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Stretching single fibrin fibers hampers their lysis

Wei Li [a], Tomas Lucioni [a], Rongzhong Li [a,b], Keith Bonin [a], Samuel S. Cho [a,b], Martin Guthold [a,*]

[a] Department of Physics, Wake Forest University, Winston-Salem, NC 27109, United States
[b] Department of Computer Science, Wake Forest University, Winston-Salem, NC 27109, United States

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ABSTRACT

Blood clots, whose main structural component is a mesh of microscopic fibrin fibers, experience mechanical strain from blood flow, clot retraction and interactions with platelets and other cells. We developed a transparent, striated and highly stretchable substrate made from fugitive glue (a styrenic block copolymer) to investigate how mechanical strain affects lysis of single, suspended fibrin fibers. In this suspended fiber assay, lysis manifested itself by fiber elongation, thickening (disassembly), fraying and collapse. Stretching single fibrin fibers significantly hampered their lysis. This effect was seen in uncrosslinked and crosslinked fibers. Crosslinking (without stretching) also hampered single fiber lysis.

Our data suggest that strain is a novel mechanosensitive factor that regulates blood clot dissolution (fibrinolysis) at the single fiber level. At the molecular level of single fibrin molecules, strain may distort, or hinder access to, plasmin cleavage sites and thereby hamper lysis.

Statement of significance

Fibrin fibers are the major structural component of a blood clot. We developed a highly stretchable substrate made from fugitive glue and a suspended fibrin fiber lysis assay to investigate the effect of stretching on single fibrin fibers lysis. The key findings from our experiments are: 1) Fibers thicken and elongate upon lysis; 2) stretching strongly reduces lysis; 3) this effect is more pronounced for uncrosslinked fibers; and 4) stretching fibers has a similar effect on reducing lysis as crosslinking fibers. At the molecular level, strain may distort plasmin cleavage sites, or restrict access to those sites. Our results suggest that strain may be a novel mechanobiological factor that regulates fibrinolysis.

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1. Introduction

Blood coagulation and fibrinolysis are antagonistic systems that control blood clot formation and blood clot dissolution. Proper functioning of both systems is critical for normal blood flow and an appropriate response to injuries. Disturbances to either system may result in excessive bleeding or thrombotic disease, such as myocardial infarction, stroke, and embolisms [1,2]. Activation and suppression of both systems are tightly controlled by numerous, sometimes interconnected, factors [2–4].

1.1. Coagulation and fibrin fiber formation

In case of trauma or injury to blood vessels, the coagulation cascade is activated and a blood clot is formed at the injury site in the blood vessel [5,6]. The final step in the coagulation cascade is the proteolytic cleavage of fibinopeptides A and B from fibrinogen by thrombin, thus converting fibrinogen to fibrin. Fibrin then polymerizes into half-staggered, double-stranded protofibrils, mainly via A:a knob-hole and D:D interface interactions [7]. Protofibrils then aggregate into a mesh of about 130 nm thick fibrin fibers (Fig. 1). This mesh of fibrin fibers constitutes the major structural component of a blood clot (Fig. 1D). A single fibrin fiber cross-section contains on the order of a hundred protofibrils. Although the interactions between protofibrils are not fully understood, there is good evidence that the protofibrils are connected by a network of flexible, interacting αC regions (Fig. 1B and C) [8–11]. Factor XIII fortifies fibrin fibers by catalyzing covalent bond formation (crosslinks) between specific glutamines and lysines in interacting fibrin molecules [12,13]. These crosslinks increase the stiffness of a clot and the stiffness of single fibers [14,15], and crosslinks make a clot more resistant to lysis [12,16].

1.2. Fibrinolysis

The fibrinolytic system breaks down blood clots to prevent permanent occlusion of blood vessels. It is controlled by numerous

* Corresponding author.
E-mail address: gutholdm@wfu.edu (M. Guthold).

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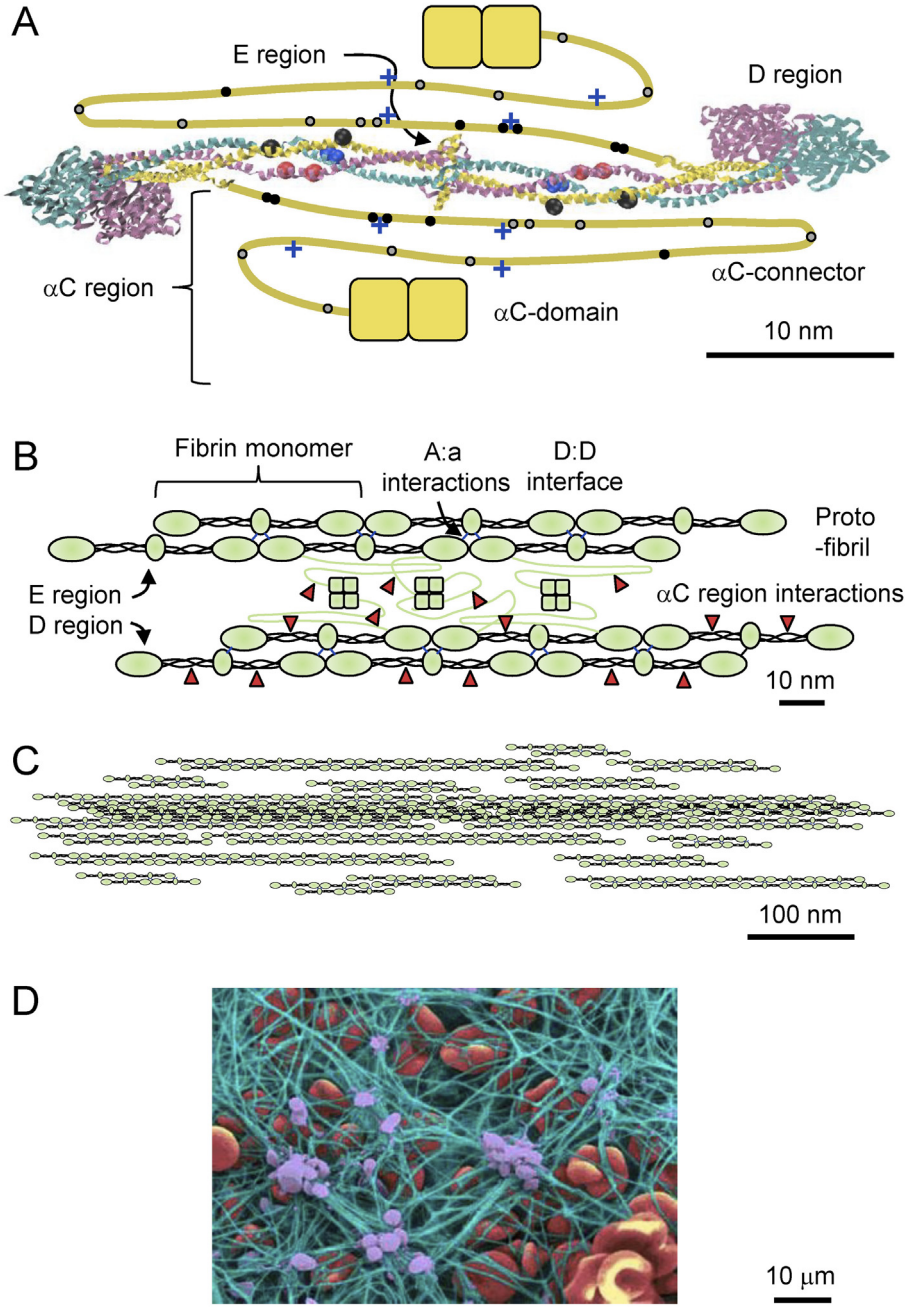

Fig. 1. Structure of human fibrinogen and plasmin cleavage sites (adapted from [53]). (A) Fibrinogen consists of six peptide chains; two Aα chains (yellow), two Bβ chains (magenta) and two γ chains (cyan). Fibrinogen resembles a double dumbbell with a central E region and two terminal D regions connected by triple helical coiled coils [54]. The α chain helix in the coiled coil folds back onto itself for about half the length of the coiled coil, and then transitions into the long, largely unstructured αC region. The αC region (α 221–610), which is drawn in by hand, consists of the unstructured, 61 nm long αC-connector (α 221–391; drawn as a yellow line) and the folded αC-domain (392–610; drawn as two yellow squares). The six plasmin cleavage sites in the coiled coil region at αLys 81, βLys122, γLys58, and αArg104, βLys133, γLys62 are indicated by spheres (α chain, black; β chain, red; γ chain, blue). The numerous possible plasmin cleavage sites in the αC-connector are indicated by black dots (lysine), or gray dots (arginine). The blue crosses indicate the four glutamines in the αC-connector that serve as donors in the α-α glutamine-lysine crosslinks. The lysine acceptors of the α-α crosslinks are in the αC-domain. (B) Schematic drawing of two double-stranded protofibrils that are connected by interactions of the αC regions as described in [8]. For clarity, not all αC regions on each fibrin molecule are shown. A typical, mature, 130 nm thick fibrin fiber contains about 100 protofibrils per cross-section connected by an extensive network of interacting αC regions. The positions of some of the plasmin cleavage sites shown in (A) are indicated by red triangles on the lower protofibril in (B). To completely sever an entire fibrin fiber, about 25% of the E-D connections (coiled coils) need to be broken and 50% of the fibrin monomers can remain intact [55]. (C) Longitudinal cross-section of a single fiber segment showing assembled protofibrils (network of αC-domains omitted for clarity). Fibrin fibers have a denser core and a less dense periphery [53]. (D) False color scanning electron micrograph showing a blood clot: fibrin fibers, green; platelets, purple; erythrocytes, red (image, courtesy of Y. Veklich & J. W. Weisel). Note different scale of scale bars in figures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chemical and enzymatic factors, including activators, inhibitors, cofactors, and receptors. Fibrinolysis is also influenced by clot architecture and fibrin fiber structure [3,4,17–19]. The key enzyme in fibrinolysis is plasmin, a serine protease that cleaves fibrin at multiple sites, thereby dissolving a clot (Fig. 1). Plasmin is the activated form of plasminogen; activation occurs when tissue plasminogen activator (tPA) cleaves plasminogen at the Arg561-Val562 peptide bond. This activation is significantly enhanced (by two to three orders of magnitude), when both plasminogen and tPA are bound to fibrin [3]. Activation is effectively inhibited by

plasminogen activator inhibitor-1 (PAI-1). In blood, any free plasmin is rapidly inactivated by α_2-antiplasmin. Several additional enzymatic activation and inhibition pathways in fibrinolysis exist as reviewed in [3,4].

Plasmin specifically cleaves on the carboxyl side of lysines or arginines. However, the sequence specificity of plasmin has not been fully established yet; in fact, plasmin seems rather promiscuous and there are many potential plasmin cleavage sites in fibrin [1,20–23]. Despite this multitude of possible sites, only a few major sites are initially cleaved in fibrin (Fig. 1). Specifically, the αC-domains are removed first. There are at least ten Lys or Arg peptide bonds in the αC region that are targeted, resulting in a heterogeneous set of early degradation products [3]. However, these initial cuts do not completely sever a fiber. Completely severing a fiber requires cleavage of all three peptide chains in the coiled-coil connector between the E and D regions within a fibrin monomer (cleavage at αLys 81, βLys122, γLys58, or αArg104, βLys133, γLys62, see Fig. 1). Moreover, both fibrin monomer strands in a protofibril and all protofibrils in a fiber need to be severed through the same cross-section (not at random points) for the whole fibrin fiber to break [3].

1.3. Some current open questions in fibrinolysis

The observation that there are many potential cleavage sites, but that only a few of those are initially cut, implies that there are factors, other than just primary amino acid sequence, that affect cleavage. In fact, it is still an open question which factors determine cleavage site selection. It is likely that cleavage is strongly affected by the spatial arrangement and accessibility of the substrate amino acids. Fibrinolysis is not only controlled by the enzymes directly involved in lysis, but also by additional factors, such as the network structure and composition of a clot. For example, high prothrombin levels increase thrombin and result in abnormal fibrin network generation, which resists fibrinolysis [24]. Clots with a dense network of thinner fibrin fibers lyse slower than coarse clots with thicker fibers [25], and lysis is delayed by clots containing DNA and histones [26]. Moreover, changes at the molecular level of fibrin can influence lysis, as crosslinking of fibrin fibers by factor XIII (FXIII) increases lysis time in both static and flow systems [16].

Furthermore, *force-induced, mechanical* modifications, often termed 'mechanobiological signaling', may be a factor that controls fibrinolysis. Recently, Varju et al. and Adhikari et al. found that strained fibrin clots lyse slower than clots under unstretched conditions [27,28]. This could be the reason why retracted clots are resistant to fibrinolysis [29,30]. This finding may imply that structural changes at the molecular level in the fibrin fibers prevent plasmin from cutting or being activated [27]. The notion that mechanical strain is utilized to affect lysis would make sense from a physiological point of view. Fibers are stretched when a clot retracts [29], when platelets interact with fibrin fibers [31], and when blood flow exerts mechanical forces on fibrin fibers [32,33] – and this strain may be a novel factor that regulates fibrinolysis [34].

1.4. Goal of current work

The overall goal of our work was to test if stretching *single* fibers has an effect on their lysis. An affirmative outcome would suggest that stretching may be a novel mechanobiological factor in clot lysis. We first performed two established assays (the colorimetric plasmin activation assay and the receding clot assay) to demonstrate that our plasmin preparations were fully active. Using our newly developed suspended fibrin fiber lysis assay [35], we then investigated the effect of stretching on the lysis of *single* fibrin fibers. In this assay, lysis manifests itself as fiber thickening (disassembly) and fraying, and significant fiber lengthening instead of as complete fiber severance. We found that stretching single fibrin fibers significantly hampers fibrin lysis – to a similar extent as FXIII crosslinking, which is a recognized clot stabilization factor. Thus, stretching fibrin fibers may be one of the key elements that protects fibers from lysis.

1.6. Clinical significance

Thrombosis is the most common underlying pathology of ischemic heart disease, stroke, and venous thromboembolism, with the first two diseases accounting for 25% of deaths worldwide [36]. Treatment options, such as anticoagulation, mechanical clot retrieval and thrombolysis aim to hamper clot formation, remove clots, or induce lysis. Given the continuing high morbidity and mortality of thrombotic diseases, it is clear that our understanding of thrombosis and treatment options needs to be improved. Slower lysis has been associated with poorer diagnoses in thrombotic disease and, thus, a better understanding of the factors that affect the lytic rate may ultimately also have clinical significance [1,4,37,38].

2. Materials and methods

2.1. Colorimetric plasmin activation assay

For the plasmin activation assay [39], 157.5 μl dimethyl sulfoxide (DMSO) was added to 5 mg N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (Sigma-Aldrich, St. Louis, MO) to get a 50 mM substrate solution. Plasmin was activated by mixing tPA (10 μg/ml) and plasminogen (3.28 mg/ml) at a volume ratio of 3:1 for time periods ranging from 0 to 50 min. At each time point, a 1 μl aliquot was mixed with 97 μl of assay buffer (0.1 M $NaPO_4$, pH 7.8, made from Na_2HPO_4 and NaH_2PO_4; Sigma-Aldrich, St. Louis, MO) and 2 μl of substrate solution, and the absorption at a wavelength of 410 nm was recorded as a function of time (time after plasminogen and tPA was mixed).

2.2. Plasmin activity assay (receding clot assay)

2.2.1. Fibrin clot formation

An 18 μl mixture was made from non-fluorescently labeled, purified fibrinogen (concentration 1.5 mg/ml) and Alexa 546 fluorophore-labeled fibrinogen (concentration 1.5 mg/ml) (Life Technologies Corporation, Carlsbad, CA) with a volume ratio of 3:1. For the crosslinked fibrin sample, 2 μl of a mixture of thrombin (final concentration 0.1 NIH units/ml) and FXIII (final concentration 9 Loewy units/ml, Enzyme Research Laboratories, South Bend, IN) were added into the 18 μl fibrinogen mixture. For the uncrosslinked fibrin sample, 2 μl of thrombin (final concentration 0.1 NIH units/ml) was added into the 18 μl fibrinogen mixture. The clot was formed on a microscope cover glass slide (No. 1.5, 24 mm × 60 mm, Fisherbrand, Pittsburgh, PA).

After a one hour incubation in a wet atmosphere at room temperature, a small square glass cover slip (No. 1, 18 mm × 18 mm, Fisher Scientific, Pittsburgh, PA) was placed on top of the clot, gently pressed down, and two layers of double-sided tape (Scotch, 3M Inc., St. Paul, MN) were used on two sides of the cover slip to attach the cover slip to the clot substrate. The clot was sandwiched between the clot substrate (the microscope cover glass slide) and the cover slip with the two layers of double-sided tape serving as a spacer. A 10 μl mixture of tPA (10 μg/ml) and plasminogen (3.28 mg/ml) was then injected between the glass cover slip and the clot substrate at one side of the sandwiched fibrin clot. Fluorescence images of the lysed fibrin clot were taken every 4 min at the

same place with a 10× lens. In this assay, lysis is manifested by a receding clot boundary as the clot is lysed.

Clot height is defined as the height from the bottom edge of each image to the lysis front, as shown in Fig. 4. The bottom edges are aligned, so lysis progress (the receding clot front) can be clearly seen. Clot height was measured from the images using ImageJ (http://imagej.nih.gov/ij/).

2.3. Preparation of highly Stretchable, striated substrate

A drop (~1 cm in diameter, ~2 mm in height) of hot fugitive glue, a styrenic block copolymer (Surebonder AT-10154 Hot Melt, Hotmelt.com, Edina, MN 55439, www.hotmelt.com), was deposited onto a microscope cover glass slide (No. 1.5, 24 mm × 60 mm, Fisherbrand, Pittsburgh, PA) from the Surebonder PRO100 Hot Melt Gun (Hotmelt.com, Edina, MN 55439, www.hotmelt.com). Immediately afterward, a rectangular PDMS (Polydimethylsiloxane) stamp with imprinted grooves and ridges was pressed into the glue, and let sit for 4 min to completely cool down before being peeled off. To stretch the fugitive glue substrate, it was peeled off the glass, stretched manually to the desired length, and then anchored again to the glass by wrapping its ends with an about 15 cm long, flexible adhesive string. The adhesive string was created by stretching Adhesive Squares (1/2 inch × 1/2 inch Adhesive Square™ RS Industrial, Inc., Buford, GA) to a 15 cm long string (Fig. 2). Additional details and tests of the biocompatibility of this substrate are described in [35]. This substrate allowed us to simultaneously stretch multiple, single fibers by the same amount. Using an inverted fluorescence microscope, we were able to image the suspended fibrin fibers during the suspended fiber lysis experiment. Upon addition of activated plasmin, we could observe what effect stretching had on lysis. Our set-up is a further development of a set-up by Bucay et al., which was used to investigate the lysis of single fibrin fibers that were suspended over fixed (not stretchable) microridges in a substrate [40]. A schematic and photographs of our stretchable, striated substrate are shown in Fig. 2.

2.4. Preparation of suspended fibrin fiber sample, and stretching of fibrin fibers

A 20 µl mixture of 18 µl purified fibrinogen (1 mg/ml, Enzyme Research Laboratories, South Bend, IN) and 2 µl of thrombin (final concentration: 0.1 NIH units/ml, Enzyme Research Laboratories, South Bend, IN) was placed into the unstretched fugitive substrate well and incubated in a wet atmosphere at room temperature for 1 h. After that, the substrate was rinsed with fibrin buffer (140 mM NaCl, 10 mM Hepes, 5 mM $CaCl_2$, pH 7.4), and then 2 µl 20 nm carboxyl coated fluorospheres (100 times dilution) (Invitrogen, Carlsbad, CA) were added to the solution. After 10 min of incubation, the sample was rinsed and kept in fibrin buffer. The fugitive substrate was then stretched to the desired length (the fibrin fibers were also stretched to the same extent). During this entire process, the fiber sample was kept under buffer.

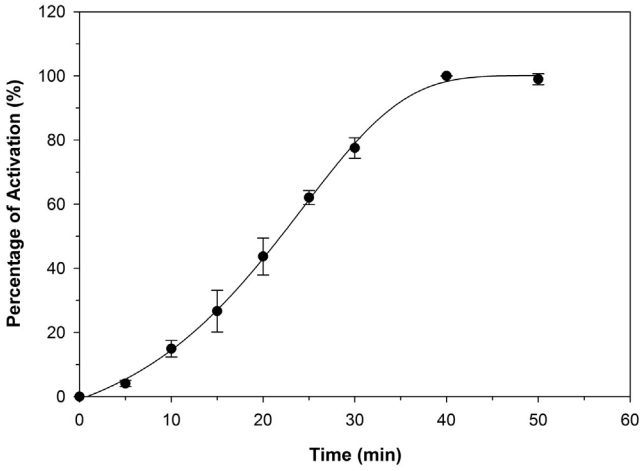

Fig. 3. Time-dependent activation of plasminogen. The activation rate of plasminogen by tPA was determined by using N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate as substrate. Full activation (100%) was achieved at 40 min, and the percentage of activation at other time points were normalized to the percentage at 40 min. All assays were performed in triplicates. Error bars are the standard error of the mean of the three samples.

The following definition of substrate stretching and fiber stretching were used. Substrate stretching (substrate strain) is defined as $\Delta L_S = ((L_{S,stretched} - 13.5\ \mu m)/13.5\ \mu m) \cdot 100\%$; where $L_{S,stretched}$ is the spacing between ridges after stretching and 13.5 µm is the spacing before stretching. The spacing between the ridges before stretching (13.5 µm) is predetermined by the PDMS stamp. The spacing between ridges after stretching was

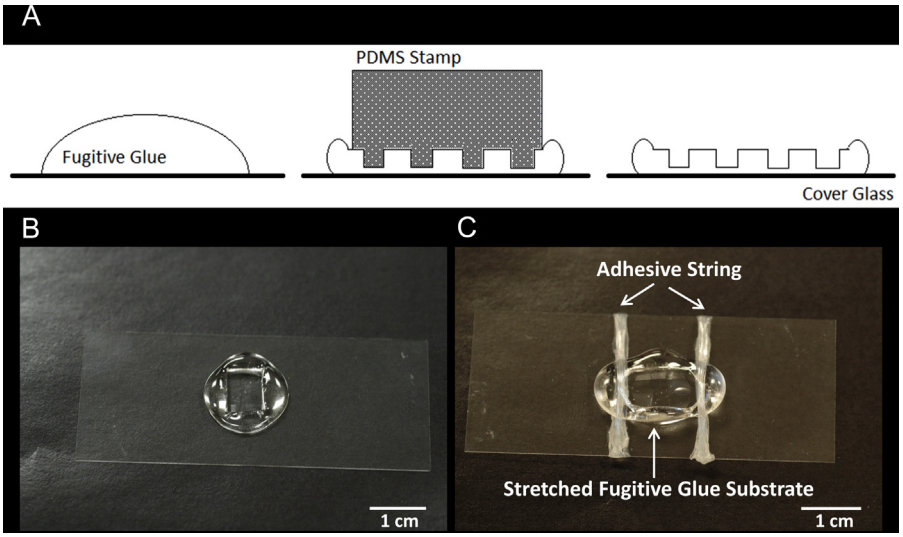

Fig. 2. Stretchable substrate. (A) Schematic of the process for making the substrate, side view. The ridges in the fugitive glue substrate are 6 µm high, 6.5 µm wide, and separated by 13.5 µm wide grooves [56]. Photographs of the unstretched substrate (B) and anchored, stretched substrate (C) on a glass cover glass. Fluorescence microscopy micrographs of fibrin fibers on a stretched substrate are shown in Fig. 5.

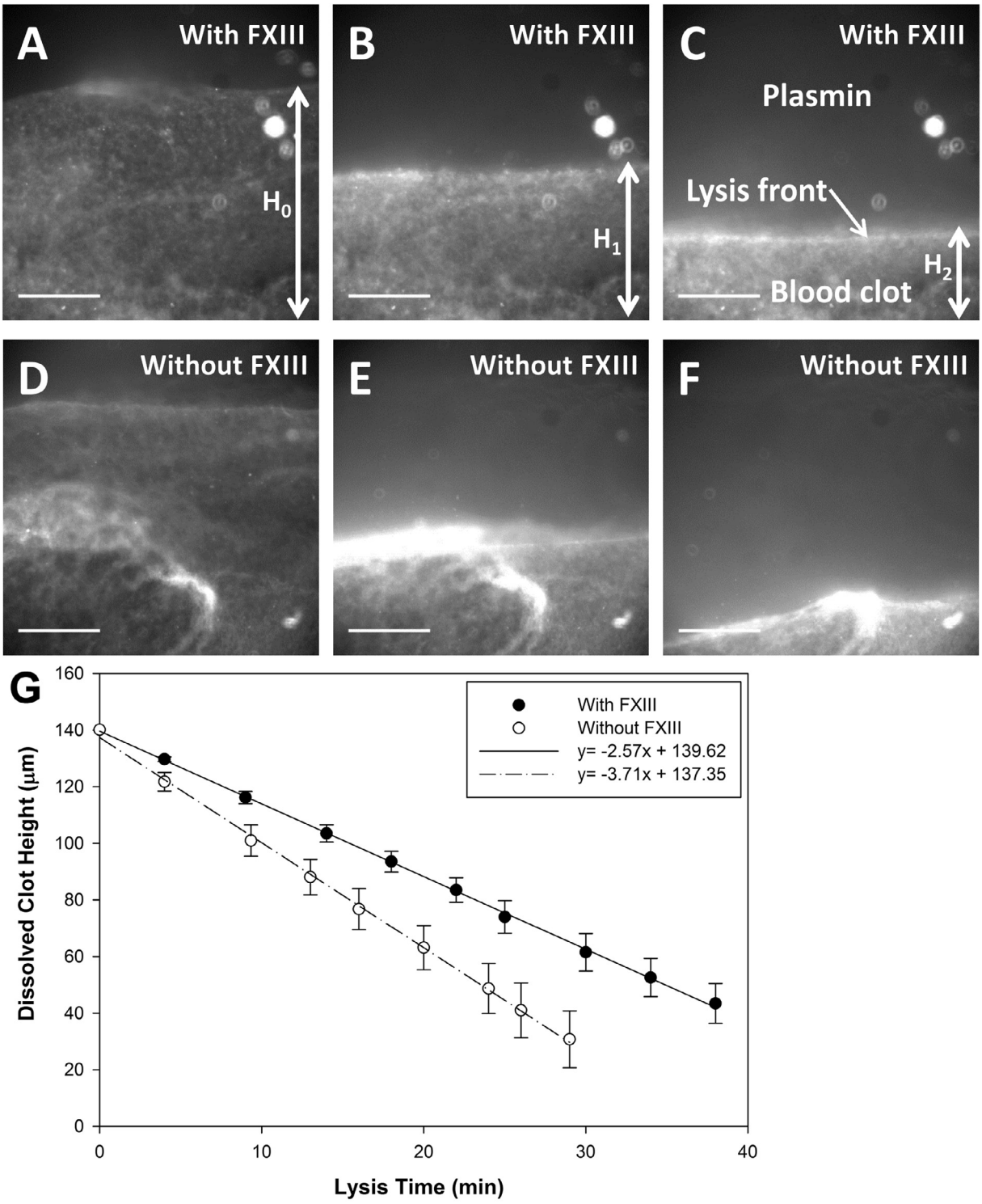

Fig. 4. Receding clot experiments. (A)–(C) Lysis of FXIII-crosslinked clot; (D)–(F) lysis of uncrosslinked clot. (A), (D) are before adding activated plasmin; (B), (E) are 14 min and (C), (F) are 28 min after adding activated plasmin. The arrows H_0, H_1 and H_2 in (A)–(C) indicate the height of the clot from the bottom of the image frame to the lysis front (top of the clot). The scale bar is 50 μm. The images were taken with a 10× lens. (G) Plot of clot height vs. time; the slope represents the lytic rate, which was 2.6 μm/min for crosslinked clots and 3.7 μm/min for uncrosslinked clots. Error bars are the standard error of the mean of the three samples.

determined from microscopy images (such as Fig. 5). The absolute error in these quantities is estimated to be less than 10% (due to well-calibrated microscopes), and the relative error between measurements is also estimated to be less than 10%.

We assumed fiber stretching to be the same as substrate stretching. Therefore, we made the following implicit assumptions: 1) Fibers are firmly anchored to the ridges. This is a good assumption because both fugitive glue and fibrin fibers are sticky, and we did not see fibers slip in any of the images. 2) Fibers run perpendicular to the ridge. We only analyzed fibers that ran mostly perpendicular across the ridges, allowing for a deviation of about 10° or less. This allowance introduces an error of less than 2%, as this error scales as $(1–1/\cos\theta)$, where θ is the deviation from 90° (perfectly perpendicular).

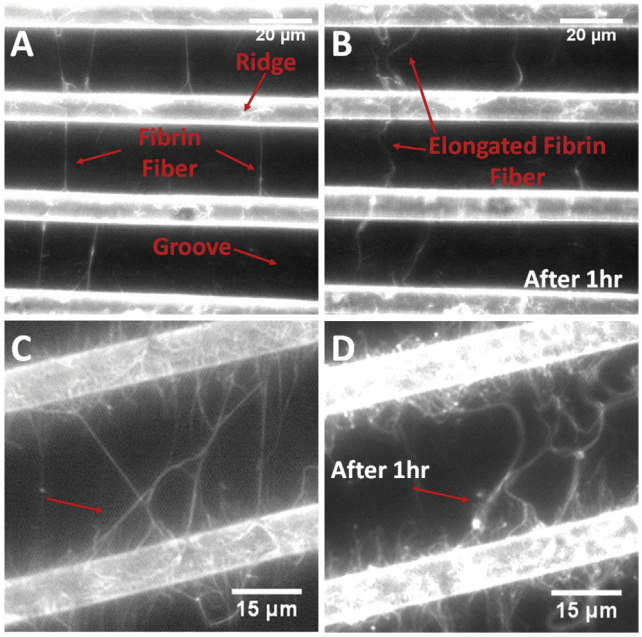

Fig. 5. Lengthening, thickening, and fraying of stretched single fibrin fibers upon lysis. In (A) and (B), the substrate was stretched to a strain of 56%; the width of the grooves is 21 μm. In (C) and (D), the substrate was stretched to a strain of 168%; the width of the grooves is 36.2 μm. In all images, the unstretched width was 13.5 μm. (A) Before adding plasmin, all fibers appeared straight, similar to suspended fibers seen in [14,41]. (B) One hour after adding plasmin, all previously straight fibers now appeared elongated and curvilinear; few fibers seemed to be completely transversely cut. (C) A small mesh of fibrin fibers before adding plasmin. (D) One hour after adding plasmin, fibrin fibers appear thicker and frayed (see arrow), in addition to being elongated.

The number of fibers that can be monitored in parallel (i.e., at the same time) depends on the stretching percentage of the fiber. Usually for unstretched fibers, there are around 50 fibers in a field of view, while for 250% strain, there are around 15 fibers in a field of view (the number of fibers in the field of view is reduced by a factor of 2.5, and fibers at the edges may not be fully visible). This number is further reduced by analyzing only fibers that run perpendicularly across the ridges.

2.5. Lysis of suspended fibrin fibers

5 μl recombinant human tPA (10 μg/ml, Aniara, West Chester, OH) and 5 μl plasminogen (3.28 mg/ml, Athens Research & Technology, Athens, GA) were mixed and incubated for 1 h before applying to the fibrin fibers. Fig. 3 shows that plasminogen is fully activated by tPA after this incubation. The 10 μl mixture was then applied to the fibrin fiber sample (tPA, final concentration of 0.48 μg/ml and plasminogen, final concentration of 0.16 mg/ml).

2.6. Inverted Fluorescence microscopy

Fluorescence images (for the purpose of visualizing the fluorescent fibrin fibers) were taken every 5 min at the same location with an inverted optical microscope system (Axiovert 200, Zeiss, Thornwood, NY, USA) in epifluorescence imaging mode, a Hamamatsu EM-CCD C9100 Camera (Hamamatsu Photonics KK, Japan) and IPLab software 3.6.5 (Scanalytics, Fairfax, VA). The sample was illuminated with a 100 W Hg lamp. Excitation light was first passed through a neutral density filter to reduce light intensity, and thus photo bleaching. A standard FITC filter cube (Chroma Technology Corp., Rockingham, VT) was used to filter excitation and emission light. Images were recorded with a Plan Neofluar 40× objective lens (N.A. 0.75) or 100× infinity-corrected Fluar oil immersion objective lens (N.A. 1.3).

2.7. Confocal microscopy

Fibrin fibers were formed on the unstretched substrate (Sections 2.1 and 2.2). Additionally, 200 nm carboxylate coated fluorospheres (100 times dilution) (Life Technologies Corporation, Carlsbad, CA) were added to the substrate, so that the top of the ridges and the bottom of the grooves could be distinguished. The sample was imaged with a Zeiss LSM 710 confocal microscope (Zeiss, Thornwood, NY, USA) one hour after adding the activated plasmin. The fibers were imaged with a 63× oil immersion lens and a series of vertical stack images (z-stacks) were collected every 0.5 μm over 13 μm (from below the bottom of the groove to above the top of the ridge).

2.8. Lengthening of fibrin fibers due to lysis and fibrin fiber length analysis

Since fibrin fiber lysis manifested itself as fiber lengthening, a custom-written Wolfram Mathematica 10 (Champaign, IL) program was used to accurately and unbiasedly determine fibrin fiber length before and after lysis. A detailed description of the fiber length determination is provided in the supplement. The absolute fiber elongation due to lysis is $\Delta L_{lysis,\ abs.} = L_{lysed} - L_{initial}$, where L_{lysed} is the fiber length after lysis, and $L_{initial}$ is the *stretched* fiber length before lysis. These lengths were determined by fitting a fourth order polynomial of the form $a + b{\cdot}x^{-1} + c{\cdot}x^{-0.5} + d{\cdot}x + e{\cdot}x^2 + f{\cdot}x^3 + g{\cdot}x^4$ to the fiber contour in our fluorescence microscopy images (for details, see supplement). We manually specified the starting and ending points in the image. The program then finds the center points along the fibers automatically and fits the 4th order polynomial to these points.

2.8.1. Justification for using 4th order polynomial

Our goal was to connect the existing fiber points with a smooth curve that mimics the path of the fiber. Typically, the higher order the polynomial, the smoother and the better the fit. However, polynomials of too high an order may introduce additional curves that are not real. We tested different order polynomials and a 4th order polynomial gave fits that mimicked the path of the fiber well without extraneous curves, as judged by visual inspection. We also tested the fit for different spline functions, some underestimating and some overestimating the length of the fiber. The length obtained from the fourth order polynomial fit was about the average of the various spline fits, and, thus, seemed like the most reasonable choice for fitting the fiber. For objectivity, we used this polynomial for all length measurements of unlysed fibers ($L_{initial}$) and lysed fibers (L_{lysed}).

Our measure of lysis is the percent elongation due to lysis, defined as $\Delta L_{lysis} = ((L_{lysed} - L_{initial})/L_{original}){\cdot}100\%$, as plotted in Fig. 8. Note, that in this definition the denominator, $L_{original}$, is the *original, unstretched* length of the fiber. An alternative definition could use the *stretched* length of the fiber, $L_{initial}$, in the denominator, so that $\Delta L_{lysis}' = ((L_{lysed} - L_{initial})/L_{initial}){\cdot}100\%$. We plotted this definition of lysis in Fig. S1 (supplement). It should be noted that in the latter definition, the percent elongation due to lysis decreases with increasing fiber stretching since $L_{initial}$ is in the denominator. Thus, the hampering effect on lysis due to stretching is more pronounced in the latter definition.

3. Results

We hypothesized that stretching single fibrin fibers would slow their lysis, implying that lysis may be controlled by mechanical

strain. To test this hypothesis, we first developed a transparent, grooved, slightly adhesive, and highly stretchable substrate, allowing us to simultaneously stretch multiple, single fibers by the same amount, lyse, and image them.

3.1. Plasmin activation

Before we investigated the lysis of suspended single fibers, we confirmed in a colorimetric assay that the used plasmin was fully activated by tPA (Fig. 3). In this assay, tPA and plasminogen were first mixed and added to a solution containing N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide, a chromogenic substrate for plasmin. Upon cleavage by plasmin, this substrate absorbs light at 410 nm. Fig. 3 shows that plasminogen was fully activated by tPA after 40 min. In all subsequent lysis experiments, we only used fully activated plasmin; that is, plasmin that was tPA-activated for at least 40 min (typically one hour).

3.2. Receding clot assay

In addition to the colorimetric plasmin activation assay, we also performed a receding clot assay to confirm normal plasmin activity in crosslinked and uncrosslinked clots (Fig. 4). Plasminogen was activated by tPA for 1 h. The activated plasminogen (plasmin) was then added to a clot that was formed between two glass cover slips. Plasmin digested the clot, as can be seen by the receding clot/plasmin interface (Fig. 4A–F). Clots that were crosslinked by FXIII were digested at a slower rate. The lytic rate for FXIII-crosslinked clots was 2.6 μm/min, whereas it was 3.7 μm/min for uncrosslinked clots (Fig. 4G). Thus, crosslinking slowed down the lytic rate by a factor of 1.4.

3.3. Lysis of single suspended stretched fibers

After having confirmed plasmin activity, we investigated whether stretching fibrin fibers has an effect on their lysis. We formed fibrin fibers on the stretchable, striated substrate (Fig. 2), and added activated plasmin for one hour. Before adding plasmin, we observed individual fibers suspended over the ridges in the substrate (Fig. 5A, C). Most fibers were straight, running horizontally across the groove, and looked like typical suspended fibrin fibers, as we have reported before [14,41]. After plasmin incubation for one hour, all fibrin fibers significantly lengthened (quantitative analysis, see Fig. 8) and they had a curvilinear appearance, but they were not completely severed (Fig. 5B, D). Additionally, fibrin fibers appeared thicker and frayed after lysis (Fig. 5D). Thus, in this suspended fiber assay, lysis was manifested as fiber elongation, thickening, and fraying, rather than a complete transverse cut (Fig. 5). This is somewhat unusual since fibers do get completely cut in whole clot assays. However, this lengthening has also been observed by Bucay et al. in their lysis assay of single suspended fibers [40], and will be discussed further in the Discussion section. Thickening (disassembly) and fraying of fibrin fibers upon lysis has also been observed by Weisel's group [25,42,43].

To get a quantitative measure of fiber thickening, we determined the thickness before and after lysis. Though, it should be noted that the fibers are close to the resolution limit of the microscope, and the fiber thickness is an estimate and not an accurate measure. We added figures in the supplement of representative fibers before and after lysis, and a plot of apparent fiber thickness before and after lysis, from which it is clear that the apparent thickness of the fibers increases. The apparent thickening could be due to the fibers actually being thicker due to fraying, or the fibers could be more mobile, or both. The following observations suggest that that actual fiber thickness increases. 1) If the apparent thickness increase would be exclusively due to more mobile fibers, fibers in a network or fibers close to the ridge should be less thick since they would be less mobile (being confined in a network or attached to a ridge). However, this is not what is seen in the images (see supplement). Fibers appear thicker regardless of whether they are part of a network, by themselves in the middle of the groove or close to the ridge. 2) If fibers were mobile, the images should appear blurry. However, we see thicker fibers with clearly focused central fibrils and a fuzzy periphery (see supplement).

In addition to the quantitative experiments using plasminogen activated by tPA that are described in detail here, we also performed qualitative lysis experiments with plasminogen that was activated by uPA and by streptokinase, and we tested commercially available (already activated) plasmin (conditions, see supplement). All of these enzymes gave similar qualitative results; that is, we saw elongation of fibrin fibers upon lysis, and few discernable transverse cuts.

3.3.1. Z-stack images of lysed fibers

Z-stack images from a confocal microscope showed that, upon lysis, some fibrin fibers drop to the bottom of the groove, and remain there (Fig. 6). Previous inverted microscopy and AFM manipulation experiments show that *prior* to lysis fibrin fibers run horizontally across the grooves without dropping down [14,41]. Therefore, a possible sequence of events upon lysis is that fibers get weakened and elongated, causing them to drop to the bottom of the groove. Since fibrin is sticky, they likely get stuck there. Although lysis may still proceed, it is not noticeable any longer since excised pieces will remain stuck to the bottom of the groove.

3.3.2. Fiber elongation (lysis) vs. stretching

Apparent fiber elongation was, therefore, used as our measure of lysis; all unlysed fibers appear straight, whereas lysed fibers appear curvilinear and elongated. To quantify fiber lysis, we simply determined fiber elongation due to lysis. We used a custom-written Mathematica program to fit a 4th order polynomial to the unlysed and lysed (elongated) fiber to accurately and unbiasedly determine their lengths, $L_{initial}$, and L_{lysed} (Fig. 7). The percent elongation, and our measure of lysis, is $\Delta L_{lysis} = ((L_{lysed} - L_{initial})/L_{original}) \cdot 100\%$.

We determined this measure of lysis, ΔL_{lysis} (fiber elongation upon lysis normalized (divided) by the original length), as a function of fiber stretching. Fiber stretching (substrate stretching) ranged from 0% (unstretched fibers) to 250%. For each stretching percentage, we determined ΔL_{lysis} of numerous fibers, resulting in a distribution of ΔL_{lysis} values. In Fig. 8 we plotted the peak values of the ΔL_{lysis} distributions vs. fiber stretching.

Four main conclusions can be drawn from these plots. 1) Lysis (ΔL_{lysis}) decreases with fiber stretching for both uncrosslinked

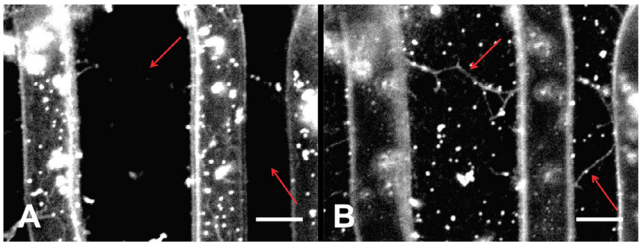

Fig. 6. Confocal microscopy images of fibrin fibers after one hour of lysis. Images of the same fiber were taken with the focal plane at the top of the ridges (A), and with the focal plane at the bottom of the grooves (B). The images were taken with a 63× oil immersion lens. The red arrows indicate the position of two fibers; the fibers are only visible when the focal plane is at the same level as the bottom of the grooves. Scale bar is 5 μm.

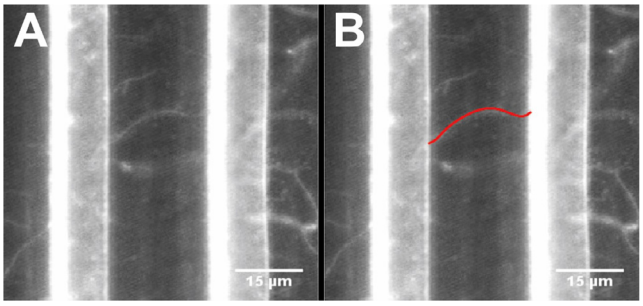

Fig. 7. Fitting of polynomial to lysed (lengthened) fiber. The width of the groove in this image is 17 μm. (A) One hour after fiber lysis. (B) Fitting of polynomial (red line) by the custom-written Wolfram Mathematica program. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

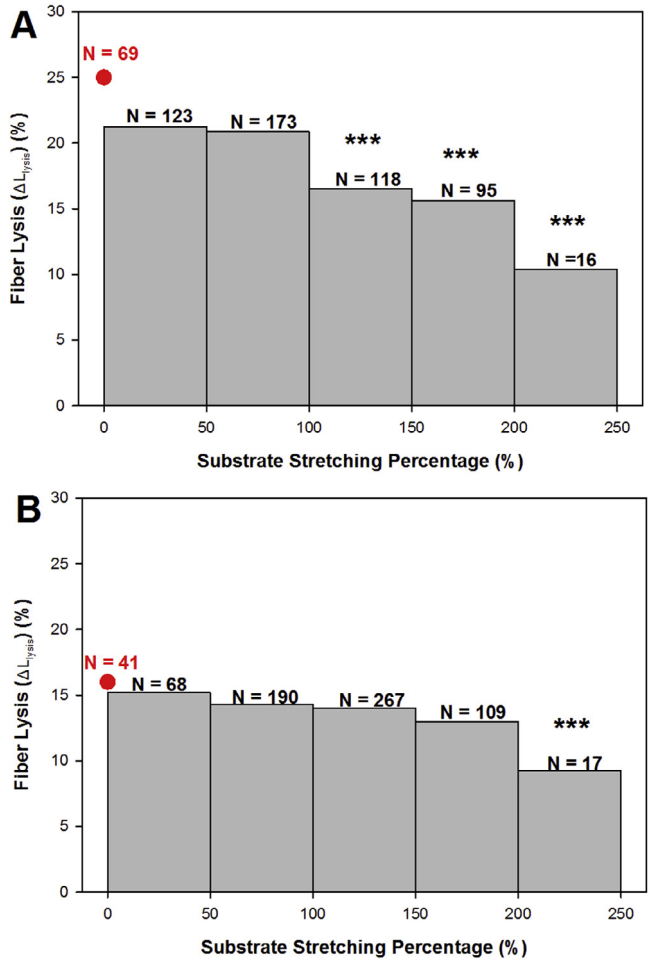

Fig. 8. Relationship between fiber lysis (as determined by fiber elongation, ΔL_{lysis}) and substrate stretching (fiber stretching). (A) Uncrosslinked fibrin fibers (without FXIII), (B) FXIII-crosslinked fibers. The red dot in each plot is the unstretched state (0% stretching). N represents the number of fibers included in each bar of the histogram. In all fibers, lysis manifests itself as fiber elongation, $\Delta L_{lysis} = ((L_{lysed} - L_{initial})/L_{original})\cdot 100\%$. ΔL_{lysis} decreases as substrate stretching increases. Fiber strain is assumed to be the same as substrate strain. Statistical significance was tested by comparing lysis for each stretched fiber data point (each column) with lysis of the unstretched fibers (red dot); * indicates $p < 0.05$, and *** indicates $p < 0.01$ in a *t*-test. (An alternative definition of lysis, $\Delta L_{lysis}' = ((L_{lysed} - L_{initial})/L_{initial})\cdot 100\%$, is plotted in Fig. S1 (supplement)).

fibers (Fig. 8A) and FXIII-crosslinked fibers (Fig. 8B). 2) This effect is more pronounced for uncrosslinked fibers. Upon stretching fibrin fibers 200% - 250%, lysis was decreased by a factor of 2.5 (from 25% to 15%) for uncrosslinked fibers, and by a factor of 1.7 for crosslinked fibers (from 16% to 9%). 3) Crosslinking also reduces lysis significantly. Going from uncrosslinked fibers to crosslinked fibers – comparing the unstretched data points (0% stretch on x-axis in Fig. 8A and B) – crosslinking reduces lysis by a factor of 1.6 (from 25% to 16%). It is noteworthy that this factor is similar to the factor of 1.4 by which crosslinking slows down the lytic rate in the receding clot assay (Fig. 4). 4) Stretching fibers by large amounts (200% - 250%) hampers fiber lysis more than crosslinking.

The fiber length in Fig. 8 was determined from looking at inverted microscopy images (not from confocal microscopy images). This means that we used the 2D projection of the three-dimensional path of the fiber for our length analysis. This does introduce a systematic error since the lysed, curvilinear fibers are longer in reality than our measurement. In essence, we are systematically underestimating our measure of lysis, $\Delta L_{lysis} = ((L_{lysed} - L_{initial})/L_{original})\cdot 100\%$. Adjusting Fig. 8 for this error would merely shift up the plot of ΔL_{lysis} vs. fiber stretching along the ΔL_{lysis} axis. However, this should not affect the four conclusions from this plot mentioned above.

In an alternative definition of lysis, the original length of the fiber (before stretching) could be used in the denominator, so lysis would be defined as $\Delta L_{lysis}' = ((L_{lysed} - L_{initial})/L_{initial})\cdot 100\%$, where $L_{initial}$ is the stretched length of the fiber before lysis. Supplementary Fig. S1 shows a plot of $\Delta L_{lysis}'$ vs. fiber stretching. Using this alternate definition, the hampering effect of stretching on lysis is more pronounced.

4. Discussion

4.1. Stretchable substrate and suspended fibrin fiber assay

We fabricated a highly stretchable, striated, and transparent substrate to investigate the effect of stretching on single fibrin fiber lysis. The stretchable substrate had the advantages that tens of individual fibers could be investigated simultaneously, and that the amount of stretching could be relatively easily varied and controlled. Studying single fibers, rather than a 'bulk clot', is significant, because it eliminates the effects of clot network structure and architecture. A caveat of our suspended fiber assay is that lysis has to be defined as fiber elongation, ΔL_{lysis}, rather than fiber severance, as is typically observed in whole clots [25,43]. An elongated fiber that often collapsed to the bottom of the grooves was the final state, not an intermediate state, in our experiments. We tested various combinations of different concentrations of tPA with plasminogen, uPA with plasminogen, streptokinase with plasminogen and already activated plasmin, as well as different concentrations of thrombin (see table S1 in supplement). Furthermore, we tried longer plasmin incubation periods (overnight). All of these experiments gave the same qualitative result – elongated, rather than severed fibers. We showed by confocal microscopy that most lysed fibers drop to the bottom of the groove. Lysis may stop once a fiber is attached to a surface, or, if lysis continues, the resulting fiber fragments may stick to the surface and appear like a whole (elongated) fiber. This fiber elongation upon lysis was also observed in Bucay's suspended fibrin fiber assay; though, this group also observed severed fibers. A possible explanation might be that their grooves were deeper, so some elongated fibers did not reach and stick to the bottom of their grooves [40].

It could also be that not all peptide bonds required for complete fiber severance are cleaved in our assay. The two major general protein areas that are cleaved by plasmin are 1) the αC region, and 2) the coiled coils [3]. We believe that the cleavages in the αC region can still be made, since this region is more accessible in all current models of fibrin fibers. In fact, since the αC region

is thought to be important for lateral fiber assembly [10,44,45], severing some of the αC regions may result in elongated, but not completely severed fibers, as was seen in our assay. We furthermore observed that lysed fibers are thicker (Fig. 5). Similar observations have been made by Weisel's group [25,42,43]. This thickening indicates a disassembly of fibrin fibers, which may result from a cleavage of the network of αC regions between protofibrils. Complete severance of the coiled coils may be hampered in our assay. It could be that fibrin fibers might be pre-strained as they are suspended over the ridges, and this may possibly limit access of plasmin to all cleavage sites, or it may distort plasmin binding sites.

4.2. Molecular mechanism

Fibrin fibers are highly extensible as they can be strained 150% (crosslinked fibers) and 230% (uncrosslinked fibers) before breaking. They are also highly elastic, as they can be strained up to 50% (crosslinked fibers) and 120% (uncrosslinked fibers) before they incur permanent damage [14,41]. The molecular mechanisms for the high extensibility and elasticity are still not fully understood, but Fourier transform infrared spectroscopy (FTIR) experiments and molecular dynamics (MD) simulations suggest that protein unfolding, such as the conversion of the α-helical coiled coils into β-strands may play a role [46,47]. Since we used activated plasmin, the activation binding sites on fibrin for tPA and plasminogen should only play a minor role in explaining our data. Rather, it is likely that stretching limits access to or distorts plasmin cleavage sites. There are several lines of evidence from computational modeling suggesting that some of the plasmin cleavage sites in the coiled coils (αLys 81, βLys122, γLys58, or αArg104, βLys133, γLys62) unfold during stretching. Zhmurov et al. found that force-induced molecular elongation is followed by unfolding of the α-helical coiled coils and extension of γ chain nodules [46]. Lim's group showed that under stretching, fibrin(ogen) unfolding in the triple helical region, starts in the middle of the coiled coils, which is the region for plasmin cleavage [48]. Köhler et al.'s simulation showed that plasmin cleavage of the coiled coils might be facilitated by a bent conformation and hampered by a straight (stretched) conformation [49]. Our data are consistent with the notion that some of the peptide bonds, which need to be cut to completely sever the three peptide chains in the coiled coil (αLys 81, βLys122, γLys58, or αArg104, βLys133, γLys62), are blocked from lysis upon stretching. It could be that some plasmin sites in the αC region are still accessible and cleavable. Not cleaving the sites in the coiled coils, while cleaving some sites in the αC region may result in the observed elongated, thickened, but not severed, fibers.

Fibrin fibers have a very open internal structure consisting of about 80% solvent (water) and only 20% protein. The pore size in fibrin fibers may be large enough for proteins, such as FXIII, tPA, or plasmin, to move and diffuse inside a fiber [50,51]. It may also simply be the case that the pore size decreases as fibers are stretched, thus, slowing down the movement of these proteins and lysis.

4.3. Cross-linking vs. stretching

FXIII catalyzes the formation of γ-glutamyl-ε-lysine bonds (crosslinks) between the γ and α chains of adjacent fibrin molecules. Reciprocal γ-γ crosslinks form across abutting D regions between γGln 398 or 399 and γLys 406. α-α crosslinks can form between several different glutamine donors in the flexible, unstructured αC-connector (αGln 221, 237, 328, 366, see Fig. 1) and several lysine acceptors in the globular αC-domain (αLys 418, 448, 508, 539, 556, 580, 601), leading to an extensive network of interacting and crosslinked αC regions between fibrin monomers and protofibrils [8,12,13,45]. These covalent bonds increase the stiffness of fibrin fibers and clots. In addition, they slow lysis. This phenomenon was observed at the whole clot level [16,52] and also here at the single fiber level. In our receding clot assay, FXIII crosslinking decreased the lytic rate by a factor of 1.4 and at the single fibrin level by a factor of 1.6. Extensive stretching had a larger effect as it reduced lysis by a factor of 2.5 (uncrosslinked fiber, 2.5-fold stretch), and a factor of 1.7 (crosslinked fiber, 2.1-fold stretch).

4.4. Physiological relevance

Blood clot formation and fibrinolysis are tightly controlled processes that are regulated by numerous factors. Too much or too little of either clot formation or clot lysis can be life-threatening or lead to debilitating diseases. Mechanobiological signaling – a process, in which a mechanical stimulus leads to a biological effect – may be a novel factor to control fibrinolysis. Blood clots experience strain from different sources, for example from blood flow, from platelets and cells, and when a clot retracts. It has also been suggested that fibrin fibers are pre-strained [40]. From a physiological point of view, fibrinolysis should be inhibited at the beginning of clot formation, right after an injury occurred, since that is when the clot is needed. In this situation, blood is still flowing, and the exerted strain from blood flow might prevent lysis. Once a clot is fully established and stabilized, wound healing commences, strain is reduced, and, at that point, a clot may be broken down. Our findings on single fiber lysis agree with the observation that stretched whole clots lyse slower [27,28], and that retracted clots lyse slower [29,30].

Thrombosis is the main underlying factor of several serious diseases, such as ischemic heart disease, stroke, and thromboembolism – diseases which account for over a quarter of all deaths worldwide [36]. In addition, these diseases cause significant disability. There is a range of treatment options available that include impairing clot formation (anticoagulation), clot lysis, or clot retrieval. All treatment options have serious side effects, such as bleeding. Improved treatment options would, therefore, have an immediate positive impact on a large patient population. A deeper and better understanding of all the factors controlling clot formation and fibrinolysis would aid in the development of improved treatment options for thrombotic diseases.

We found that clot lysis by plasmin causes single fibrin fibers to thicken and to lengthen. This finding may imply that plasmin initially cuts some of the numerous connections between protofibrils within a fiber (see Fig. 1). Namely, a partial cutting of the network of αC regions may cause the observed thickening and lengthening of the fibers, thereby loosening and opening up the internal structure of a fiber. We also found that stretching single fibers hampers their lysis. This may suggest that stretching a clot should be avoided when efficient clot dissolution is desired. It may also suggest that clots are more stable in an environment with higher stretching forces. A slower rate of fibrinolysis is associated with poorer diagnoses in thrombotic disease [1,4,37,38].

5. Conclusion

We used a stretchable substrate made from fugitive glue (a styrenic block copolymer) and a suspended fibrin fiber lysis assay to investigate the effect of stretching on single fibrin fiber lysis. The key findings from our experiments are: 1) fibrin fibers thicken and elongate upon lysis; 2) stretching fibrin fibers significantly reduces their lysis; 3) this effect is more pronounced for uncrosslinked fibers; 4) this hampering effect on lysis induced by stretch-

ing may be more significant than the hampering effect induced by FXIII crosslinking. Stretching fibrin fibers may distort plasmin cleavage sites or hinder access to plasmin cleavage sites. Both mechanisms may contribute to stretch-induced impaired lysis. Our results suggest that stretching may be a novel mechanobiological factor that regulates fibrinolysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2017.07.037.

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