arg-MCA (0.4 mM).[1] Citrated whole blood (500 μL) was recalcified by first adding Boc-Asp(OBzl)-Pro-Arg-MCA (6.75 μL, 10 mM in DMSO) and then a solution of $CaCl_2$ (31.2 μL, 200 mM).[8] The recalcified blood or plasma sample was placed into a 1 mL plastic syringe blocked with BSA and connected to the capillary with Teflon tubing. The patterned capillary was kept under NaCl solution (150 mM) in a Petri dish in a 37 ˚C incubator. The blood or plasma was flowed into the capillary at a rate of 1.39 μL min^{-1} to

64.32 μL min^{-1} by using a syringe pump (Harvard PHD 2000, Harvard Apparatus, Holliston, MA). For fully developed laminar flow through a circular capillary, the shear rate ($\dot{\gamma}$) can be calculated from the volumetric flow rate (Q) and the radius of the capillaries (R) as $\dot{\gamma} = 4 \times Q/(\pi \times R^3)$.[9] Thrombin generation was monitored by fluorescence microscopy to detect the cleavage of Boc-Asp(OBzl)-Pro-Arg-MCA.[10] The formation of fibrin and the aggregation of platelets were monitored by brightfield microscopy.

Monitoring fibrin formation with Alexa 488 labeled fibrinogen

Fibrinogen labeled with Alexa 488 was added to 1mL PRP to a concentration of 100 μg/mL, and the sample was incubated at room temperature for 30 min.[11] This PRP sample was flowed into a microcapillary (I.D. = 450 μm) at a shear rate of 20 s^{-1}. After aggregation of platelets occurred, excess fibrinogen was rinsed away with saline, leaving the crosslinked fibrin mesh. Fluorescence microscopy was used to detect the presence of fibrinogen/fibrin labeled with Alexa 488.

Image acquisition and preparation

Fluorescence and brightfield images of fibrin labeled with Alexa 488 were acquired at 37 ˚C by using a digital camera (C4742, Hamamatsu Photonics, Japan) mounted to a Leica DMI6000 B epi-fluorescence microscope (Leica Microsystems GmbH, Germany) with a 20 × 0.7 NA objective. The confocal image of the bilayer patch was acquired by a digital camera (C9100, Hamamatsu Photonics, Japan) mounted on a Visitech VT Infinity 3 multi-point confocal system (VisiTech International Ltd., United Kingdom) attached to Leica DMI 6000 B microscope with a 20 × 0.7 NA objective at 24 ˚C. All the other fluorescence and brightfield images were acquired by using a digital camera (C4742, above) mounted to a Leica DMI 6000 B epi-fluorescence microscope with a 10 × 0.4 objective at 37 ˚C.

Image analysis was performed as previously described.[1] The original grayscale fluorescence images were collected and false-colored by using MetaMorph® software (Molecular Devices Corporation, Downingtown, PA). For each figure, the levels of all

wavelengths were adjusted to the same values. Images were overlaid by using Adobe Photoshop software. The confocal images were collected and false-colored by using SimplePCI® software (Hamamatsu Corporation, Sewickley, PA), and the brightness and contrast were adjusted to be better visualized in grayscale by using Adobe Photoshop software.

Supplemental experiments

At an above-threshold shear rate, coagulation does not initiate downstream of TF patches.

In experiments where coagulation did not initiate on the patch of tissue factor (TF), we wished to confirm that coagulation did not initiate downstream of the patch. We also monitored coagulation downstream of the patches in all experiments. As an example, we performed the following control experiment. Platelet rich plasma (PRP) was flowed into a microcapillary (I.D. = 450 μm) over a 200 μm patch of tissue factor (TF) at a shear rate of 80 s^{-1} and flow velocity of 4.5 mm/s at 37 ˚C. Details and procedures are described in the experimental section. Fibrin formation and platelet aggregation were monitored both at the patch and 3 mm downstream from the patch by using brightfield microscopy. No fibrin formation or platelet aggregation was observed on the patch which is in agreement with the results as in Figure 5A of the main text. At shear rate of 40 s^{-1} and flow velocity of 2.25 mm/s, clotting initiated no further than 100 μm downstream from the patch. If the sole role of increased shear rate and flow rate were to move the site of initiation of coagulation further downstream, then this initiation would have occurred within 200 μm of the patch or closer. These results agree with the previous study on effects of shear in propagation of clotting,[12] where clotting downstream from a channel was not observed.

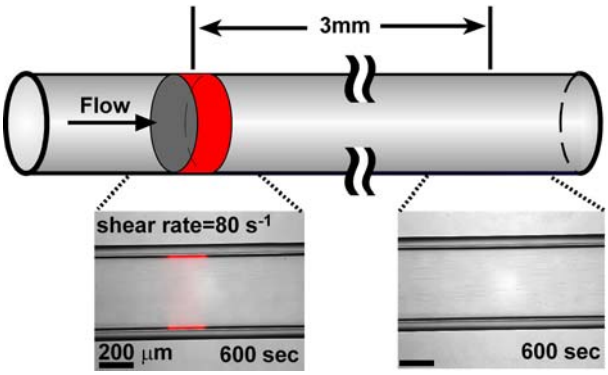

Figure S1. Coagulation of PRP did not initiate 3 mm downstream of the patch of TF at a high shear rate of 80 s^{-1}. PRP was flowed into a microcapillary patterned with a patch (red) of TF in an inert background (gray). Brightfield images show that coagulation of PRP did not initiate at the patch or downstream of the patch within 600 sec (10 min).

Control experiments for shear force

High shear force could wash TF away from a patch, preventing the initiation of coagulation. However, two control experiments ruled out this alternative mechanism. In one experiment the capillary was extensively rinsed with a saline solution at a shear rate of 2000 s^{-1}, which generated a shear force of approximately 40 times higher than NPP at a shear rate of 40 s^{-1}.[13] When NPP was flowed into this capillary at a shear rate of 5 s^{-1}, coagulation initiated on a 200 μm patch of TF in approximately 210 sec. In a second control experiment, NPP was flowed over a 200 μm patch of TF at an above-threshold shear rate of 40 s^{-1} for 300 sec, and then the shear rate was reduced to a below-threshold shear rate of 5 s^{-1}. Coagulation initiated on the patch in 100 sec, indicating that the TF stimulus was still present and active.

Control experiment of whole blood without sodium citrate

Whole blood with CTI (100 μg / mL) was flowed into the device within 6 min after blood was collected from a donor. No sodium citrate was added to the blood sample. When whole blood was flowed over a 200 μm patch of TF, the clot time of whole blood was

noticeably longer at a high shear rate of 120 s^{-1} than at a low shear rate of 20 s^{-1} (Figure S2).

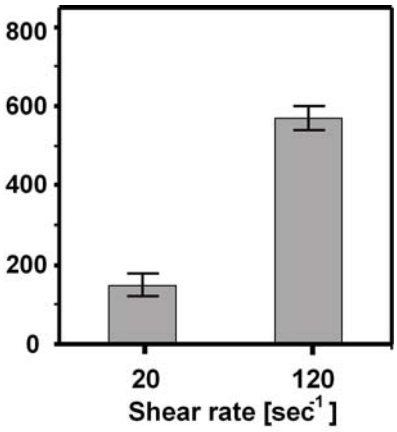

Figure S2. Whole blood without sodium citrate initiated clotting faster at a low shear rate than at a high shear rate. Error bars represent maximum and minimum (n = 2).

Supplemental Figure

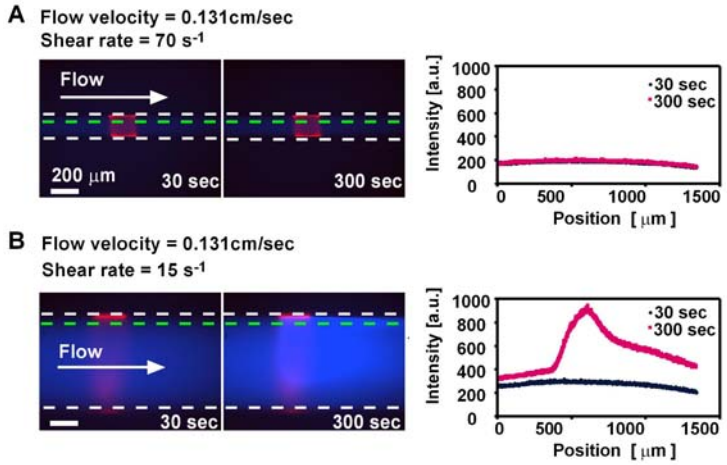

Figure S3. Initiation of coagulation of blood plasma depends on shear rate, not flow velocity. (A-B) Blue and red fluorescence is explained in the caption of Figure 1. Time-lapse, fluorescence images demonstrate that with same flow velocity, coagulation of NPP did not initiate in a capillary with an inner diameter of 150 μm at a high shear rate of 70 s^{-1} (A), but coagulation initiated in a capillary with an inner diameter of 700 μm at a low shear rate of 15 s^{-1} (B). The plots to the right in panels A and B show blue fluorescence intensity (thrombin) of a linescan along the green dashed line drawn in the fluorescence images on the left. The image of the capillary with an inner diameter of 700 μm gave rise to increased background fluorescence in panel B due to increased out-of-focus background fluorescence of the uncleaved fluorescent substrate in this thick capillary.

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