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Blood clots form rapidly in the event of vascular injury to prevent blood loss. They may also form in undesired places causing heart attacks, strokes and other diseases. Blood clots can rupture, and fragments of the clot may lodge in distal blood vessels causing, for example, ischemic strokes or embolisms. Thus, there has been great interest to understand the mechanical behavior and failure mechanisms of blood clots and their constituents. To develop a mechanically realistic model of a blood clot, knowledge of the mechanical properties of its constituents is required [1]. The major structural component providing mechanical strength to the clot is a mesh of fibrin fibers. Principally, three pieces of information are needed to develop realistic (fibrin fiber) network models [2]: 1) The architecture of the network; 2) the properties of the single fibers; 3) the properties of the fiber branch points.

The architecture of the fibrin fiber network in a blood clot may be obtained from microscopy images. Recently, there has been significant activity in determining the mechanical properties of individual fibrin fibers [3-5]. Here, we investigated the strength and failure of crosslinked and uncrosslinked fibrin fiber branch points (Figure 1). We report two distinct methods of failure; rupture at the joint, and rupture along a fiber. In fully crosslinked fibrin fibers, rupture occurred most often along the fiber. Conversely, in uncrosslinked fibers, failure occurred most often due to the detachment of a leg at the branch point. Perhaps the most unexpected finding was that neither crosslinked nor uncrosslinked joints showed failure due to continuous unzipping of the joint, rendering joints rather resilient to failure. It appears that unzipping may be prevented by a triangular branch point architecture that was revealed in many branch points when they were strained as well as the twisting of protofibrils during aggregation [6]. Finally, we report the strength (stress at failure) of fibrin fiber branch points.

Fibrin is formed by thrombin-mediated cleavage of fibrinopeptides A and B from soluble fibrinogen. Fibrin monomers self-assemble into half staggered protofibrils, which associate to form fibers. During the formation of fibrin fibers, factor XIIIa, covalently rigidifies molecule-molecule interactions through three types of crosslinks. 1) Gamma-gamma crosslinks which form between the gamma-nodule of abutting molecules; 2) Alpha-alpha crosslinks which form multiple bonds between the alpha-C regions of adjacent molecules; and 3) the less frequent alpha-gamma crosslinks. Crosslinking of

fibrin films and fibers increases the modulus (stiffness) while making fibrin fibers less extensible [7-9].

During clot formation the fibrin fibers branch and grow forming a network with interspersed platelets and red blood cells. The fiber joints, or branch points, in this network are mostly formed by the intersection of three fibers at a node [10, 11]. On a molecular level, there are two models as to how these joints form; the trimolecular joint model and the tetramolecular joint model [12, 13].

To examine fibrin joints a striated substrate was prepared as previously reported [3] using a micromolding technique [14] and Norland Optical Adhesive-81 (Norland Products, Inc. Cranbury, NJ). Fibrin fibers were polymerized directly on the substrate with a human fibrinogen concentration between 0.5 and 1 mg/ml (American Diagnostica, Stamford, CT, FXIII depleted) and a thrombin concentration between 0.05 and 0.1 u/ml (Enzyme Research Laboratories, South Bend, IN). To form crosslinked fibers joints, 25 Loewy u/ml of FXIII were added during polymerization (Enzyme Research Laboratories, South Bend, IN). The fibrin fibers were labeled with 24 nm carboxyl, yellow-green fluospheres for visualization (Invitrogen, Carlsbad, CA). To test the extent of crosslinking, samples were prepared in parallel and subjected to SDS polyacrylamide gel electrophoresis. To within the sensitivity limits of the gel, crosslinking was complete ($\geqq$ 90 %).

Mechanical manipulation of joints were done with a combined AFM/fluorescence microscope (ThermoMicroscope Explorer AFM, Zeiss Axiovert 200, Hamamatsu EM-CCD C9100 camera, 3rd Tech NanoManipulator) as described in [3, 7] . The AFM tip applied a force to one leg of the joint. Fluorescence microscopy images were recorded and used to determine failure mechanism and measure initial length and strain at rupture of each leg of the joint (Figure 1 A, B).

Straining fibrin fiber joints resulted in two methods of joint failure; rupture along a fiber, and rupture at the node (movies of failure mechanisms; see supplement). Additionally we observed detachment of a fiber at the ridge; however, these data were excluded since they result from the experimental setup and have no physiological relevance.

Uncrosslinked and crosslinked joints showed different rupture behaviors. Uncrosslinked joints ruptured 68.5% of the time at the node and 31.5% of the time along a fiber. Conversely, crosslinked joints ruptured 39.5% of the time at the node and 60.5% of the time along a fiber (Figure 1 C, D). This suggests that individual crosslinked fibrin fibers are weaker than crosslinked joints, while the opposite is true for uncrosslinked fibrin fibers. Failure of crosslinked fibers before failure of joints may also be due to the lower extensibility of crosslinked fibers, 147% to 217% [4, 7], as compared to uncrosslinked fibers, 226% [3]. Also, the difference between crosslinked and uncrosslinked fibrin joints suggests that FXIIIa crosslinking provides a mechanism which fortifies and strengthens fiber branch points. Thus, natural, fully crosslinked clots may mainly fail by fiber rupture rather than rupture of branch points.

Perhaps most surprising is the finding that joints are so resistant to failure. We expected that especially uncrosslinked fiber joints would easily unzip, since bonds implicated in lateral aggregation are weak [15] . However, we did not observe such unzipping events. A possible explanation might be that fibrin protofibrils aggregate in a helical manner around the fiber, limiting fiber size [6] and preventing two dimensional unzipping.

Unzipping may be further prevented by a triangular architecture, in which three cross-struts prevent unzipping of the joint (Figure 1E-G). In this architecture, observed in 27% of both crosslinked and uncrosslinked joints, each fiber branches into the two additional fibers involved in the joint, resulting in a triangle at the branch point. The formation of this architecture suggests that fiber polymerization occurs in all directions, since all three fibers of the joint participate in the formation of the triangle. This is consistent with the model of fibrin branching proposed by Ryan et al. in which fibrin oligomers form and join together both lengthwise and laterally resulting in branching points and lateral aggregation [10]. In about half of the joints showing triangular architecture, the shape is visible prior to manipulation. In the other half, it is not visible until manipulation has begun. Two possible explanations for the inconsistency of visible architecture are; 1) triangular architecture may be visible in the early stages of joint formation and as the joint matures the triangle closes, 2) fibers with visible triangular architecture may have a larger joint which is therefore visible where as most joints may

be to small to see. In cases of triangular architecture one cross-strut would often rupture without complete failure of the joint.

The results of joint manipulation suggest that crosslinking, twisting of protofibrils during aggregation and the method of lateral aggregation and branching in fibrin, which may results in triangular architecture, all help to strengthen fibrin fiber joints. Crosslinked and uncrosslinked joints display remarkable resilience to rupture. On average, the legs of crosslinked and uncrosslinked joints can be stretched to a strain of 132% and 146%, respectively, before rupture. Using a modulus of 8 MPa and 4 MPa for crosslinked uncrosslinked fibers, this corresponds to a rupture stress (strength) of 11 MPa and 7 MPa, respectively [7]. Aside from decreasing extensibility and increasing stiffness, crosslinking also decreases the likelihood of joint failure before fiber failure. The properties of the joints and as well as the single fibers [7] can now be used to construct a realistic model of a blood clot.

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Authorship Contributions:

CRC, EAS, CDL, and MG designed, performed, analyzed and interpreted research; CRC and MG wrote paper

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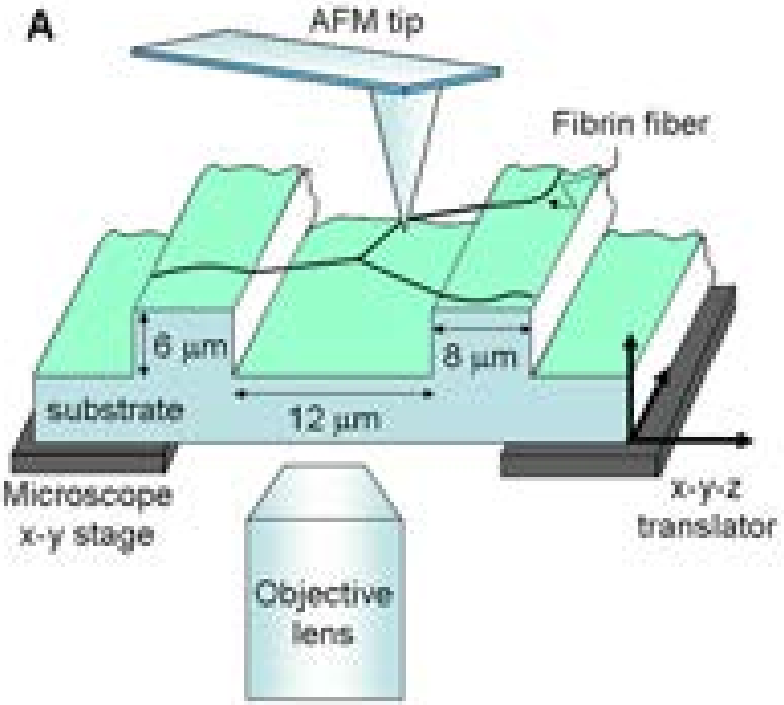
Figure Caption:

Figure 1. A) Schematic view of the experimental set-up. The AFM tip is located above the fibrin fiber joint which is formed on a striated substrate. The inverted fluorescence microscope allows visualization and movie collection of the manipulation from below. **B)** Movie frame of a fibrin fiber joint manipulation. The ridges of the striated surface are the bright horizontal bars. The location of the AFM tip is represented by a green asterisk. **C) & D)** Histograms of the maximum extensibility of the uncrosslinked and crosslinked joints. The data is color coded by failure mechanism. The maximum extensibility before detachment at the node is depicted in blue and the maximum extensibility before fiber rupture is depicted in red. The error in the strain is due to error in the measurement of the length of the fiber and is approximately 8%. All manipulations were done in fibrin buffer ((140 mM NaCl, 10 mM Hepes, 5 mM $CaCl_2$, pH 7.4). **E-G)** Movie frames of a fibrin fiber joint manipulation in buffer showing triangular joint architecture. The triangular structure becomes visible as the joint is stretched. (A small piece of fiber from a previous manipulation can be seen stuck to the AFM tip.)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x-y stage
Objective
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x-y-z
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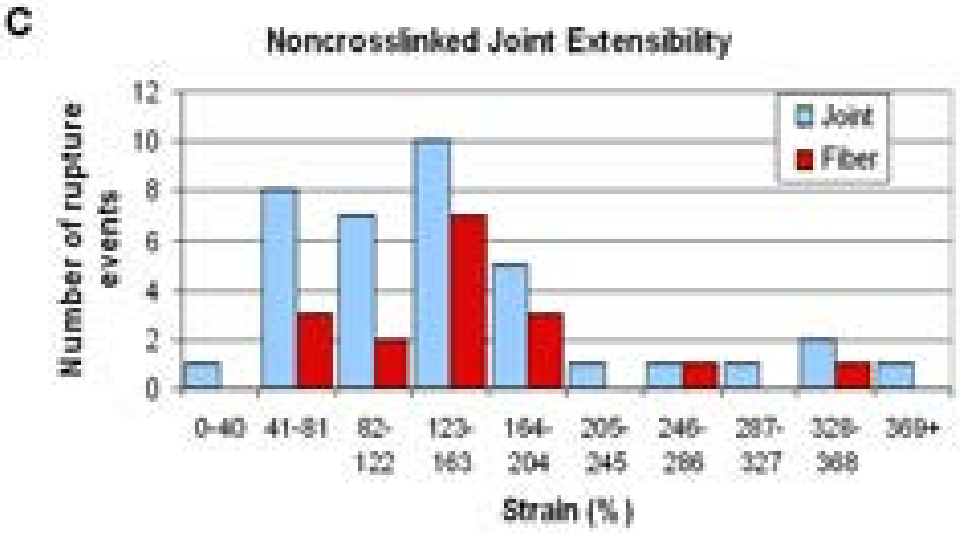
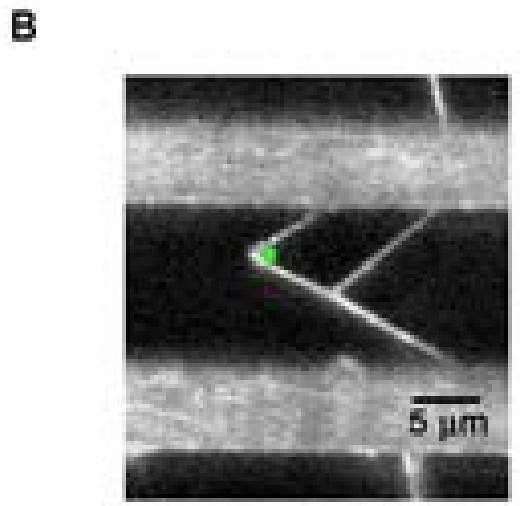

C
Noncrosslinked Joint Extensibility
Number of rupture events
Joint
Fiber
0-40
41-81
82-122
123-163
164-204
205-245
246-286
287-327
328-368
369+
Strain (%)


B
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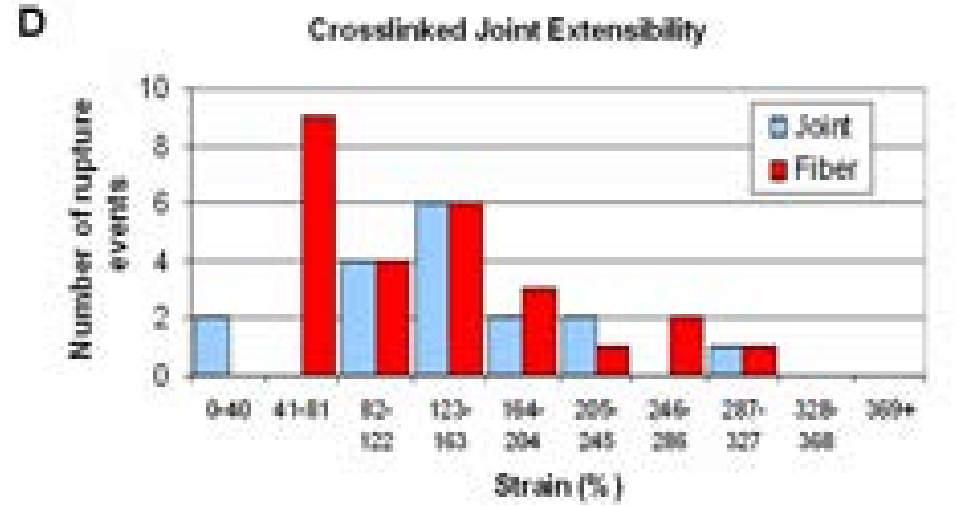
D
Crosslinked Joint Extensibility
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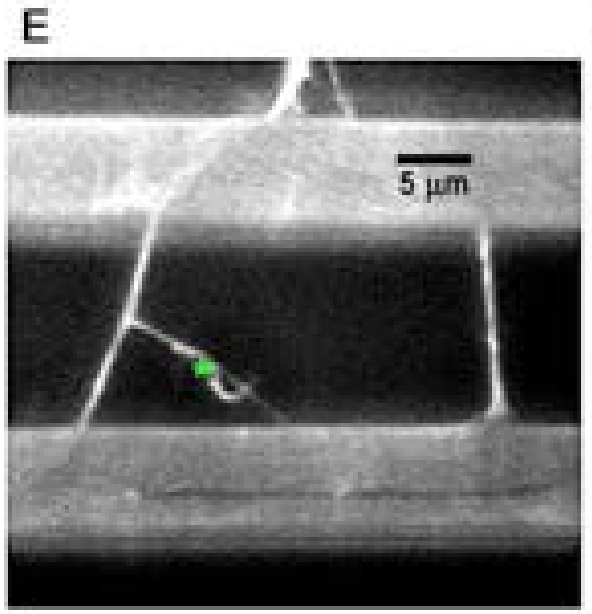
Joint
Fiber
0-40
41-81
82-122
123-163
164-204
205-245
246-286
287-327
328-368
369+
Strain (%)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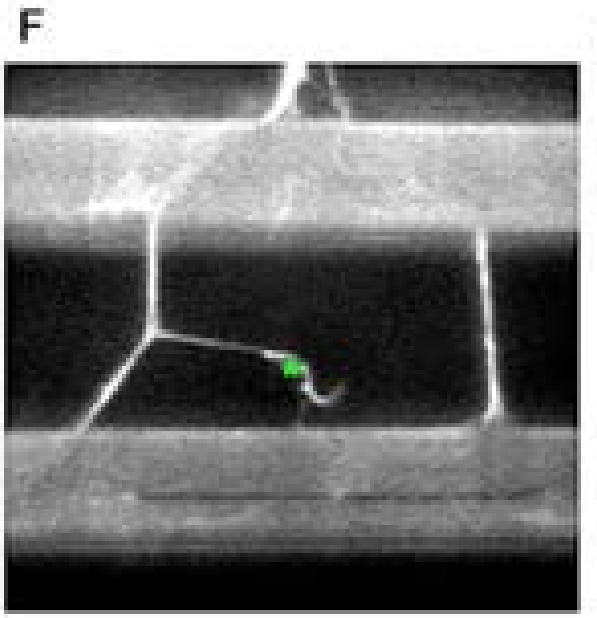
5 µm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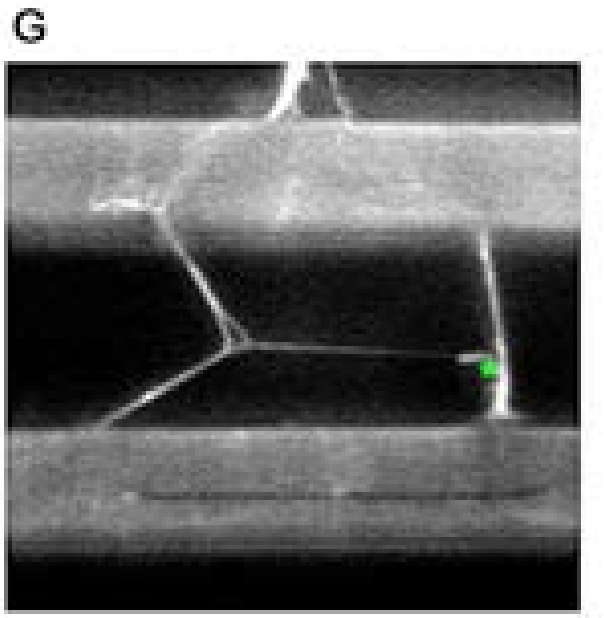
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