Microscale spatial heterogeneity of protein structural transitions in fibrin matrices

Frederik Fleissner, Mischa Bonn, Sapun H. Parekh*

Following an injury, a blood clot must form at the wound site to stop bleeding before skin repair can occur. Blood clots must satisfy a unique set of material requirements; they need to be sufficiently strong to resist pressure from the arterial blood flow but must be highly flexible to support large strains associated with tissue movement around the wound. These combined properties are enabled by a fibrous matrix consisting of the protein fibrin. Fibrin hydrogels can support large macroscopic strains owing to the unfolding transition of α-helical fibril structures to β sheets at the molecular level, among other reasons. Imaging protein secondary structure on the submicrometer length scale, we reveal that another length scale is relevant for fibrin function. We observe that the protein polymorphism in the gel becomes spatially heterogeneous on a micrometer length scale with increasing tensile strain, directly showing load-bearing inhomogeneity and nonaffinity. Supramolecular structural features in the hydrogel observed under strain indicate that a uniform fibrin hydrogel develops a composite-like microstructure in tension, even in the absence of cellular inclusions.

INTRODUCTION

Fibrin is the primary filamentous protein component in blood clots during hemostasis. Blood clots need to be sufficiently strong to prevent further bleeding but must be sufficiently flexible to support large strains (1). Under shear strains of more than ~20%, clots exhibit nonlinear elasticity—an increase in elasticity with strain amplitude, known as strain hardening. This mechanism allows a blood clot to be flexible when relaxed and to become robust and resistant under large external forces (1, 2). Following hemostasis, fibrin degradation occurs as the skin is rebuilt toward the end of wound healing. It has been shown that fibrin degradation is significantly slower (by more than 10-fold) in “tight” clots and when fibrin is under tensile strain (3).

Fibrin monomers have a coiled-coil structure consisting of six α helices. To form the hydrogel, fibrin monomers self-assemble, resulting in double-stranded protofibrils with monomers staggered relative to one another by roughly half a protein length (45 nm) (4). Multiple protofibrils further assemble into larger fibers with a final thickness ranging from 50 to 200 nm and a fiber length ranging from 0.3 to 4.8 μm (2, 5). These fibers are the primary structural unit of fibrin hydrogels. At an even larger scale, fibers form branches and entanglements between each other until a three-dimensional network is established with a mesh size on the order of 1 μm (6, 7).

This hierarchical and complex structure of fibrin networks gives it unique mechanical properties, with the network responding to strain on several length scales. At the molecular level, the α helices can be unfolded into β sheets under tensile force, which has been shown in both single-molecule experiments (8) and strained fibrin networks. Small-angle x-ray scattering has revealed that structural transitions of the coiled-coil α helices in fibrin monomers must play a role in the elongation of a fibrin, starting at 15% tensile strain (9). Recently, attenuated total internal reflection Fourier transform infrared (ATR-FTIR) spectroscopy on fibrin clots directly showed that the secondary structure content of fibrin clots was altered under compression and tension (10). Spectral analysis of the amide I and amide III vibrational bands revealed that relaxed human fibrin gels contained 31% α helix, 37% β sheet, and 32% turns, loops, and random coils, which changed to 16% α helices and 52% β sheet structures under large (400%) extensional strain (10, 11). Combined with previous rheological studies, a physical-chemical description of fibrin at the molecular (from spectroscopy and scattering) and macroscopic (from rheology) scale in response to strain is becoming clear.

However, a description that spans the molecular scale to microscale of fibrin is comparatively absent. Because fibrin clots contain micrometer-sized platelet inclusions that exert contractile forces, a description of load bearing and structure on micrometer length scales is highly relevant to understanding the underlying physics governing stress distribution in the material. A microscopic mechanical description of collagen recently showed rich mechanical properties that could not be observed (or explained) by continuum theories using holographic optical tweezers, which highlights the importance of measuring local mechanics in protein hydrogel materials (12).

Morphological evidence of spatially heterogeneous strain is clear from ultrastructural images and confocal fluorescence of fibrin networks (9, 10, 13); however, these images do not provide any evidence of local force distribution in the network, which (as mentioned above) is related to fibrin protein structure. Therefore, by measuring spatially resolved fibrin protein structure within the hydrogel, it would be possible to directly visualize the local load distribution. This would allow identification of rich local mechanical properties in fibrin, similar to those recently discovered in collagen.

Observation of spatially heterogeneous protein structure as a function of load requires measuring protein structure at the submicrometer scale (ideally on the fiber length scale, which is ~100 to 300 nm) combined with defined mechanical deformations. Raman spectroscopy is ideally suited for this purpose because it is capable of probing amide I and amide III molecular vibrations similar to FTIR, it has a much higher (~400 nm) spatial resolution, and it does not suffer from the intense water absorption in aqueous samples that can mask amide vibrations (14). Spontaneous Raman spectroscopy has been used to quantify secondary structure in various biopolymers, including whelk egg capsules (15), keratin (16), and collagen fibers (17), but not as a function of external strain.

Unfortunately, the signal intensity in spontaneous Raman is limited, as only 1 of 10^16 photons is inelastically Raman-scattered (14, 18). Thus, it is challenging to perform spectral imaging without excessively long...
measurement times that could complicate interpretation for even slightly viscoelastic materials. One way to overcome the limitations of spontaneous Raman is through the use of nonlinear Raman scattering such as coherent anti-Stokes Raman scattering (CARS). Here, the Raman signal is generated in a four-wave mixing process where the signal strength is resonantly enhanced by up to six orders of magnitude (14). This is realized by the spatial and temporal overlap of two laser beams where the energy difference between the two lasers defines the Raman frequency that is probed. Quantitative band analysis of broadband CARS (BCARS) spectra is possible with established routines; thus, CARS provides Raman-like vibrational spectra at increased speed (19).

Here, we use hyperspectral BCARS (20–23)—in which an entire vibrational spectrum (800 to 4000 cm−1) is acquired in a single acquisition at each spatial location—to determine spatially resolved secondary structure in fibrin hydrogels. We combine the BCARS approach with tensile measurements of fibrin gels to quantify mechanically induced secondary structural changes at submicrometer spatial length scales. Our results show that the secondary structure in fibrin becomes increasingly heterogeneous with increasing tensile load, with micrometer-sized regions primarily showing an α helix next to similarly sized β sheet regions.

RESULTS

Fibrin networks strain-stiffen both in shear and tension

Before investigating the structural properties of fibrin hydrogels using BCARS, we measured the viscoelastic properties of our hydrogels with shear rheometry and tensile testing. Prestrain sweeps on both partially cross-linked fibrin samples (defined here as fibrin gels polymerized from as-received fibrinogen) and FXIIIa (fibrin stabilizing factor XIIIa)—cross-linked fibrin samples (defined here as fibrin cross-linked with saturating FXIIIa) were conducted to measure the so-called differential shear modulus K of the materials (2, 24, 25). Our partially cross-linked fibrin samples have trace amounts of FXIII as it copurifies with fibrinogen, but the amount of FXIII is still less than that found in cross-linked samples (fig. S1) (26). Figure 1A shows prestrain sweeps in shear for both types of gels and shows a characteristic plateau storage modulus, K′, at low strains of 320 and 420 Pa for the partially cross-linked and cross-linked samples, respectively. The plateau modulus for our fibrin gels was comparable to that observed in other studies (2, 27), and prestrain sweeps of fibrin hydrogels with c_fibrin = 15 mg/ml show the characteristic increase in K′ α c_fibrin 11/5 as well as K′ convergence at large prestrains (fig. S2). The onset of nonlinear elasticity in shear occurred at a strain of ~40 and 50% for partially cross-linked and cross-linked samples, respectively, on the basis of the intersection of linear fits to the high- and low-strain region for each curve. We performed additional shear creep recovery experiments for both partially cross-linked and fully cross-linked hydrogels (fig. S3). These measurements show that additional FXIII led to a decreased dissipative response and faster response dynamics to steady mechanical perturbation compared to partially cross-linked fibrin.

We also performed tensile tests on the hydrogels as an additional mechanical characterization. From these data, we see a critical strain of ~35% extension where the normalized force-strain curve changes from a shallow slope at low strain to a large slope at high strain (Fig. 1B). However, these measurements do not show substantial differences between partially cross-linked fibrin and cross-linked fibrin hydrogels. Together, the shear and tensile measurements do not show substantial differences in the nonlinear mechanics of the fibrin hydrogels. Nevertheless, the shear measurements—both rheology and creep recovery—show a clear increase in the linear differential modulus and reduced dissipative response, as expected with additional cross-linking of fibrin (26, 28, 29).

Helix and sheet structures are orthogonal under tensile strain

In our BCARS measurements of protein structure, we apply uniaxial tension to fibrin hydrogels, which defines a clear anisotropy in the material along the loading direction. Fibrin fibers are known to align under increasing strain, which should occur before any tension-based protein unfolding (9). Previous molecular dynamics simulations have shown that the orientation of the coiled-coil helices (present at low strains) and sheet structures (present only at large strain) is organized such that their...
stabilizing hydrogen bonds are orthogonal to each other. Thus, we initially focus on identifying the orientation of the β sheet with respect to the uniaxial load to maximize our sensitivity for strain-induced β sheets.

To determine the secondary structure in fibrin gels in situ, we acquire hyperspectral BCARS data sets of fibrin (one spectrum at each spatial position) and decompose the vibrational amide I spectra (1570 to 1700 cm$^{-1}$) from each resonantly retrieved Raman-like spectrum to determine the contribution of α helix, β sheet, and random coil structures in each spectrum (see “CARS data processing” in Materials and Methods). The amide I vibration (corresponding to the NH-coupled C=O vibration) is present in any protein; however, because of the local hydrogen bonding that stabilizes α helices and β sheets, the amide I vibrational resonance shape is distinct for each secondary structure. α helices have a peak at 1640 cm$^{-1}$, whereas β sheets have a peak at 1667 cm$^{-1}$. In an α helix, the C=O group in one peptide is hydrogen-bonded to the secondary amines of another peptide bond in a direction that is parallel to the helical axis. Corresponding hydrogen bonds that stabilize β sheets are formed orthogonal to each β strand (30). The characteristic spectra of these structures, an additional mode from random coils, and two tyrosine ring breathing mode vibrations (31) were used to fit the amide I region of each spectrum. Before decomposition, spectra were normalized by the amount of protein in each spectrum as given by the CH$_3$ vibration (2934 cm$^{-1}$) that arises from protein side chains (31). After spectral decomposition, the fractional area of each component relative to the total area was quantified to determine the percentage contribution of each structural motif in every spectrum (Fig. 2, A and B). Similar decomposition of Raman spectra has been shown to correspond to higher than 95% with structural percentages determined by x-ray diffraction (32).

When stretching the sample to 60% strain, we observe a change in the spectral shape (Fig. 2B) and opposing trends for the two peaks with respect to the sample rotation angle (Fig. 3B). The polar plot of the peak related to β sheets shows an elliptic orientation with a major axis nearly orthogonal to the laser axis. The second peak, indicative of α helices, shows an ellipticity with a major axis parallel to the laser polarization. Figure 3B shows that the contribution of β sheet structures to the amide I vibration is maximized when the loading axis and laser polarization are nearly orthogonal (angle, ~80°), whereas for α helices, the contribution is maximized when the laser and loading axes are parallel. This can be generalized to note that the stabilizing hydrogen bonds within the β sheet and α helices in fibrin are orthogonal to one another under load, which is consistent with the α helix and β strand elements lying along the loading direction, as previously postulated (33). Furthermore, the angle-integrated (total) contribution for α helix decreases by 11% under strain, whereas for β sheet, it increases by 19%, showing that the amount of β sheet increases under tension and assumes a preferential orientation.

![Fig. 2. Phase-retrieved and CH$_3$-normalized BCARS spectrum of fibrin.](A and B) Never-loaded (A) and 80% strained (B) fibrin hydrogel. The amide I band was decomposed with a sum of five Lorentzians: green, blue, and red peaks that represent structural species indicated and two smaller ring modes (depicted in gray) that are related to tyrosine rings. The contribution of each species to the amide I band was determined by the fractional area under each component. Black lines indicate the raw data, and orange lines represent the Lorentzian fits.
Strain-induced increase in β sheet content is more pronounced in cross-linked hydrogels

Following identification of the β sheet motif orientation relative to the loading direction, we measured the secondary structural changes as a function of tensile strain in both cross-linked and partially cross-linked fibrin. In the following measurements, the laser polarization was fixed nearly perpendicular to the loading direction, which was chosen to maximize the sensitivity to new β sheets formed with increasing strain [indicated by the angle between the major axis in the polar plot relative to the laser in Fig. 3B (red)]. We stretched gels from their initial length to 110% strain. For larger strains, many of the gels broke or started to slide; however, all gels withstood 110% strain without failure. The contribution of α helix and β sheet structures is plotted against the local strain, by averaging over the 30 most protein-rich spatial pixels in a field of view of 5 μm × 5 μm (Fig. 4). Because all spectra were normalized to protein concentration, we combined the spectra from five independent hydrogel samples and binned the measurements along the strain axis into 20% increments, starting from 10%. The local strain in the sample was quantified by measuring the displacement of polystyrene beads in the field of view from bright-field images (see Materials and Methods). The local strain, calculated from different pairs of polystyrene bead displacements in the field of view, varied by less than 5%. Therefore, the strain was assumed to be applied uniformly over the sample. As the tensile load stretches the gel relative to the fixed mounting point, the field of view, and hence the analyzed collection of pixels, included in our calculation of secondary structure is not the same for each strain level. Consistent with previous results by Brown et al. (9), showing water expulsion with tensile strain, we observe increased fibrin concentration (inferred from an increased CH intensity) with increasing strain on fibrin hydrogel samples (fig. S5).

The contribution of the peak centered at 1667 cm⁻¹, representative of β sheet structure (32), increased for both hydrogel samples starting at ~30% strain—excluding the initial jump from 0 to 10% strain that arises from sample handling, as will be explained below. We observed that partially cross-linked hydrogels yielded a lower amount of β sheet over the entire range of strains compared to additionally FXIIIa–cross-linked fibrin. Looking at each of the curves in Fig. 4A, the amount of β sheet sharply increases from 30 to 90% strain (more so for FXIIIa–cross-linked samples) and flattens out at 90% strain in both samples. The SEM increases under load relative to the “true” 0% measurement. From decomposition of the amide I band, we determine that the amount of β sheet increased to a final content of 42% for partially cross-linked gels and 52% for cross-linked gels. The contribution of the peak centered at 1640 cm⁻¹, representative of α-helical structure (31), decreased with increasing deformation for both samples (Fig. 4B). Coincident with a strong increase in β sheet content at 30% strain, a decrease in α helix content was seen starting at the same strain (neglecting the small decrease from 0 to 10% strain). The α helix content in the partially cross-linked gel seems to stabilize at 25%, whereas the cross-linked gel does not stabilize at 110% strain. The measured contribution from random coils, represented by a peak centered at 1650 cm⁻¹, remained largely constant at all strains (Fig. S6), consistent with previous infrared data (10).

As a true 0% strain measurement, BCARS hyperspectral maps of fibrin that were directly formed between two coverslips were acquired and processed for both partially cross-linked and FXIIIa–cross-linked hydrogels, which are shown at 0% in both plots of Fig. 4. The true 0% measurement shows the largest contribution for α helices and the smallest contribution for β sheets. Therefore, we surmise that the initial change in secondary structure seen from 0 to 10% strain contains effects from sample handling, which is unavoidable in our experiment. Minimal deformation of fibrin hydrogels (less than 10% in shear) has been shown to cause fibrin unfolding parallel to the loading axis (2), and tensile strains as small as 15% can initiate fibrin unfolding (9). Both of these effects...
would lead to an increase in β sheet content at 10% strain in our measurements compared to the true 0% measurement where no handling occurs.

For both α helix and β sheet structures, it is clear that the absolute change in each secondary structure motif is smaller at all strains for partially cross-linked fibrin than that for the FXIIIa–cross-linked gel. Another noticeable trend is the larger error bars at larger strains in both motifs, in both types of gels. Because the data for Fig. 4 were determined by pooling all spectra from partially cross-linked or cross-linked samples at each strain, it is challenging to determine whether the fibrin gel exhibits more structural (and, therefore, load-bearing) heterogeneity at larger strains or whether the larger scatter comes from increased measurement noise. We note that the SD of the CH₃ signal relative to the mean at each strain (indicative of protein concentration heterogeneity) changes only slightly in the cross-linked gel and does not change at all in the partially cross-linked gels (fig. S7). Furthermore, the uncorrelated spatial features between protein content and β sheet when never loaded (fig. S8) strongly suggest that structural heterogeneity develops in the gel because of increasing load that is independent of any measurement uncertainty.

**Tensile strain increases structural heterogeneity**

To examine whether the spatial distribution of secondary structure in fibrin hydrogels becomes more heterogeneous under strain, we acquired hyperspectral data from 20 μm × 20 μm regions (41 pixels × 41 pixels) to create images depicting the contribution of β sheet, α helix, and random coil structural elements in both gel formulations. The spatial pixel spacing in each image is 0.5 μm, which is roughly the same size as the largest fiber diameters in the gel. We note that all gray pixels in the maps are fluid or polystyrene pixels that do not contain any detectable protein signature (based on the absence of CH₃ vibration).

The image of β sheet content—related to the distribution of local force—in a never-loaded, partially cross-linked gel (0% strain) is somewhat homogeneous, with ~37% β sheet content on average (Fig. 5A). The α helix content and random coil content look equally homogeneous (figs. S9 and S10). Figure 5B shows the corresponding histogram of β sheet contribution for all spatial pixels in the map. This histogram depicts the relative spatial homogeneity with a mean (μ) of 37.2% and an SD (σ) of 2.8% when fit with a Gaussian distribution. By stretching the hydrogel to 50% local strain, more β sheet content appears on average, as expected. The β sheet map (Fig. 5C) now shows more heterogeneity. The histogram of all the protein-containing pixels demonstrates that the distribution of β sheet has broadened when compared to the never-loaded case (μ = 38.0, σ = 3.2; Fig. 5D). For 100% local strain, the β sheet heterogeneity is even more pronounced (Fig. 5E), as quantified by the increasing SD in the Gaussian fit of the histogram (μ = 43.2, σ = 6.6%; Fig. 5F). Coupled with similar decreasing heterogeneity in the spatial distribution of α helix content and increasing heterogeneity in the random coil structure with increasing strain (figs. S9 and S10), these data demonstrate that fibrin structural heterogeneity increases under load in a nontrivial manner. Looking closely at the high-strain β sheet maps, for multiple experiments, we observe that a supramolecular structure with ~4 to 6 μm scale appears (fig. S11). The random coil and α helix contributions seem to be complementary to the β sheet. We note that similar results to Fig. 5 were obtained with cross-linked gels (fig. S12). This indicates that fibrin has a structural (and load-bearing) disorder on the microscale that becomes apparent under uniform strain and is not present when never loaded.

**DISCUSSION**

All tested fibrin hydrogels strain-stiffened in shear rheology measurements (Fig. 1). The cross-linked network had a 30% larger plateau shear modulus than the partially cross-linked network, whereas the nonlinear elasticity (both onset and moduli) was very similar for both samples, which is consistent with previous measurements (26, 28). Although the nonlinear elasticity was similar for the two networks, additional FXIIIa cross-linking resulted in substantially increased tension-induced β sheet content in the gel (Fig. 4), similar to that seen previously by Brown et al. (34). The β sheet content for gels was ~30% when never loaded and increased to 42 and 52% at high tensile strain for partially cross-linked and FXIIIa–cross-linked gels, respectively. The results for secondary structural content of the partially cross-linked gel at high strain are nearly identical to those obtained (via ATR-FTIR) by Litvinov et al. (10) on “naturally
It is well known that CARS (and generally all vibrational) signal strength depends both on the orientation of the molecular vibration (axis of polarizability) with respect to the laser polarizations and on the concentration of vibrational oscillators in the focal volume (35, 36). Our results from Fig. 3 demonstrate that rotating the fibrin gel sample relative to a constant laser polarization reveals the specific orientation of the α-helical and β sheet motifs in the gel under strain. Fibrin(ogen) proteins contain multiple α helices, specifically between the two D-domains and the E-domain of the protein. These helices have been shown to lie along the long axis of the protein, as this would result in hydrogen-bonded C=O vibration being nearly orthogonal to the β strands (33).

From previous knowledge about the hierarchical assembly of fibrin molecules into protofibrils, fibers, and entangled gels and the semiflexible nature of fibrin gel elasticity (2), we expect that protofibrils will align to a unidirectional load (15). Therefore, it is possible that the Raman signal in the amide I region is affected by two coupled effects: (i) reorientation of fibrin fibers (and constituent proteins), leading to reorientation of secondary structural elements and corresponding reorientation of the hydrogen-bonded C=O moieties in the proteins, and (ii) structural transitions from α helix to β sheet with increasing load. From integrating the polar plot traces in Fig. 3B over all angles, it is evident that the total β sheet contribution to the amide I spectrum increased by 19% when the network was strained by 60% compared to the never-loaded network, whereas the total α helix contribution decreased by 11%. The loading axis was fixed at 80° relative to the laser polarization to measure force-induced structural transitions and spatial heterogeneity. At this orientation, an increase in β sheet contribution from 32 to 42% was observed under 60% tensile strain (Fig. 3). If one only accounts for new β sheet formation (on average, a 19% increase), the signal at 80° orientation would have increased from 32 to 38%, which means that the remaining 4% comes from reorientation of existing β strands. The native fibrinogen molecule is known to contain a small amount of disordered β strands in the outer D-domains, which assume no particular orientation with respect to the long axis of the protein and may potentially reorient under load (37, 38). Nevertheless, most of the additional β sheet signal at the 80° orientation came from β strands created by strain-induced transitions from α helices to β sheets.

Looking at the trends in structural changes from our BCARS measurements, we observe that cross-linked gels exhibit greater increase in β sheet (and greater decrease in α helix) content. It is known that FXIII addition leads to the formation of more tightly coupled protofibrils, which increases the bending rigidity of fibers, leading to a larger plateau modulus (as observed in Fig. 1) (2, 26). The gel’s nonlinear elasticity is believed to originate from the resistance to extension of protofibrils themselves in the following way. First, the αC-domains in fibrin monomers that connect protofibrils can be elongated. Second, forced unfolding of the coiled coils (α → β) within the monomers is possible (2, 9). Covalent bonds catalyzed by FXIIIa enhance the γ-chain connections as well as the α-chain linkage (39). Helms et al. (40) proposed that γ-γ cross-linking might change the pattern of stress propagation from a dominating γC-domain deformation toward an unfolding mechanism. Instead of routing the stress back and forth via αC-domain connections, the strong γ-γ linking in FXIIIα-cross-linked gels channels the stress through the coiled-coil region of monomers in protofibrils. This pathway would result in enhanced unfolding of coiled-coil α helices that connect the D- and E-domains into β sheets (28, 40). Our data support this hypothesis as additional FXIIIα cross-linking increases the strain-induced changes in secondary structure compared to the partially cross-linked gels. We observe similar changes in secondary structure with strain in samples with reduced mesh size [partially cross-linked fibrin gels (15 mg/ml)] to those in cross-linked samples (7.5 mg/ml) (figs. S13 and S14). This indicates that decreasing the mesh size also results in greater structural transitions at a given tensile strain in partially cross-linked fibrin.

BCARS imaging of secondary structure showed increasing β sheet content and substantial spatial-structural heterogeneity in strained samples when compared to never-loaded samples. From our structural images over 20 μm × 20 μm regions, we observed that discrete sections of the fibrin mesh exhibit large β sheet content under uniaxial strain, whereas others show very little β sheet content. Complementary heterogeneity was found in the random coil and α helix structural content (figs. S9 and S10). Considering previous electron micrographs of fibrin gels under 400% tensile strain (10), as well as confocal micrographs of fluorescently labeled fibrin under shear strain (41), it is clear that not all fibers align to the load. Correspondingly, a unidirectional deformation will cause only parts of the gel to unfold, whereas other parts may remain relaxed. Thus, it is plausible that some fibers will not actively resist the load. On the basis of autocorrelation of multiple β sheet structural images at greater than 85% strain, our data reveal that β sheet bands, separated by ~4 to 6 μm, occur within strained fibrin gels (fig. S11). These β sheet bands are parallel to the loading direction and identify regions that bear larger forces compared to neighboring helix-dominated regions. This length scale is
and expulsion of water (tissue plasminogen activator due to exposed hydrophobic regions of fibrin). The mechanism assumes that fibrin unfolding leads to a loss of binding sites for the plasminogen (shown that, in addition to pore size and fiber density, the spatial structure observed in the β sheet map shows that an initially uniform fibrin gel becomes a structured material on the microscale under external tensile strain. With conflicting reports in the literature about the reversibility of secondary structural changes, future experiments are aimed at determining whether these structural transitions and the heterogeneity observed in this study are reversible after removing all strain.

Fibrin composites—fibrin plus red blood cells and platelets—are one of the most important biocomposite materials. The spatial structure observed in the β sheet map shows that an initially uniform fibrin gel becomes a structured material on the microscale under external tensile strain. With conflicting reports in the literature about the reversibility of secondary structural changes, future experiments are aimed at determining whether these structural transitions and the heterogeneity observed in this study are reversible after removing all strain.

The suggested mechanism assumes that fibrin unfolding leads to a loss of binding sites for the tissue plasminogen activator due to exposure of hydrophobic regions of fibrin and expulsion of water. Following this logic, the structural heterogeneity observed in our experiments strongly suggests that fibrin degradation occurs in a similar spatially heterogeneous pattern. External loads, as well as contraction of platelets, may cause load-bearing parts of the fibrin network to unfold and thus become more resistant to lysis. Consequently, only regions that are not load-bearing—that is, those in the native conformation—would be removed in the beginning of fibrinolysis. This would be an intrinsic mechanism to regulate degradation of the entire fibrin mesh because it would maintain clot stability during clot turnover and skin rebuilding.

CONCLUSION

BCARS microscopy of fibrin hydrogels was used to determine mechanically induced changes in secondary structure. The identification of orthogonal α helix and β sheet hydrogen bonds experimentally confirms the proposed geometry of these structures in previous work. Spectra of FXIIIa—cross-linked fibrin under load showed a reduced amount of α helix, as well as increased β sheet content, compared to partially cross-linked gels. From structural maps, we directly observe the heterogeneity of secondary structure in the hydrogel under unidirectional tensile loads, which shows clear nonuniform force distribution. The combination of structural transitions and heterogeneity in the fibrin structure under load gives additional insight into the fundamental mechanisms of elasticity of fibrin gels and how local fibrin structure may help to maintain stability throughout wound healing.

MATERIALS AND METHODS

Hydrogel preparation

Fibrin hydrogels were prepared as described by Piechocka et al. Human fibrinogen monomers (FIB-3), human thrombin (HT 1002a), and human fibrin stabilizing factor (HFXIII) were obtained from Enzyme Research Laboratories. Fibrinogen was diluted in 20 mM Hepes and 150 mM NaCl at pH 7.4. To promote cleavage by thrombin, 5 mM CaCl₂ was added to the buffer to ensure thrombin activation. Hydrogels were mixed to achieve final concentrations of fibrinogen (7.5 mg/ml) and thrombin (1.05 U/ml). This protocol resulted in full polymerization of fibrin, as judged by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (fig. S15).

For additional cross-linked gel, FXIIIa was activated by thrombin at the same unit concentration to form FXIIIa. The solution was kept at 37°C for 10 min before further usage to allow complete cleavage of FXIII. To form stabilized fibrin hydrogel, FXIIIa with a final concentration of 8 U/ml was added to a gel with fibrinogen (7.5 mg/ml) (28, 46).

RESEARCH ARTICLE

For all gel solutions (partially cross-linked or cross-linked), 3-μm-diameter polystyrene microspheres (Polyscience GmbH) were doped at low concentration. In an area of 50 μm × 50 μm, typically ~25 beads could be found. This allowed postprocessing calculation of the local gel deformation in the hydrogels under load. The final mixture was pipetted into glass molds (thickness, 150 μm) and allowed to polymerize in an incubator (100% humidity, 37°C, and 5% CO₂) for at least 2 hours.

Rheology and tension

Shear rheology of hydrogels was performed on a commercial shear rheometer (ARES, Rheometric Scientific) with parallel plate geometry. Data acquisition was done in TA Orchestrate software (TA Instruments). Fibrin hydrogels were prepared by polymerizing fibrin solution between two circular cover glasses (diameter, 24 mm; Menzel) with a 150-μm gap and sealed with silicon oil (Baysilone, medium viscosity; Bayer) to prevent drying of the gel. The two cover glasses were fixed to the steel plates of the rheometer with double-sided adhesive tape (tesa SE). A normal contact force of 0.1 N was applied to the sample, which resulted in a gap spacing of approximately 175 μm between the plates. Prestrain sweeps were executed by changing the prestrain from 1 to 500%, superposed by an oscillating strain with an amplitude smaller than 10% of the prestrain value. For tensile tests of fibrin gels, a material testing machine (Z005, TestXpert II, Zwick Roell) equipped with a load cell (ZeFD1, HBM) was used. The initial sample geometry was approximately 5 mm × 20 mm × 0.2 mm. Fibrin gels were physically clamped and stretched at a constant rate of 10 mm/min until samples broke.

BCARS microspectroscopy

We used a nanosecond-based BCARS system for microspectroscopy of fibrin hydrogels, as depicted in fig. S16. The details of this setup have been extensively described by Billecke et al. (47), and additional details are provided in the Supplementary Materials.

Sample handling and strain application to fibrin hydrogels

A small piece of polymerized fibrin (~0.5 mm × 5 mm × 150 μm) was cut by a scalp from the mold, carefully picked with a precision tweezer and placed on two coverslips such that the two ends of the gel could be fixed on the coverslips with super glue (LOCTITE 454, Henkel). Care was taken to ensure that no glue was in the center of the gel where the measurements took place. After gluing, the sample was sandwiched between two additional coverslips and surrounded by buffer solution to ensure that the sample was fully hydrated throughout the measurement. The sandwich was transported to the microscope and mounted as shown in the zoomed image in fig. S16.

Fibrin samples were raster-scanned in-plane with a step size of 0.5 μm. For most data presented here, an area of 5 μm × 5 μm was scanned to acquire 121 spectra for statistical evaluation and mapping. The exposure time for each spectrum was set to 1 s to obtain a sufficient signal-to-noise ratio.

Uniaxial strain was applied by translating one coverslip, to which the gel was glued, with respect to the fixed coverslip, by a known amount relative...
to the original length of the gel (fig. S11). The local displacement in the hydrogel where the BCARS spectra were acquired was determined by quantifying embedded microsphere displacement in bright-field images at each strain. Bead tracking was done using ImageJ (National Institutes of Health).

**CARS data processing**

Because raw BCARS contains both resonant and nonresonant components, recorded spectra must be processed into Raman-like spectra to allow quantitative analysis (19). To extract the resonant, Raman-like component from the CARS spectra, we used a Kramers-Kronig transform described by Parekh et al. (23) and Liu et al. (48).

Further data processing to determine secondary structure was done in MATLAB (R2012a, MathWorks). To account for variations in hydrogel thickness and nonsystematic variations in the experimental setup, all spectra were normalized by the peak value of the CH$_3$-stretching mode at 2934 cm$^{-1}$, which is proportional to the amount of protein in the focus. The contribution to the Raman signal related to $\alpha$ helix, $\beta$ sheet, and random coil secondary structures was found by decomposition of the amide I band. We found that five different peaks were necessary to fit the amide I band. We found that five different peaks were necessary to fit the amide I region between 1570 and 1730 cm$^{-1}$: 1640 cm$^{-1}$ for $\alpha$ helices, 1650 cm$^{-1}$ for random coils, 1667 cm$^{-1}$ for $\beta$ sheets, and two minor peaks at 1612 and 1600 cm$^{-1}$ for tyrosine ring modes (31). Each peak was defined as a Lorentzian function with a given linewidth, constrained (but floating) center frequency, and floating (but positively constrained) amplitude. The fitting was executed on the normalized spectra using least squares with a Levenberg-Marquardt algorithm.

To identify polystyrene-containing pixels, we searched spectra for strong peaks at 988 cm$^{-1}$ (ring breathing phenyl ring), 1029 cm$^{-1}$ (CH$_3$ in-plane bending mode), and 1597 cm$^{-1}$ (ring breathing phenyl ring) (31). Any spectra that showed spectral features from polystyrene microspheres were excluded from further processing.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/7/e1501778/DC1

**Supplementary Data**

Supplementary Methods

Supplementary References and Notes

**REFERENCES AND NOTES**

1. J. V. Shah, P. A. Janmey, Strain hardening of fibrin gels and plasma clots. *Rheol. Acta* **36**, 262–268 (1997).
2. I. K. Plechcocka, R. G. Bacabac, M. Potters, F. C. MacKintosh, G. H. Koenderink, Structural hierarchy governs fibrin gel mechanics. *Biophys. J.* **98**, 2281–2289 (2010).
3. A. S. Adhikari, A. H. Mekhdjian, A. R. Dunn, Strain tunes proteolytic degradation and diffusive transport in fibrin networks. *Biomacromolecules* **13**, 499–506 (2012).
4. W. E. Fowler, R. R. Hantgan, J. Hermans, H. P. Erickson, Structure of the fibrin protofibril. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4872–4876 (1981).
5. E. A. Ryan, L. F. Mockos, J. W. Weisel, L. Lorand, Structural origins of fibrin clot rheology. *Biophys. J.* **77**, 2813–2826 (1999).
6. J. W. Weisel, The mechanical properties of fibrin for basic scientists and clinicians. *Biophys. Chem.* **112**, 267–276 (2004).
7. M. W. Moessner, K. R. Siebentil, D. A. Mehl, The structure and biological features of fibrinogen and fibrin. *Ann. N. Y. Acad. Sci.* **936**, 11–30 (2001).
8. B. B. C. Lim, E. H. Lee, M. Sotomayor, K. Schulten, Molecular basis of fibrin clot elasticity. *Structure* **16**, 449–459 (2008).
9. A. E. X. Brown, R. I. Litvinov, D. E. Discher, P. K. Purohit, J. W. Weisel, Multiscale mechanics of fibrin polymer: Gel stretching with protein unfolding and loss of water. *Science* **325**, 741–744 (2009).
10. R. I. Litvinov, D. A. Faizullin, Y. F. Zuev, J. W. Weisel, The $\alpha$-helix to $\beta$-sheet transition in stretched and compressed hydrated fibrin clots. *Biophys. J.* **103**, 1020–1027 (2012).
11. E. Bramant, E. Benedetti, A. Sagripanti, F. Papineschi, Determination of secondary structure of normal fibrin from human peripheral blood. *Biopolymers* **41**, 545–553 (1997).
12. C. A. R. Jones, M. Cibula, J. Feng, E. A. Knack, D. H. McIntyre, H. Levine, B. Sun, Micromechanics of cellularized biopolymer networks. *Proc. Natl. Acad. Sci. U.S.A.* **112**, E5117–E5122 (2015).
13. S. Münster, L. M. Jawaerth, B. Fabey, D. A. Weitz, Structure and mechanics of fibrin clots formed under mechanical perturbation. *J. Thromb. Haemost.* **11**, 557–560 (2013).
14. A. Downes, A. Elffick, Raman spectroscopy and related techniques in biomedicine. *Sensors* **10**, 1871–1889 (2010).
15. M. J. Harrington, S. S. Wasko, A. Masic, F. D. Fischer, P. Pratzl, Pseudelastic behaviour of a natural material is achieved via reversible changes in protein backbone conformation. *J. R. Soc. Interface* **9**, 2911–2922 (2012).
16. R. Paquin, P. Colombo, Nanomechanics of single keratin fibres: A Raman study of the $\alpha$-helix $\rightarrow$ $\beta$-sheet transition and the effect of water. *J. Raman Spectrosc.* **38**, 504–514 (2007).
17. L. Galvis, J. W. C. Dunlop, G. Duda, P. Pratzl, A. Masic, Polarized Raman anisotropic response of collagen in tendon: Towards 3D orientation mapping of collagen in tissues. *PLOS One* **8**, e63518 (2013).
18. E. O. Potma, X. S. Xie, Detection of single lipid bilayers with coherent anti-Stokes Raman scattering (CARS) microscopy. *J. Raman Spectrosc.* **34**, 642–650 (2003).
19. J. P. R. Day, G. Rago, K. F. Domke, K. P. Velikov, M. Bonn, Label-free imaging of lipopolipid bioactive molecules during lipid digestion by multiplex coherent anti-Stokes Raman scattering microscopy. *J. Raman Spectrosc.* **38**, 504–514 (2007).
20. L. Galvis, J. W. C. Dunlop, G. Duda, P. Pratzl, A. Masic, Polarized Raman anisotropic response of collagen in tendon: Towards 3D orientation mapping of collagen in tissues. *PLOS One* **8**, e63518 (2013).
21. M. J. Harrington, S. S. Wasko, A. Masic, F. D. Fischer, P. Pratzl, Pseudelastic behaviour of a natural material is achieved via reversible changes in protein backbone conformation. *J. R. Soc. Interface* **9**, 2911–2922 (2012).
22. R. Paquin, P. Colombo, Nanomechanics of single keratin fibres: A Raman study of the $\alpha$-helix $\rightarrow$ $\beta$-sheet transition and the effect of water. *J. Raman Spectrosc.* **38**, 504–514 (2007).
23. L. Galvis, J. W. C. Dunlop, G. Duda, P. Pratzl, A. Masic, Polarized Raman anisotropic response of collagen in tendon: Towards 3D orientation mapping of collagen in tissues. *PLOS One* **8**, e63518 (2013).
24. M. L. Gardel, J. H. Shin, F. C. MacKintosh, L. Mahadevan, P. Matsudaira, D. A. Weitz, Elastic behavior of a natural material is achieved via reversible changes in protein backbone conformation. *J. R. Soc. Interface* **9**, 2911–2922 (2012).
25. M. L. Gardel, J. H. Shin, F. C. MacKintosh, L. Mahadevan, P. Matsudaira, D. A. Weitz, Scaling of F-actin network rheology to probe single filament elasticity and dynamics. *Phys. Rev. Lett.* **93**, 188102 (2004).
26. N. A. Kurniawan, J. Grimbergen, J. Koopman, G. H. Koenderink, Factor XIII stiffens fibrin clots by causing fiber compaction. J. Thromb. Haemost. 12, 1687–1696 (2014).

27. H. Duong, B. Wu, B. Tawil, Modulation of 3D fibrin matrix stiffness by intrinsic fibrinogen–thrombin compositions and by extrinsic cellular activity. Tissue Eng. Part A 15, 1865–1876 (2009).

28. J.-P. Collet, J. L. Moen, Y. I. Veclikh, O. V. Gorkun, S. T. Lord, G. Montalescot, J. W. Weisel, The αC domains of fibrinogen affect the structure of the fibrin clot, its physical properties, and its susceptibility to fibrinolysis. Blood 106, 3824–3830 (2005).

29. G. W. Nelb, C. Gerth, J. D. Ferry, Rheology of fibrin clots: III. Shear creep and creep recovery of fine lighted and coarse unligated clots. Biophys. Chem. 5, 377–387 (1976).

30. L. Pauling, R. B. Corey, The pleated sheet, a new layer configuration of polypeptide chains. Proc. Natl. Acad. Sci. U.S.A. 37, 251–256 (1951).

31. Z. Movasaghi, S. Rehman, I. U. Rehman, Raman spectroscopy of biological tissues. Appl. Spectrosc. Rev. 42, 493–541 (2007).

32. M. Berjot, J. Marx, A. J. P. Alix, Determination of the secondary structure of proteins from the Raman amide I band: The reference intensity profiles method. J. Raman Spectrosc. 18, 289–300 (1987).

33. A. Zhmurov, O. Kononova, R. I. Litvinov, R. I. Dima, V. Barsegov, J. W. Weisel, Mechanical transition from α-helical coiled coils to β-sheets in fibrinogen. J. Am. Chem. Soc. 134, 20396–20402 (2012).

34. J. H. Brown, N. Volkmann, G. Jun, A. H. Herschen-Edman, C. Cohen, The crystal structure of modified bovine fibrinogen. Proc. Natl. Acad. Sci. U.S.A. 97, 85–90 (2000).

35. W. M. Tolles, J. W. Nibler, J. R. McDonald, A. B. Harvey, A review of the theory and application of coherent anti-Stokes Raman spectroscopy (CARS). Appl. Spectrosc. 31, 253–271 (1977).

36. X.-J. Cheng, X. S. Xie, Coherent anti-Stokes Raman scattering microscopy: Instrumentation, theory, and applications. J. Phys. Chem. B 108, 827–840 (2004).

37. R. F. Doolittle, Structural basis of the fibrinogen–fibrin transformation: Contributions from X-ray crystallography. Blood Rev. 17, 33–41 (2003).

38. I. Azpiazu, D. Chapman, Spectroscopic studies of fibrinogen and its plasmin-derived fragments. Biochim. Biophys. Acta 1119, 268–274 (1992).

39. R. A. S. Aniêng, T.-S. Lai, J. W. Weisel, C. S. Greenberg, P. J. Grant, Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. Blood 100, 743–754 (2002).

40. C. C. Helms, R. A. S. Aniêng, S. Uitte de Willige, K. F. Standeven, M. Guthold, α–α Cross-links increase fibrin fiber elasticity and stiffness. Biophys. J. 102, 168–175 (2012).

41. M. A. Kotlarchyk, S. G. Sheim, M. B. Alvarez-Elizondo, L. C. Estrada, R. Singh, L. Valdevit, E. Kniazeva, E. Gratton, A. J. Putnam, E. L. Botvinick, Concentration independent modulations of local micromechanics in a fibrin gel. PLOS One 6, e20201 (2011).

42. C. Storm, J. J. Pastore, F. C. MacKintosh, T. C. Lubensky, P. A. Janmey, Nonlinear elasticity in biological gels. Nature 435, 191–194 (2005).

43. K. M. Weigandt, D. C. Pozza, L. Porcar, Structure of high density fibrin networks probed with neutron scattering and rheology. Soft Matter 5, 4321–4330 (2009).

44. J. W. Weisel, R. I. Litvinov, The biochemical and physical process of fibrinolysis and effects of clot structure and stability on the lysis rate. Cardiovasc. Hematol. Agents Med. Chem. 6, 161–180 (2008).

45. I. Varjú, P. Sotonyi, R. Machovich, L. Szabó, K. Tenekedijiev, M. M. C. G. Silva, C. Longstaff, K. Kolev, Hindered dissolution of fibrin formed under mechanical stress. J. Thromb. Haemost. 9, 979–986 (2011).

46. E. Potier, J. Noailly, C. M. Sprecher, K. Ito, Influencing biophysical properties of fibrin with buffer solutions. J. Mater. Sci. 45, 2494–2503 (2010).

47. N. Billecke, G. Rago, M. Bosma, G. Eijkel, A. Gemmick, P. Leproux, G. Huss, P. Schrauwen, M. K. C. Hesselink, M. Bonn, S. H. Parekh, Chemical imaging of lipid droplets in muscle tissues using hyperspectral coherent Raman microscopy. Hitoschem. Cell Biol. 141, 263–273 (2014).

48. W. Liu, C. R. Carlisle, E. A. Sparks, M. Guthold, The mechanical properties of single fibrin fibers. J. Thromb. Haemost. 8, 1030–1036 (2010).

Acknowledgments: We are grateful to J. Baio, A. Liu, G. Koenderink, D. Bonn, K. Koyun, K. Kremer, K. Daoulas, and J. Hunger for helpful and critical discussions; V. Balos for initial protocols for fibrinogen cleavage; and the Molecular Imaging Laboratory for general support. F. Gericke and A. Hanewald provided excellent technical support for setup construction and rheological measurements, respectively. Funding: F.F. is supported by a PhD fellowship from the Max Planck Graduate Center, and S.H.P. acknowledges financial support from the Deutsche Forschungsgemeinschaft (grant #PA 2526/1-1) and the Marie Curie Foundation (grant #CIG322284). Author contributions: S.H.P. conceived the study. F.F. and S.H.P. designed and built the experimental setup; F.F. performed all experiments. F.F., M.B., and S.H.P. analyzed the data. F.F., M.B., and S.H.P. wrote the paper and discussed the results. S.H.P. supervised the study. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 7 December 2015
Accepted 13 June 2016
Published 8 July 2016
10.1126/sciadv.1501778

Citation: F. Fleissner, M. Bonn, S. H. Parekh, Microscale spatial heterogeneity of protein structural transitions in fibrin matrices. Sci. Adv. 2, e1501778 (2016).