

Strength and failure of fibrin fiber branchpoints

C. R. CARLISLE,* E. A. SPARKS,* C. DER LOUGHIAN† and M. GUTHOLD*

*Department of Physics, Wake Forest University, Winston-Salem, NC, USA; and †Ecole Normale Supérieure de Lyon, Université Claude Bernard Lyon, France

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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 in understanding 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]: (i) the architecture of the network; (ii) the properties of the single fibers; and (iii) the properties of the fiber branchpoints.

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 branchpoints (Fig. 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 because of the detachment of a leg at the branchpoint. Perhaps the most unexpected finding was that neither cross-linked nor uncrosslinked joints showed failure due to extended unzipping of the joint, indicating that joints are rather resilient to failure. It appears that, in some cases, continuous unzipping may be prevented by a triangular branchpoint architecture, in which fibers (cross-struts) reach across all three legs in a Y-shaped junction. This architecture was revealed in many branchpoints as they were strained. This triangular architecture

is consistent with a model in which polymerization occurs in all directions at the branchpoints, as proposed by Ryan *et al.* [6]. Twisting of protofibrils during aggregation may also prevent unzipping of branchpoints [7]. Finally, we report the strength (stress at failure) of fibrin fiber branchpoints.

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 crosslink: (i) γ – γ crosslinks, which form between the γ -nodules of abutting molecules; (ii) α – α crosslinks, which form multiple bonds between the α C regions of adjacent molecules; and (iii) the less frequent α – γ crosslinks. Crosslinking of fibrin films and fibers increases the modulus (stiffness) while making fibrin fibers less extensible [8–10].

During clot formation, the fibrin fibers branch and grow, forming a network with interspersed platelets and red blood cells. The fiber joints, or branchpoints, in this network are mostly formed by the intersection of three fibers at a node [6,11]. At a molecular level, there are two models of how these joints form: the trimolecular joint model, and the tetramolecular joint model [12,13].

To examine fibrin joints, a striated substrate was prepared by a micromolding technique, as previously reported [3]. Fibrin fibers were polymerized directly on the substrate, with a human fibrinogen concentration between 0.5 and 1 mg mL⁻¹ (American Diagnostica, Stamford, CT, USA; FXIII-depleted), and a thrombin concentration between 0.05 and 0.1 units/mL (Enzyme Research Laboratories, South Bend, IN, USA). To form crosslinked fiber joints, 25 Loewy units/mL of FXIII were added during polymerization (Enzyme Research Laboratories). The fibrin fibers were labeled with 24-nm carboxyl yellow–green fluospheres for visualization (Invitrogen, Carlsbad, CA, USA). To test the extent of crosslinking, samples were prepared in parallel and subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis. To within the sensitivity limits of the gel, crosslinking was complete ($\geq 90\%$).

Mechanical manipulations of joints were performed with a combined atomic force microscope/fluorescence microscope (ThermoMicroscope Explorer atomic force microscope, Zeiss Axiovert 200, Hamamatsu EM-CCD C9100 camera, 3rd Tech NanoManipulator), as described in [3,8]. The atomic force

Correspondence: Martin Guthold, Department of Physics, 7507 Reynolda Station, Wake Forest University, Winston-Salem, NC 27109, USA.

Tel.: +336 758 4977; fax: +336 758 6142.

E-mail: gutholdm@wfu.edu

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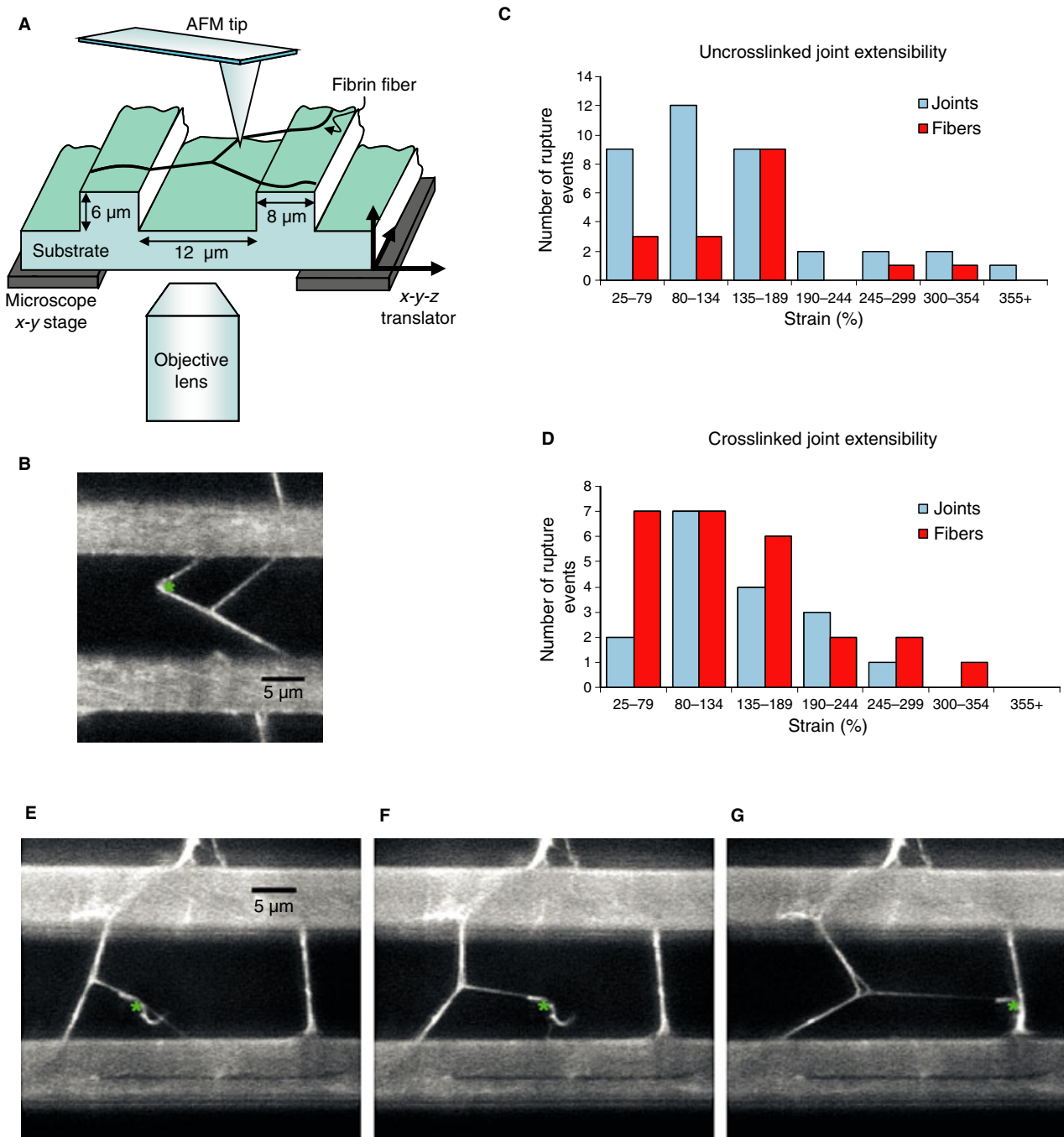


Fig. 1. (A) Schematic view of the experimental setup. The atomic force microscope (AFM) tip is located above the fibrin fiber joint, which is formed on a striated substrate. The inverted fluorescence microscope allows visualization and movie collection of the manipulation from below. (B) Movie frame 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 are 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 performed in fibrin buffer (140 mM NaCl, 10 mM HEPES, 5 mM CaCl₂, 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.)

microscope 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 (Fig. 1A,B).

Straining fibrin fiber joints resulted in two methods of joint failure: rupture along a fiber, and rupture at the node (for movies of failure mechanisms, see supplement). Additionally, we observed detachment of a fiber at the ridge; however, these

data were excluded, as they result from the experimental setup and have no physiologic 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 (Fig. 1C,D). This suggests that individual crosslinked fibrin fibers are weaker than crosslinked joints, whereas 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 (147–217%) [4,8] than of uncrosslinked fibers (226%) [3]. Also, the difference between crosslinked and uncrosslinked fibrin joints suggests that FXIIIa crosslinking provides a mechanism that fortifies and strengthens fiber branchpoints. Thus, natural, fully crosslinked clots may mainly fail by fiber rupture rather than by rupture of branchpoints.

Perhaps most surprising is the finding that joints are so resistant to failure. We expected that uncrosslinked fiber joints, in particular, would easily unzip, as bonds implicated in lateral aggregation are weak [14]. However, we did not observe extended unzipping events. A possible explanation might be that fibrin protofibrils aggregate in a helical manner around the fiber, limiting fiber size [7] and preventing two-dimensional unzipping. Unzipping may be further prevented by a triangular architecture, in which three cross-struts prevent continuous, extended unzipping of the joint (Fig. 1E–G). In this architecture, observed in 27% of both crosslinked and uncrosslinked joints, each fiber is connected via cross-struts to the two additional fibers involved in the joint, resulting in a triangle at the branchpoint. The formation of this architecture suggests that fiber polymerization at joints occurs in all directions, as all three fibers of the joint participate in the formation of the triangle. We believe that this interpretation is consistent with the model of fibrin branching proposed by Ryan *et al.*, in which branchpoints originate at the point at which protofibrils or filaments diverge. Additional protofibrils are then added to form a mature branchpoint [6]. 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 the joint is stretched. The triangular architecture may only be visible in the early stages of joint formation, and as the joint matures, the triangle closes. In cases of triangular architecture, one cross-strut would often rupture without complete failure of the joint.

The results suggest that crosslinking, twisting of protofibrils during aggregation and aggregation in all directions at the joints, which may result 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 strains of 132% and 146%, respectively, before rupture. Using moduli of 8 MPa and 4 MPa for crosslinked and uncrosslinked fibers, respectively, this corresponds to rupture stresses (strengths) of 11 MPa and 7 MPa, respectively [8]. As well as decreasing extensibility and increasing stiffness, crosslinking also decreases the likelihood of joint failure before fiber failure.

The properties of the joints and the single fibers [8] can now be used to construct a realistic model of a blood clot.

Addendum

C. R. Carlisle, E. A. Sparks, C. Der Loughian and M. Guthold designed, performed, analyzed and interpreted the research; C. R. Carlisle and M. Guthold wrote the article.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Joint rupture at the node.

Fig. S2. Joint rupture along a fiber.

Movie S1. Joint rupture at the node.

Movie S2. Joint rupture along a fiber.

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Subclinical deep venous thrombosis observed in 10% of hemophilic patients undergoing major orthopedic surgery

C. HERMANS,* F. HAMMER,† S. LOBET* and C. LAMBERT*

*Division of Haematology and †X-Ray Department, Cliniques Universitaires Saint-Luc, Université Catholique de Louvain, Brussels, Belgium

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Deep venous thrombosis (DVT) is a common postoperative complication in patients undergoing major orthopedic surgery of the lower limbs, such as total hip replacement (THR), total knee replacement (TKR) or hip fracture surgery (HFS). In the absence of thromboprophylaxis, subclinical venous thrombosis rates as high as 60% have been reported when using systematic bilateral phlebography after orthopedic surgery. As a result, routine pharmacological thromboprophylaxis with low-molecular-weight heparin (LMWH) or an alternative antithrombotic agent is strongly recommended in patients undergoing these procedures [1].

With the availability of efficient and safe clotting factor concentrates, THR, TKR as well as ankle arthrodesis are frequently performed in subjects with hemophilia suffering from chronic hemophilic arthropathy [2]. Yet, pharmacological prophylaxis of venous thromboembolism (VTE) in this patient group remains controversial. With the exception of retrospective case reports and small series, the incidence of VTE disease in hemophilic patients after major orthopedic surgery is still unclear. Despite the concern that pharmacological thromboprophylaxis might increase bleeding complications in these patients, no properly sized study has objectively evaluated the need, appropriate timing, dosage and duration of low-molecular weight heparin (LMWH) prophylaxis in this setting.

Correspondence: Cedric Hermans, Haemostasis and Thrombosis Unit, Division of Haematology, Cliniques universitaires Saint-Luc, Avenue Hippocrate 10, B-1200 Brussels, Belgium.
Tel.: +32 2 764 1785; fax: +32 2 764 8959.
E-mail: cedric.hermans@uclouvain.be

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We conducted a prospective study in order to evaluate by imaging techniques the incidence of subclinical DVT in consecutive hemophilic patients referred to our Centre for major orthopaedic surgery between June 2002 and June 2008. Twenty patients with an average age of 43 ± 14 years (range: 18–74) suffering from severe ($n = 16$), moderate ($n = 4$), or mild ($n = 2$) hemophilia A, or mild hemophilia B ($n = 2$) were included in the study. In total, 29 major orthopedic surgical procedures were performed, among which five THRs, 15 TKRs, four ankle arthrodeses, one decompressive laminectomy for lumbar stenosis and four femoral osteosyntheses. Screening for DVT was performed by unilateral (13) or bilateral (16) compression ultrasonography (US) between 4 and 14 days after surgery.

None of the patients had a personal or family history of venous thrombosis or pulmonary embolism (PE). All patients were treated with continuous infusion of recombinant clotting factor (F)VIII or FIX concentrate during a period ranging from 12 to 16 days delivered by a central venous catheter inserted pre-operatively after a bolus infusion of FVIII or FIX concentrate. No heparin was administered. Coagulation factor levels were maintained between 80% and 100% during the first 5 post-operative days, and then above 50% until discharge. None of the patients received pharmacological thromboprophylaxis. All wore grade 1 compression above-knee stockings, except for the operated TKR side. A single infusion of an antifibrinolytic agent (1 g tranexamic acid) was given in the operating room during most surgeries, with rehabilitation starting the day after surgery by mobilization of the operated limb. Extensive US-Doppler (Model IU 22, Koninklijke Philips Electronics N.V., Eindhoven, equipped with 4- to 12-MHz linear transducers) of the lower limb evaluating proximal veins and calf veins was performed by the same experienced radiologist on either the operated side(s) or both sides between Day 4 and Day 14 after surgery.