Single Particle Tracking of ADAMTS13 (A Disintegrin and Metalloprotease with Thrombospondin Type-1 Repeats) Molecules on Endothelial von Willebrand Factor Strings*‡§1,2

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Background: ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type-1 repeats) cleaves pro-thrombotic ultra-large von Willebrand factor (VWF) strings.

Results: Customized single particle tracking enabled visualization of single ADAMTS13 enzymes that bind to long platelet-decorated VWF strings.

Conclusion: ADAMTS13 readily bind to multiple available sites on VWF strings.

Significance: Single molecule imaging can be used to study interactions between enzymes and large biopolymers in flow.

von Willebrand factor (VWF) strings are removed from the endothelial surface by ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type-1 repeats)-mediated proteolysis. To visualize how single ADAMTS13 molecules bind to these long strings, we built a customized single molecule fluorescence microscope and developed single particle tracking software. Extensive analysis of over 6,000 single inactive ADAMTS13E225Q enzymes demonstrated that 20% of these molecules could be detected in at least two consecutive 60-ms frames and followed two types of trajectories. ADAMTS13E225Q molecules either decelerated in the vicinity of VWF strings, whereas sometimes making brief contact with the VWF string before disappearing again, or readily bound to the VWF strings and this for 120 ms or longer. These interactions were observed at several sites along the strings. Control experiments using an IgG protein revealed that only the second type of trajectory reflected a specific interaction of ADAMTS13 with the VWF string. In conclusion, we developed a dedicated single molecule fluorescence microscope for detecting single ADAMTS13 molecules (nm scale) on their long, flow-stretched VWF substrates (μm scale) anchored on living cells. Comprehensive analysis of all detected enzymes showed a random interaction mechanism for ADAMTS13 with many available binding sites on the VWF strings.

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4 The abbreviations used are: VWF, von Willebrand factor; UL, ultra-large; ADAMTS, a disintegrin and metalloprotease with thrombospondin type-1; SM, single molecule; SFMFM, SM fluorescence microscopy; SPT, single particle tracking; BOEC, blood outgrowth endothelial cell; EM, electron multiplying; CCD, charge-coupled device; DIOC, 3,3′-dihexyloxycarbocyanine iodide.
Visualizing Single ADAMTS13 Enzymes on VWF Strings

Although technically challenging, such studies provide new insights that cannot be obtained with classical bulk experiments, where only average parameters are measured. Single molecule measurements using atomic force microscopy and optical tweezers (17, 21, 22) have demonstrated that force-induced unfolding of single VWFA2 domains occurs above a certain shear threshold. Interestingly, these studies also revealed that complete unfolding of single VWFA2 domains is not necessary for cleavage by ADAMTS13 as a partially unfolded intermediate is also a suitable substrate (17, 22).

Previously, we have shown that platelet-decorated VWF strings are cleaved multiple times by ADAMTS13, with a preference for sites that become exposed after local elongations of the string (23). Here, we aimed to gain insight into the ADAMTS13’ binding mode on these mobile VWF strings by directly visualizing how and where single inactive ADAMTS13E225Q enzymes bind to these VWF substrates using SMF microscopy (SMFM). SM measurements have been extensively applied to track proteins in membranes of living cells (24, 25) or lipid bilayers (26), following motor proteins along the cytoskeleton (27, 28) and DNA-binding proteins on DNA (29–32). It is important to note that, because the A2 domain is only necessary for cleavage by ADAMTS13 as a partially unfolded intermediate is also a suitable substrate (33).

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EXPERIMENTAL PROCEDURES

Blood Sample Collection

Blood was drawn from healthy volunteers who gave informed consent. The study was approved by the Institutional Clinical Review Board of the KU Leuven (Leuven, Belgium) and performed according to the Declaration of Helsinki.

Preparation of Washed Platelets

Washed platelets were prepared as described previously (23). Platelets were fluorescently labeled by incubation with 100 nM 3,3’-dihexyloxacarbocyanine iodide (DIOC, Invitrogen) for 10 min before use. All steps were performed at room temperature (RT).

Endothelial Cell Culture

Blood outgrowth endothelial cells (BOECs) were isolated from the blood of healthy volunteers, characterized by immunofluorescent staining and grown in EBM-2/EGM-2 medium (Lonza, San Diego, CA) as described (34, 35).

ADAMTS13 Production and Purification

Recombinant human ADAMTS13 (rADAMTS13) and catalytically inactive ADAMTS13 (rADAMTS13E225Q) were expressed using an inducible T-Rex system (Invitrogen) and purified as described previously (36). rADAMTS13 and rADAMTS13E225Q concentrations were determined using a sandwich ELISA with normal human pooled plasma (n = 20) as a reference (37). Purity was estimated by SDS-PAGE with subsequent Simply BlueTM staining (Invitrogen) (36).

Fluorescent Labeling of Proteins

Purified rADAMTS13, rADAMTS13E225Q, and mouse monoclonal IgG1 control antibody (Ab, Dako Cytomation, Glostrup, Denmark) were dialyzed against 0.15 m bicarbonate buffer (pH 8.3). A 2-fold molar excess of the Atto647N-NHS ester (Atto-tec, Germany) was added. After a 1-h incubation at RT, free dye was removed by dialysis against HBS (50 mM HEPES, pH 7.4, 5 mM CaCl₂, 1 mM ZnCl₂, and 150 mM NaCl) or PBS buffer for rADAMTS13/rADAMTS13E225Q or IgG control, respectively. The concentrations of ADAMTS13 solutions were determined as described (37).

Activity of Fluorescently Labeled ADAMTS13

Activity of rADAMTS13 and rADAMTS13-Atto647N was evaluated using a fluoroogenic VWF substrate (FRET-VWF73, Peptides International, Louisville, KY). In brief, FRET-VWF73 was excited at 355 nm and emission was measured at 460 nm every minute for 1.5 h. Fluorescence intensities were depicted in function of time. The slope of the resulting curve was used as a measure of enzyme activity. Activity of rADAMTS13-Atto647N (0.125 nM) was compared with the activity of unlabeled rADAMTS13 (0.125 nM), which was set as 100%. rADAMTS13 in the presence of EDTA (5 mM) was used as a negative control.

Immobilization of rADAMTS13-Atto647N on Single Molecule Clean Slides

Conventional coverslides (Menzel-gläser, Thermo Fisher Scientific) were cleaned by sonication at RT successively in acetone (spectrophotometric grade; Sigma) for 15 min, 1 M sodium hydroxide (Sigma) for 5 min (twice) and Milli-Q (MQ) water (Merck Millipore, Billerica, MA) for 15 min (twice). Coverslides were rinsed with MQ between consecutive steps and finally dried with argon (air liquid) and incubated in an UV-ozone photoreactor (UVP PR-100, UVP, Upland, CA) for at least 1 h. rADAMTS13-Atto647N was captured on coverslides using the in-house developed monoclonal anti-human ADAMTS13 Ab 20A5 (37), washed with PBS, 0.1% Tween 20, and blocked with PBS, 3% milk. After incubation with 0.1 μg/ml of rADAMTS13-Atto647N for 30 min, coverslides were washed three times with PBS, 0.1% Tween 20, mounted in a chamber, and kept moist during measurements by adding PBS, 0.3% milk. Negative controls included non-coated coverslides or coverslides only coated with Ab and/or PBS, 3% milk.

Degree of Labeling of ADAMTS13-Atto647N

rADAMTS13-Atto647N and rADAMTS13E225Q-Atto647N molecules immobilized on coverslides were continuously excited with laser light and emission light was detected with a cooled electron multiplying (EM) charge-coupled device (CCD) camera (ImageEM, Hamamatsu Photonics, Hamamatsu Japan) and sequential Simply BlueTM staining (Invitrogen) (36).
Visualizing Single ADAMTS13 Enzymes on VWF Strings

Parallel Flow Chamber Experiments to Study the Binding of ADAMTS13E225Q to VWF Strings

Confluent BOEC monolayers were grown on collagen-coated coverslides, stimulated with 25 μM histamine (Sigma) for 10 min at RT, and mounted in a parallel-plate flow chamber as described previously (23). BOECs were perfused with DIOC-labeled platelets for ~2 min followed by fluorescently labeled rADAMTS13E225Q-Atto647N (6 nM) in HBS for an additional 3 min (2.5 dyne/cm²). A customized microscope set up was developed to visualize single rADAMTS13E225Q-Atto647N enzymes (see below). Videos were recorded 60 to 90 s after initiation of flow for up to 5000 frames (240 s) using Hokawo software (Hamamatsu Photonics).

Single Molecule Microscope Set Ups

To detect single rADAMTS13-Atto647N molecules under static conditions, an IX-71 inverted fluorescence microscope (Olympus, Tokyo, Japan) was equipped with various epifluorescence objectives (×20 air (numerical aperture (NA) 0.5); ×40 oil (NA 1.3); ×60 oil (NA 1.4); and ×100 oil (NA 1.3); Olympus) and one cooled EM-CCD camera (ImageEM, Hamamatsu Photonics). Using two lenses, an additional ×3.3 magnification was included before this EM-CCD to enable visualization of single ADAMTS13 molecules. A 644-nm laser (Spectra Physics, Newport, RI) was used to excite rADAMTS13-Atto647N immobilized on coverslides. The excitation light was guided to the sample using a 650-long pass dichroic mirror (z650rdc). A 665-long pass filter for Atto647N in front of CCD1 (F1) and a 700/75m-band pass filter in combination with a 665-long pass filter for Atto647N in front of CCD1 (F1). The enzyme image was magnified 3.3 times by using a set of lenses (M) in front of the camera.

FIGURE 1. Single molecule fluorescence microscope set up. Schematic representation of excitation and emission paths of the customized microscope setup. The 491 (blue) and 644 nm (red) lasers, to excite DIOC (platelets) and Atto647N (rADAMTS13), respectively, were combined using mirrors and a 505-long pass dichroic mirror (DM1). After expansion, the laser beams were directed toward the sample present on the microscope stage using a set of mirrors and a 488/633 dual band mirror dichroic (DM2). Emission light arising from the sample was collected by the objective. The far red fluorescent and green fluorescent light, respectively, emitted by Atto647N and DIOC, was separated using a 650-long pass dichroic mirror (DM3) and selectively directed toward two simultaneously acquiring EM-CCD cameras. Additional filters were used to reject unwanted fluorescence: a 527/30-band pass filter for DIOC in front of CCD2 (F2) and a 700/75m-band pass filter in combination with a 665-long pass filter for Atto647N in front of CCD1 (F1). The enzyme image was magnified 3.3 times by using a set of lenses (M) in front of the camera.

Overlay of the Two Channels

A sample with TetraSpeck (Invitrogen) fluorescent beads was first used to align the two detection channels, such that the focal planes are the same and the excited area is centered in both cameras. These beads are stained with multiple fluorescent dyes and are therefore detected in both green and red channels. To overlap the fluorescence images acquired in both channels images of TetraSpeck beads were taken with both EM-CCD cameras simultaneously. The images of the ADAMTS13-Atto647N detecting channel (red channel) and the DIOC-labeled platelets detecting channel (green channel) were post-experimentally overlaid with about 18 nm precision using internal reflection fluorescence (39) mode to reduce emission arising from the molecules passing in solution. Samples were illuminated with a thin, laminated excitation sheet using a highly inclined laser beam. Emission light emerging from the sample was split by a 650-long pass dichroic mirror (z650rdc). A 527/30-band pass filter for DIOC (HQ527/30m) and filters for Atto647N as described above (HQ665LP and HQ700/75m) were used to selectively detect fluorescent signals from DIOC-labeled platelets and rADAMTS13-Atto647N (Fig. 1). Filters were obtained from Chroma Technology Corporation (Bellow Falls, VT). Movies were acquired with 30-ms integration time at 32 or 16 Hz. View fields are ~41 to 41 μm² (80 × 80 nm² per pixel) and 120 × 120 μm² for ADAMTS13 and platelets, respectively. TetraSpeck™ fluorescent 100-nm microspheres (Invitrogen) were used to align the two cameras and overlay fluorescence images from both channels.

City, Japan) (cf. single molecule microscope set ups). Videos were recorded using Hokawo software (Hamamatsu Photonics) for a maximum 5000 frames at integration times of 30, 50, or 200 ms. The fluorescent intensity of single spots was followed in function of time using a routine developed in Matlab (version 2010, The MathWorks, Inc., Natick, MA). Dye molecules bleach upon continuous excitation and this is visible as sudden drops in fluorescence intensity. Hence, the number of labels attached on a single molecule can be determined by counting the number of stepwise decreases in fluorescence as a function of time (38).

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customized software. Briefly, the beads were first automatically detected in both channels using an intensity threshold (Fig. 2, A and B, red circles) and the accurate position of these beads was determined by fitting the point spread function of the emitted fluorescence with a two-dimensional Gaussian function. Spots corresponding to the same bead were assigned by successively selecting spots (green numbered circles) in the DIOC (A) and Atto647N (B) channel. Ten beads were chosen randomly (A, DIOC channel; inset: magnified view; B, Atto647N channel) and an overlay of the positions of the selected beads was plotted to verify whether the beads were correctly selected in both channels (C), black points and red circles, respectively, depict the beads selected in the Atto647N and DIOC channels.

Identification of Platelet-decorated VWF Strings—Fluorescently labeled platelets were automatically detected using an intensity threshold. Due to differences in fluorescence intensity of labeled platelets this threshold was manually selected for each acquired movie. After smoothing of the images, platelets were automatically detected in all frames of a movie and their centroid was determined from the area of the images where the fluorescence signal was above the threshold. For visualization purposes, selected platelets were encircled (Fig. 3A, right panel, green circles). After automatic detection, manual frame-by-frame correction (up to 5000 frames per movie) was carried out. Non-detected platelets were added and background spots falsely detected as platelets were deleted (Fig. 3A, striped and full arrows, respectively). A single spot corresponds to a platelet if a fluorescent signal (with a size of ≈2 μm) is observed for multiple frames.
Observing movement of the platelets in flow is used as an additional criterion.

Identification of Enzymes—Analysis of enzyme movies was performed using a homemade routine for single molecule localization (33). After adequate image processing, points were automatically detected using an intensity threshold. The center of the spot was determined with nanometer accuracy (about 40 nm) by fitting the diffraction-limited point spread function with a two-dimensional Gaussian curve. For visualization purposes, detected enzymes were encircled in all frames of a movie (Fig. 3, B and C, right panel, green circles). After automatic detection, manual frame-by-frame correction (up to 5000 frames per movie) was performed. Non-detected points were added and enzymes passing through flow (visible as lines) and detected multiple times by the software program were deleted (Fig. 3, B and C, full and striped arrows, respectively). Finally, density plots of enzymes were constructed by adding up the number of enzymes located in each pixel of all corrected frames from an enzyme movie. This allows analyzing how many times a fluorescent spot (representative for an enzyme) is detected at a specific location during the entire movie. A spot is defined as a single enzyme if a fluorescent signal is observed and the fluorescence signal is visible as a diffraction-limited spot.

Correction for VWF String Motion—After rigorous frame-by-frame correction enzymes and platelet movies were overlaid (cf. overlay of the two channels). As platelet-decorated VWF strings exhibit movement in flow, a Matlab routine was developed to overlay images and simultaneously correct for string motion caused by hydrodynamic flow. This was done to guarantee that the observed movements of single ADAMTS13 enzymes over the VWF string are not a result of string motion. String motion was identified by calculating the difference in the position of the detected platelets (distances in number of pixels) in subsequent frames of the image sequence. The motion of the string was corrected by fixing the position of the platelets, i.e., the changes in the average position of the platelets was compensated by moving the image in the opposite direction in all images of both platelet and enzyme movies. For example, if the platelets in an image sequence moved in average three pixels up than all images are moved three pixels down to ensure that the position of the platelets remains the same. Importantly, only average string motion is accounted for and not stretching, which is observed as increases in interplatelet distances. This correction was performed in both the raw image data as well as for the ADAMTS13 trajectories generated with SPT software (see below).

Calculation of ADAMTS13 Trajectories—Enzyme trajectories were calculated, verified, and corrected using various custom-made Matlab routines. An enzyme trajectory is defined as an enzyme visible in 2 or more consecutive frames.
First, detected enzymes were automatically connected through their X-Y positions if spots were detected within a distance of 13 pixels (corresponds to about 1 μm). Resulting rADAMTS13E225Q trajectories could be observed in function of time. As enzymes can go out-of-focus for several frames due to string motion, and hence are not detected, a semiautomatic software routine was developed to connect enzymes that belong to the same trajectory but are not visible during some frames (Fig. 4, A and B). During the re-connection process, one-by-one visualization of the trajectories allowed us to link enzymes and their trajectories at the same location that disappeared for a few frames, whereas at the same time assuring that we did not wrongfully connect different enzymes that appeared within a distance of 5 pixels. The software program depicts the final frame of the trajectory where a fluorescent spot was detected and the next frame where a spot was detected again within a distance of 5 pixels (1 pixel = 80 nm) and a maximum of 100 frames between observations (frame rate 32 or 16 Hz). Enzymes were either reconnected or not upon inspection of the images (Fig. 4, A and B). To confirm the fact that different enzymes were observed, in case of doubt, the corresponding frames were verified in the original enzyme movie. Afterward, all trajectories were manually checked by overlapping the enzyme images on the string images and falsely connected trajectories were disconnected using a specifically designed Matlab routine. Generated trajectories were visualized and disconnected where necessary by scanning through enzyme trajectories frame-by-frame (Fig. 4C).

Movies were acquired with 30-ms integration time at 32 or 16 Hz, meaning samples were exposed to the laser every 30 ms and images were acquired either every 30 or 60 ms. Data analysis was performed by adjusting the frame rate to 60 ms (16 Hz) to allow comparisons between the behavior of rADAMTS13E225Q-Atto647N (movies acquired either with a rate of 32 or 16 Hz) and IgG-Atto647N (movies acquired with a rate of 16 Hz).

**Statistical Analysis**

Statistical analysis was performed using GraphPad software (GraphPad Prism 5, La Jolla, CA). Data were compared using the unpaired, two-tailed Student’s t test. p values lower than 0.05 were considered statistically significant. Values are represented as mean ± S.E.

**RESULTS**

Detection of Single Fluorescently Labeled rADAMTS13 Molecules under Static Conditions—Purified rADAMTS13 (data not shown) was labeled with the bright and photostable Atto647N dye to guarantee a strong fluorescent signal. Labeling rADAMTS13 with a 2-fold molar excess of Atto647N had no effect on rADAMTS13 functionality, as the activity of rADAMTS13-Atto647N did not differ from that of non-labeled
rADAMTS13 (101.7 ± 12.7% activity of WT rADAMTS13 (mean ± S.E.; n = 7)). A custom-made IX-71 inverted fluorescence microscope was built to realize single molecule detection of rADAMTS13 enzymes (Fig. 1). Briefly, the microscope consisted of a powerful 644-nm laser to excite labeled rADAMTS13 molecules, a highly sensitive EM-CCD camera, and a ×3.3 magnification before this camera. As VWF strings (μm scale) can easily be detected with low magnifications, we evaluated the minimal magnification required for enabling single rADAMTS13 molecule detection (Fig. 5, A–D). Using ×20 or 40 objectives, either bright spots (corresponding to rADAMTS13 aggregates, Fig. 5, A and B) or weak fluorescent spots were detected. These weak signals corresponded to single rADAMTS13 molecules but were too faint to guarantee efficient single molecule detection (Fig. 5 B). The intensity of fluorescent spots corresponding to single enzymes was higher when using either ×60 or 100 objectives (Fig. 5, C and D). The single molecule nature of the signals using ×60 objectives was verified by analyzing the intensity profiles of 127 randomly selected fluorescent spots (Fig. 5C, representative experiment). The fluorescent signal of the detected particles disappeared either in one (65%, Fig. 5E, left) or two steps (30%, Fig. 5E, right). Only 5% of the labeled rADAMTS13 showed a three-step decrease of the fluorescence intensity (data not shown). These data prove that single rADAMTS13 enzymes were detected and that most molecules carried one (65%) or two (30%) Atto647N dyes. Of note, the low concentration used (0.1 μg/ml) ensured that each diffraction-limited fluorescence signal corresponded to a single molecule. In control experiments, using non-coated coverslides or coated with Ab alone, no fluorescent signals were observed (data not shown). In conclusion, SMFM allowed visualization of single ADAMTS13-Atto647N enzymes under static conditions.

Visualization of Single Fluorescently Labeled rADAMTS13E225Q Molecules on Platelet-decorated VWF Strings—To investigate the binding of ADAMTS13 to platelet-decorated VWF strings, a flow assay was used where BOECs were stimulated with histamine and perfused with DIOC-labeled washed platelets (green fluorescence) and Atto-647N labeled rADAMTS13 (red fluorescence). To study the mechanism of binding in the absence of proteolysis, we used the rADAMTS13E225Q mutant. This catalytically inactive ADAMTS13 variant has been shown to bind native, folded, and full-length (sheared) VWF with the same affinity as WT ADAMTS13 suggesting that the mutation
has no effect on substrate binding (36). Using inactive rADAMTS13E225Q increases the chance of visualizing interactions between rADAMTS13 and VWF strings in the limited view field (41 by 41 μm) and within the time resolution of the EM-CCD. rADAMTS13E225Q was produced, purified, and labeled as active rADAMTS13 (data not shown). Because SMFM on living cells under flow conditions is technically more challenging due to the cells’ autofluorescence and the background arising from the labeled enzymes passing in solution, the microscope was modified. Background was reduced by performing illumination in a quasi-total internal reflection fluorescence mode. In addition, a compromise was needed between high magnifications required for detection of single rADAMTS13 molecules (nm scale) and lower magnifications to guarantee correct VWF string identification (μm scale). The problem was solved using different magnifications by working with two EM-CCD cameras. The 3.3 magnification was only included before the camera detecting rADAMTS13-Atto647N (EM-CCD1) and not before the camera detecting DIOC-labeled platelets (EM-CCD2) (Fig. 1). In this way, while visualizing single enzyme molecules, it was possible to observe a wide section of the VWF string. A specific set of filters and (dichroic) mirrors was used to guide the emission of green fluorescent DIOC and far red fluorescent Atto647N light to their respective cameras (Fig. 1).

Ultimately, the customized SM microscope enabled simultaneous detection of single ADAMTS13 enzymes (Fig. 6A) and platelet-decorated VWF strings (Fig. 6B). Precise spatial overlay of image sequences acquired with the two synchronized cameras was guaranteed using a calibration sample containing TetraSpeck fluorescent microspheres (Fig. 2). Accurate superposition of enzyme and platelet images nicely showed that fluorescent spots corresponding to rADAMTS13 are observed on the platelet-decorated VWF strings (Fig. 6C and supplemental Movies S1 to S3). In total, 16 independent flow chamber assays were performed and the binding of rADAMTS13 to 31 different VWF strings was evaluated. No fluorescent spots were observed in controls with unlabeled rADAMTS13E225Q. In conclusion, nanometer scale detection of single inactive rADAMTS13 molecules on platelet-decorated VWF strings waving under flow was realized by customizing the SMFM set up.

**Distribution Analysis of Single rADAMTS13E225Q Molecules on VWF Strings**—After having developed our customized SMFM system for real-time visualization of the binding of single rADAMTS13E225Q molecules to platelet-decorated VWF strings we next wanted to analyze single ADAMTS13 interactions with the VWF string. Hence, Matlab routines were developed and rigorous manual frame-by-frame correction was performed to identify platelets and single enzymes (Fig. 3) in all frames of 16 acquired movies.

To analyze the distribution of all single molecules in the view field of one experiment, rADAMTS13E225Q density plots were generated by accumulating all frames of a movie. Such a density plot shows a heat map, which visualizes how many times a fluorescence signal (representative for an enzyme) is detected at a specific location (Fig. 7). Superposition of these density plots onto accumulated fluorescence images from the platelet channel then allows us to visualize the position of enzyme molecules on the VWF strings. Our data (Fig. 7, A–C) revealed that the majority of rADAMTS13E225Q molecules are distributed in the vicinity of a VWF string although some enzymes were also observed in areas surrounding the VWF strings. Interestingly, in two experiments, enzymes were detected along a line not coinciding with a platelet-decorated VWF string (Fig. 7A). When observing some of these enzymes in real time, it was clear that they waved in flow suggesting that these enzymes co-localized with a moving platelet-free VWF string (supplemental Movie S4). Further inspection of these density plots showed that the bulk of observed rADAMTS13E225Q molecules only appeared once in a single frame (less than 60 ms) at a specific location (dark blue pixels, Fig. 7, A–C). These dark blue colored pixels can represent either different enzymes that each could be detected for only one frame or they could arise from one
enzyme that is detected at different locations over time (but only one frame per location). At a limited number of sites, fluorescent spots could be detected at the same location in multiple frames, consecutive and non-consecutive, (Fig. 7, A–C, green and red pixels). This phenomenon represents either a single enzyme that remained attached to that site for multiple frames or multiple enzymes that were consecutively detected at the same location but at different time points. In conclusion, density plots show that the majority of detected enzymes coincide with the VWF strings and that at certain locations multiple fluorescence signals, representative for enzymes, were detected.

SPT of rADAMTS13E225Q Molecules on VWF Strings—To be able to track the path of the molecules diffusing over VWF strings, dedicated SPT software was developed. rADAMTS13E225Q trajectories were calculated using a SPT routine in Matlab and simultaneously adjusted for VWF string motion by an additional routine. Finally, enzyme trajectories were verified and manually corrected where needed (Fig. 4). Enzymes visible in 2 or more consecutive frames were considered to follow a trajectory.

Comprehensive analysis of all 6,173 enzymes on the 31 analyzed VWF strings and the surrounding area demonstrated that we could measure a trajectory in 20% of the detected rADAMTS13E225Q molecules (1,240 enzymes, Fig. 8, A and B). Trajectories were also observed for strings with no platelets attached (data not shown) and occasionally (<1%) outside the area containing a VWF string. We distinguished two main types of trajectories: In total one-third of enzymes that showed a trajectory slowed down along the VWF string without stopping completely (Fig. 8A and 9A). rADAMTS13E225Q trajectories in this group undergo one-dimensional motion, visible here as a stripy appearance, due to deceleration of the enzymes as a result of collisions with the string. Trajectories where decelerated molecules appeared as a diffraction-limited spot in a single frame (less than 60 ms), possibly reflecting a brief interaction.

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Visualizing Single ADAMTS13 Enzymes on VWF Strings

FIGURE 7. The majority of rADAMTS13E225Q molecules are detected along VWF strings. A–C, density plots of labeled rADAMTS13E225Q-Atto647N are generated by the accumulation of all frames of a movie allowing visualization of how many times a fluorescent spot, representative for an enzyme, is detected in each pixel (1 pixel = 80 × 80 nm). The images represented here correspond to overlays of all accumulated frames of the platelet acquiring channel (gray) and enzyme acquiring channel (colored pixels) of three independent experiments. The color bar at the right indicates how many times an enzyme is detected per pixel (80 nm). Fluorescent spots found in a single frame at a certain location are depicted in dark blue. Detection of multiple fluorescent spots at the same pixel is depicted by colors shifted more toward red. Red corresponds to the observation of 10 spots or more at that specific pixel in several frames. The scale bars correspond to 5 μm. A part of platelet-decorated VWF strings in C (white box) was enlarged (bottom images) to better observe red and green pixels. The scale bars correspond to 1 μm. These images are representative for all VWF strings analyzed (n = 31) and show platelet-decorated VWF strings (A–C), a platelet-free VWF string (A, left), and parts of the view field where no strings were observed.

FIGURE 8. rADAMTS13E225Q follows distinct trajectories. A and B, images represent the accumulated constructed trajectories from a representative movie. The movie with the platelet-decorated VWF string analyzed here is the same as the one analyzed in Fig. 7C. Enzymes followed 2 types of trajectories: they either slowed down against the platelet-decorated VWF string in multiple consecutive frames (A) or interacted with the VWF strings for two frames (120 ms) or more (B). The majority of the detected enzymes did not follow a trajectory and were only visible in a single frame (less than 60 ms) (C). Each molecule and/or trajectory is represented in a different random color. The white boxes in B are the same as in Fig. 7C and show that rADAMTS13E225Q present in the green and red pixels can correspond both to multiple molecules binding to the same site or enzymes staying for a longer period of time to one specific location. The scale bars correspond to 5 μm. D, the different types of trajectories were counted for each individual string and the surrounding area for both rADAMTS13E225Q-Atto647N (black bars) and IgG-Atto647N (white bars) molecules. The percentages of trajectories versus all detected fluorescent molecules are depicted here for rADAMTS13E225Q and IgG controls. Values represent mean ± S.E. (rADAMTS13E225Q n = 31; IgG n = 7). Statistical analysis was performed using an unpaired t test with GraphPad Prism software. *p values: *p < 0.05.
with the VWF strings, were also included in this category (Fig. 9A, supplemental Movie S5). Upon repeating the same experiment with Atto647N-labeled IgG control instead of ADAMTS13, a similar percentage of IgG molecules showed this type of trajectory (Figs. 8D and 10A). In the second type of rADAMTS13E225Q trajectories, molecules arrived, readily bound and stayed attached to the string for two frames (120 ms) or more (two-thirds of the enzymes that described a trajectory) (Fig. 8B). In this group, the detachment of rADAMTS13E225Q from the string after a short interaction time (2 to 5 frames, corresponding to 120 to 500 ms) could sometimes be observed with molecules leaving the string displaying the aforementioned typical one-dimensional motion (Fig. 9B and supplemental Movie S6). For molecules that remained attached for longer periods of time (up to seconds; Fig. 9C and supplemental Movie S7) it was not always clear whether rADAMTS13E225Q detached or whether the fluorescent signal disappeared due to bleaching of the dye. These trajectories represented a specific interaction of rADAMTS13E225Q with VWF as a significantly higher percentage of molecules (relative to all detected enzymes) arrested for at least 120 ms when compared with control IgG-Atto647N (Figs. 9D and 10B).

SPT further allowed determining whether the multiple fluorescent spots detected at one location (green to red pixels, Fig. 7) corresponded to one or more rADAMTS13E225Q molecules. Inspection of the green and red pixels depicted in the density plots (Fig. 7C, delineated by white boxes) showed that these sites corresponded both to multiple molecules that bind to nearby locations during the data acquisition as well as to a single molecule that remains attached for a longer time period (compare white boxes in Figs. 7C and 8B). The number of rADAMTS13E225Q molecules that remained attached for at least 120 ms ranged from 4 to 90 per string (median of 18, 41 ± 41 μm view field) reflecting the presence of multiple ADAMTS13 binding sites per string.

The majority of the detected rADAMTS13E225Q molecules in a view field (80% of the enzymes corresponding to 4,943
enzymes) were only visible in one frame (Fig. 8C). These enzymes were observed either as stripes or as diffraction-limited spots. Also the majority of all detected IgGs (87.0% corresponding to 1001 molecules) did not follow a trajectory (Fig. 10C), suggesting that binding of molecules detected for the time of a single frame (less than 60 ms) probably represent nonspecific interactions (87.0 ± 2.8% for IgG-Atto647N and 76.6 ± 9.3% for rADAMTS13E225Q-Atto647N, mean ± S.E., p < 0.01) (Fig. 8D).

In conclusion, constructing trajectories allowed us to distinguish 2 different rADAMTS13E225Q binding modes: short and long term interactions. Only the trajectories where rADAMTS13E225Q molecules interact with the VWF string for 120 ms or longer were observed significantly more than for the IgG control and hence represented a specific interaction with the VWF string.

**DISCUSSION**

ADAMTS13 cleaves platelet-decorated VWF strings at a discrete number of sites (23). In this study, we aimed to unravel the mode of operation of the enzyme by visualizing binding of single rADAMTS13E225Q molecules to these strings using the powerful SMFM technique. SM studies have been performed extensively to track DNA-binding proteins on flow-stretched DNA (29–32). To the best of our knowledge, SMFM hitherto had not been implemented to study movement of ADAMTS13 on its flow-stretched VWF substrates. Hence, a customized SMF microscope was built to enable visualization of single inactive rADAMTS13E225Q molecules and SPT software was designed to analyze the trajectories described by the enzymes on mobile platelet-decorated VWF strings.

The SMF microscope, equipped with objectives realizing high magnifications and powerful lasers, was customized using quasi-total internal reflection fluorescence illumination, to reduce background signals from cells and fluorescent particles passing in solution, and including two synchronized highly sensitive EM-CCDs, for simultaneous detection of small enzymes (nm) and long platelet-decorated VWF strings (μm). These adaptations to the SMF microscope enabled us, for the first time, to visualize the binding of single rADAMTS13E225Q molecules to VWF strings in real-time.

SPT analysis identified 2 classes of rADAMTS13E225Q trajectories: enzymes either slowed down along the VWF string (Fig. 11A) or they readily bound to the string and remained there for at least 120 ms (Fig. 11B). Enzymes in the first category were sometimes observed to briefly (less than 60 ms) interact with the VWF string (Fig. 11A, blue dot). Slowing down of the enzymes likely is due to a nonspecific interaction of the enzyme with the string, as a similar percentage of these events was detected in control experiments using an IgG protein similar in size. Due to changes in the flow profile, brief collisions and/or weak interactions with the VWF string, any molecule in solution can slow down in the vicinity of the VWF string. The longer lasting binding, however, represented specific interactions of rADAMTS13E225Q with the VWF string as this was observed significantly more than for the control. Our results show that ADAMTS13 readily interacts with the VWF strings indicating that ADAMTS13 molecules find a binding site quickly after interaction with available sites on the VWF string.

Performing experiments with catalytically inactive rADAMTS13E225Q allowed us to obtain insight into the location of sites on the VWF string that are sufficiently unfolded to allow binding of ADAMTS13. Multiple interaction sites were identified that appeared scattered along the sections of the VWF string (present in our limited view field) demonstrating that many binding sites for rADAMTS13E225Q are accessible in these VWF strings and this under relevant physiological shear conditions and ADAMTS13 concentrations. ADAMTS13 binding appears to be a random process where ADAMTS13 collides with the VWF string and readily binds to one of many available sites.

The number of ADAMTS13 binding sites observed here (median of 18), however, is considerably higher than the limited number of cleavage events (median of 3) observed in platelet-decorated VWF strings ranging in size between 100 and 500 μm (23). The availability of many ADAMTS13 binding sites increases the chance that ADAMTS13 finds a cleavage site and hence can ensure efficient proteolysis of prothrombotic UL-VWF multimers. As the number of enzyme binding sites on the VWF string exceeds the number of sites that are cleaved, binding will probably not be the rate-limiting step in this process. Rather complete unfolding of cleavage sites in the platelet-decorated VWF strings or local elongations are important to enable ADAMTS13 proteolysis. Of note, binding appeared to occur independent of the presence of platelets as enzymes were
also observed on platelet-free VWF strings. This is in agreement with the observation that rADAMTS13 cleaves platelet-free and platelet-decorated VWF strings at a similar rate by rADAMTS13 (6).

This SMFM study allowed us to unravel the ADAMTS13’ mode of operation by following trajectories of inactive rADAMTS13E225Q molecules and visualizing binding sites on platelet-decorated VWF strings. Performing experiments with active ADAMTS13 enzyme would shed light on the functional importance of the specific interactions. For example, this would allow us to determine how many of these interactions eventually will lead to VWF proteolysis. As previous studies demonstrated that ADAMTS13 could bind VWF in its globular, native conformation without subsequent cleavage (36), binding of ADAMTS13 to parts of the VWF strings that are still partially folded would not result in cleavage. In line with this, one might expect that not all binding events observed on VWF strings would result in proteolysis. Furthermore, this system could be used to explore the interaction mechanism between ADAMST13 and VWF. Potential uses include studying ADAMTS13 mutants to increase the insight into the role of C-terminal domains, determining the ADAMTS13’ apparent association constant, and visualizing the link between local elongations and the subsequent exposure of multiple ADAMTS13 cleavage sites.

In conclusion, we developed a customized SMFM and imaging software to evaluate the binding mechanism of rADAMTS13 molecules on their long, mobile platelet-decorated VWF substrates. Detailed analysis suggests that rADAMTS13E225Q randomly interacts with the string and readily binds to one of the (many) available sites allowing efficient proteolysis of endothelium-anchored VWF strings.

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