4. Fibrinogen Unfolding During Fibrin Gel Stretching

Fibrin is a transient extracellular matrix that forms upon vascular injury as a first step towards wound healing. Fibrin fibers can be extended several times their own length without rupture, though the origin of this extraordinary extensibility is still poorly understood. In this chapter, we study the origin of fibrin extensibility by using small-angle X-ray scattering (SAXS) in combination with tensile tests. SAXS measurements provide insight into both the axial and lateral molecular packing structure of the fibers. We show that the degree of axial and radial packing order is dependent on fibrin bundle size, which we relate to the mesh size of the network that puts an upper bound on the range of crystalline order. By comparing our SAXS measurements with full-atom simulations of protofibrils, we show that the second order reflection of the half-staggering axial repeat distance is suppressed due to the symmetric structure of the fibrinogen monomer. Upon stretching, however, several new peaks appear, including the second order reflection. At high strains of ~100%, the peaks indicative of axial order disappear, showing that stretching causes forced molecular unfolding. To trace the molecular origin of these changes, we compare our SAXS results with predicted SAXS spectra based on full-atom simulations of stretched protofibrils. Taken together, our experiments and simulations directly show that protofibril elongation is mediated by 1) elongation of the alpha-helical coiled-coil regions and 2) γ-nodule unfolding, which starts at 30% strain. These results are important to understand the reduced extensibility in case of disease-related fibrinogen mutations and the influence of FXIIIa crosslinking on fibrin fiber extensibility.

The formation of a fibrin network during blood clotting is one of the first steps towards wound healing [330]. The fibrin network serves as a plug to stop bleeding and provides a scaffold for cells that mediate wound healing. Fibrin formation is initiated by enzymatic conversion of the soluble plasma protein fibrinogen to its activated form, referred to as fibrin [441]. The fibrinogen monomer is comprised of two identical subunits, each of which is formed by three polypeptide chains denoted Aα, Bβ and γ. These chains fold into a trinodular structure that is around 46 nm in length and 4.5 nm in diameter [350]. The central E-region is formed by the N-terminal portions of all the chains, while the terminal D-regions are formed by the C-terminal portions. The D-regions consist of a number of structurally distinct domains, including the β and γ-nodules [442]. The middle portions of the Aα, Bβ and γ chains form rod-like α-helical coiled-coil connectors between the E- and D-regions. The C-terminal ends of the Aα-chains known as the αC regions form globular αC-domains that are tethered to the molecule with the flexible αC-connectors [359]. Thrombin triggers fibrin clot formation by sequentially removing two sets of fibrinopeptides known as FpA and FpB from the N-termini of the Aα and Bβ chains. Removal of the FpA peptides exposes A-knobs in the E-region that are complementary to ‘a’ holes in the D-regions, thus causing half-staggered self-assembly into two-stranded protofibrils [443]. Subsequent removal of the FpB peptides exposes B-knobs in the E-region that are complementary to ‘b’ holes in the D-regions. Both B:b interactions and interactions between the alphaC-domains of adjacent protofibrils are thought to contribute to protofibril bundling [330, 358, 444, 445]. Under near-physiological (‘coarse clot’) conditions, purified fibrin forms fibers that are bundles of several tens of protofibrils [66]. In contrast, at high pH and ionic strength, protofibril bundling is inhibited and so-called ‘fine clots’ are formed (see Chapter 2). The fibers are mechanically reinforced by activated Factor XIII (FXIII), which forms covalent bonds between the γ and α chains [446].

The mechanical properties of fibrin significantly affect the biological functions of fibrin [447, 448]. Blood clotting requires that fibrin networks are sufficiently elastic and strong that they can withstand the mechanical forces applied by flowing blood [449, 450] and the contractile forces applied by platelets [420, 451]. Moreover, fibrin networks need to provide appropriate mechanical resistance to the contractile forces applied by cells during wound healing [67, 270]. Yet, abnormally stiff networks formed in case of excessive crosslinking can also be disadvantageous since they are not easily lysed and cause thrombotic diseases [343]. Both whole plasma clots and purified fibrin networks exhibit remarkable elastic properties: they stiffen strongly and can be reversibly sheared or stretched up to strains in excess of 100% [66, 366, 389, 413]. Mechanical measurements on single isolated fibers have shown that the fibers
themselves also stiffen when stretched [282], and that they can be stretched up to four-fold their original length without breaking [283, 333, 334].

The physical basis of the exceptional nonlinear elastic behavior of fibrin is still under debate. Fibrin networks are structured across multiple scales, from the molecular (monomer) scale, to the protofibril, the fiber, and finally the network scale. It is difficult to disentangle the distinct contribution of each of these scales to the overall macroscopic response. At the network scale, the elastic properties of fibrin have been modeled by polymer theories, which treat fibrin fibers as uniform semiflexible polymers with a certain stretch and bend rigidity [39]. These models indeed predict a strain-stiffening response. However, the extreme extensibility of fibrin networks can only be understood when the internal structure of the constituent fibers is explicitly modeled.

There are two main models to explain the extensibility of fibrin fibers. The first model relies on forced unfolding of the fibrin monomers. All-atom Molecular Dynamics (MD) simulations of the force-extension behavior of single fibrin molecules showed that stretching causes molecular elongation by an interplay between unwinding of the α-helical coiled-coil connectors and unfolding of the γ-chain nodules [299, 335]. The simulations further predicted that the coiled-coil connectors undergo an α-helix to β-strand conversion, resembling the stretch-response of coiled-coil domains in intermediate filaments and myosin [452, 453]. Each coiled-coil consists of 111 or 112 amino-acid residues of the Aα-, Bβ-, and γ-chains, and can thus contribute a 23-nm molecular extension as it goes from a folded length of 17 nm to an unfolded length of 40 nm, as supported by molecular simulations and AFM measurements [426]. Unfolding the γ-chain nodules can contribute another 160 nm molecular extension [299]. However, it is unclear how intermolecular contacts between fibrin molecules packed together in a fiber will influence the unfolding process. This question is difficult to address in view of the large size and complexity of the fibers. Furthermore, it is unclear what force levels fibrin molecules will experience locally within a strained network, especially in dilute networks that are known to experience inhomogeneous (nonaffine) deformations [413, 454].

The second model to explain the extensibility of fibrin fibers has focused on their bundle-like supramolecular structure, which consists of relatively rigid protofibrils coupled by long and rather flexible carboxy-terminal extensions of the Aα-chains that protrude from the protofibrils [359]. Computational modeling of this composite structure showed that stretching of the unstructured αC-connector region of the α-chain can in principle explain the extensibility and strain-stiffening of fibrin fibers [365]. Experimental support for this model comes from force-extension measurements on fibers assembled from fibrin of different species, which demonstrated that a longer αC-chain region correlates with greater extensibility [337]. However, there is also some experimental evidence against a supramolecular origin of nonlinearity. As shown in Chapter
2, we have evidence from rheology studies that fine clots, which mostly lack a bundle organization, show the same elastomeric response as coarse clots. This observation does not change when α-α-crosslinking is inhibited. The extensibility and strain-stiffening behavior thus occur independent of the αC-chain regions and is apparently intrinsic to the protofibrils themselves, lending support to the molecular unfolding model.

A few studies have sought to directly test the molecular mechanism that underlies the elastomeric response of whole fibrin networks by combining macroscopic mechanical testing with in situ structural measurements. There are some clear hints from Small Angle X-Scattering (SAXS) studies on stretched fibrin films and gels that molecular unfolding does occur at high tensile strains. The scattering peak corresponding to the half-staggered axial repeat distance (22.5 nm) of fibrin was shown to increase in width and decrease in height at increasing strains, indicating increased disorder [389,455,456]. One study even showed disappearance of the peak at a tensile strain of 100% strain [389]. More direct evidence for force-induced changes in secondary structure comes from vibrational spectroscopy measurements, which confirmed the predicted α-helix to β-strand conversion of the coiled-coil regions in stretched and compressed fibrin gels [336]. Further qualitative evidence for this structural transition has come from staining with the β-sheet-specific dye Congo Red [366]. However, the generality of these observations is unclear and the details of the unfolding pathway, such as onset strain and sequence of domain unfolding, are unknown.

Here we aimed to test directly whether forced unfolding of fibrin molecules occurs during macroscopic fibrin network stretching, and if so, to identify the unfolding pathway. To this end, we probed the molecular packing structure of fibrin gels that were reconstituted in vitro by in situ SAXS measurements during gel stretching with a uniaxial tensile tester. SAXS is widely used for structural characterization of soft matter on small length scales (\(\sim\)1-100 nm) [457]. This range of scales nicely matches the typical length scales associated with the molecular packing structure of fibrin as well as other protein fibers like collagen [458] and silk [459]. Moreover, SAXS is non-invasive and can probe structural information in a label-free manner. These features make SAXS an ideal method to study changes in molecular structure taking place in macroscopic samples during deformation. However, it is not straightforward to identify changes in molecular structure from SAXS data for complex systems such as fibrin.

Long-range order in the molecular packing arrangement of fibrin monomers in the fibers gives rise to Bragg diffractions, which show up as peaks at characteristic distances in the SAXS spectra [295,298,460,461]. Changes in molecular structure can be inferred from changes in the positions, heights, and widths of these Bragg peaks provided that a model is available of the molecular packing structure. Unfortunately, modeling of scattering from fibrin is complicated due
to the complexity of its molecular packing structure. Fibrin fibers are known
to exhibit long-range axial packing order resulting from half-staggered overlap
of the fibrin monomers since early SAXS measurements dating back to [460] as
well as electron microscopy (EM) studies [289]. Intriguingly, third and higher-
order reflections of the 22.5 nm axial repeat distance are usually visible, but the
second order reflection is almost always lacking in SAXS spectra [298,460,461].
The origin of this peak suppression is unclear. For fibrin samples with thin
fibers, also the first order reflection of the half-staggering distance is not clearly
visible [456]. Furthermore, the cross-sectional packing structure of fibrin fibers
is still poorly understood. Some X-ray scattering and EM studies concluded
that there is no ordered, lateral packing in fibrin fibers [460,462], while others
found evidence of lateral crystallinity [298,463,464].

Here we provide SAXS data for fibrin gels obtained during stretching, to-
gether with a molecular interpretation based on a direct comparison of our
data with full atom simulations of fibrin protofibrils from our co-workers,
Artem Zhmurov (Moscow Institute of Physics and Technology), and Valeri
Barsegov (University of Massachusetts Lowell). To understand the influence
of the molecular packing structure of fibrin fibers on the SAXS spectra, we
first performed SAXS measurements on unstretched fibrin gels with varying
bundle sizes. We show that the axial molecular packing structure of fibrin
can explain the suppression of the second order reflection of the 22.5 nm axial
repeat. Further, we find that the Bragg peaks corresponding to axial order are
much more pronounced for thicker fibers and that the peak width is correlated
to the mesh size of the network. The SAXS spectra confirm a recent model de-
scribing the radial packing structure of fibrin fibers as partially ordered [295].
When the fibrin gels are subjected to uniaxial stretch, we find clear evidence
for forced molecular unfolding. The second order reflection of the 22.5 nm
axial repeat distance appears when the strain reaches \( \sim 30\% \), which coincides
with unfolding of the \( \gamma \)-nodules in simulations of single protofibril stretching.
Furthermore, peaks corresponding to axial packing order become broader and
less intense with increasing strain, indicative of increased disorder. At strains
of \( \sim 90-100\% \), the peaks disappear, but only for slow pulling rates. By com-
bing SAXS experiments with MD simulations, we can now understand the
molecular basis of fibrin’s extensibility and strain-stiffening response. Forced
unfolding may also help explain recent findings that mechanical stretch pro-
tects fibrin fibers against fibrinolysis [465–467].

4.2 Materials and Methods

4.2.1 Fibrin Polymerization

Human fibrinogen depleted of Von Willebrand Factor (VWF), fibronectin and
plasminogen (FIB3) as well as human a-thrombin were purchased from En-
zyme Research Laboratories (Swansea, UK). The fibrin networks were in all
cases covalently crosslinked by fibrinoligase (FXIIIa), which is present in the fibrinogen stock, resulting in a constant molar ratio of FXIIIa with respect to fibrinogen. Experiments were performed on so-called ’coarse clots’, which were formed in near-physiological conditions, at 37°C and in fibrin buffer containing 20 mM Hepes, 150 mM NaCl and 5 mM CaCl$_2$ at pH 7.4. Fibrin formation was initiated by adding 0.5 U/mL thrombin (final concentration) and allowed to proceed for at least 4 hours before experiments. Fibrin’s $\gamma$- and $\alpha$-chains were fully crosslinked according to SDS-PAGE analysis over the entire fibrin concentration range that was tested (4 to 8 mg/ml).

To test how the influence of fiber bundle size (expressed in terms of the average number of protofibrils per fiber, $N_p$) influences the SAXS spectra, we also prepared samples under conditions designed to either increase or decrease $N_p$.

To increase $N_p$, we used two different strategies. The first strategy used FIB3 fibrinogen that was first dialyzed into fibrin buffer (without CaCl$_2$) for 2 days. The protein concentration after dialysis was determined by measuring the absorbance at a wavelength of 280 nm with correction for scattering at 320 nm [292]. This resulted in a larger number of protofibrils in a bundle compared to the non-dialyzed case: $N_p = 120$ compared to $N_p = 84$ at $c_p = 1$ mg/ml, as determined by turbidimetry. Since we did not measure $N_p$ at the concentration used for the SAXS experiments, we assumed $N_p \sim 100$ for the 8 mg/ml samples. The second strategy, which increased $N_p$ further, was inspired by a recent study showing that removal of fibrinogen oligomers, which are generally present in solutions of purified plasma fibrinogen, results in an increase in protofibril lateral association [344]. Briefly, FIB3 fibrinogen was filtered through a 0.2 $\mu$m filter and injected at a concentration of 2.7 mg/ml onto a Superdex 200 gel filtration column, which was equilibrated with fibrin buffer (20 mM HEPES, 150 mM NaCl, pH 7.4), at room temperature. The flow rate was 0.5 ml/min (at a pressure of ~0.12 MPa). The fibrinogen monomer fraction corresponding to the second peak [344] was collected and concentrated to ~15 mg/ml by centrifugation in MacroSep centrifuge tubes (Pall Corporation) at 811 rcf. The tubes were washed with buffer before use. The fibrinogen monomer fraction was snap-frozen and stored at -80°C. This strategy gave $N_p = 366$ at $c_p = 1$ mg/ml, as measured by turbidimetry. Since we did not measure $N_p$ at the concentration used for SAXS measurements ($c_p = 4$ mg/ml), we will assume $N_p \sim 366$ for the 4 mg/ml samples. Fibrin gels from dialyzed or gel-filtered fibrinogen were allowed to polymerize for 4 hours at 37°C in fibrin buffer supplemented with 5 mM CaCl$_2$.

To decrease $N_p$ relative to the 'coarse clot' conditions, we created so-called 'fine clots' [292,338,339]. Briefly, fine clots were generated by dialyzing FIB3 fibrinogen for at least 2 days at 4°C against fine clot buffer (50 mM TRIS-HCl, 400 mM NaCl, pH adjusted to 8.5 with NaOH). The final fibrinogen
concentration was determined by measuring the solution absorbance at 280 nm with correction for scattering at 320 nm [292]. Fine fibrin clots were polymerized for 1 hour at 37°C by adding 0.5 U/ml thrombin in fine clot buffer with 3.2 mM CaCl₂. This procedure resulted in a dense network with thin fibrin fibers with \( N_p = 2 \), independent of fibrinogen concentration (see also Chapter 2).

### 4.2.2 Small Angle X-ray Scattering (SAXS) and Tensile Tests

Small Angle X-ray Scattering (SAXS) was performed at the DUBBLE Beamline (BM26B) of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) [468]. The range of wavevectors, \( q \), was calibrated using silver behenate powder as a standard (see Fig. 4.4). The sample-to-detector (P1M) distance was 2.8–3 m and the energy of the beam was 12 keV. Measurements of fibrin gels inside glass capillaries (i.e. 0% strain, ‘capillary measurements’) were performed using 2 mm borosilicate glass capillaries with 0.01 mm wall thickness (cat.nr. 4007620, Hilgenberg, Germany). Capillaries filled with buffer were taken as background. Since there can be variations in capillary thickness, we ensured that a background was taken for every capillary and on every spot we measured, before we polymerized the fibrin gels in the same capillaries. The beam dimensions on the sample were 900x700 \( \mu \)m.

For stretching experiments, a Linkam tensile tester (TST350, Linkam Scientific Instruments, UK) was installed in the beam path. The tensile tester was equipped with a 20 N force transducer with 0.001 N force resolution. The samples were subjected to a stepwise increasing strain ramp, with a pulling speed of 7 (5 samples), 10 (6 samples) or 50 (3 samples) \( \mu \)m/s. After each strain interval, we measured for 20 s at 5 or 6 different positions in the sample. For fine clot samples, we measured for 30 s up to 2 min per spot due to low scattering signal. No visual damage was observed, nor abrupt changes in the force level, during the SAXS measurements. The beam dimensions on the sample were \( \sim \)400x700 \( \mu \)m. Dog-bone shaped fibrin gels were created with two pieces of Velcro (\( \sim \)6.5x6 mm) in home-made Teflon molds with a sample length of 22 mm. These molds were cleaned with 70% ethanol, treated with a thin layer of mineral oil to facilitate removal of the fibrin gels from the mold, and preheated to 37°C before use. The Velcro pieces were also cleaned in 70% ethanol. The sample volume was 850 \( \mu \)l and the initial sample dimensions were 5.2 mm in width and 2.8 mm in thickness. About half of the Velcro area was clamped in the tensile tester. The initial slack in the gel was pulled out before the strain measurement was started and sample buffer was added every other strain step to prevent drying.
Figure 4.1: A manual sample stretcher developed in-house. The sample was clamped as indicated and manually stretched, where one knob turn translated into 1 mm stretching. When the desired distance was reached, the distance was locked (inset, red arrows) and the sample was detached from the stretcher (inset, white arrows) and transported to a fixative solution to fix the sample at that stretched distance. The total length of the stretcher was about 170 mm.

4.2.3 Imaging and Analysis of Fiber Alignment

To determine the degree of fiber alignment at increasing strain levels, a manual stretcher was developed in-house (Fig. 4.1). Alexa488 labeled fibrinogen was purchased from Life Technologies (Bleiswijk, the Netherlands), dissolved in coarse clot buffer (without CaCl$_2$) and mixed with unlabeled fibrinogen in a 1:30 down to 1:80 molar ratio. Dog-bone shaped fibrin samples of 8 mg/ml (dialyzed coarse clots) with Velcro pieces at their ends were clamped in a similar fashion as for the SAXS measurements. The pulling speeds used in SAXS experiments were approximated (≈5−40 µm/s). Buffer was applied to the sample every mm of straining. When the desired strain level was reached, the stretched distance was locked (Fig. 4.1 inset) and the sample was fixed in a 2.5% glutaraldehyde solution in fibrin buffer for at least 3 hours and up to overnight. During this time, the sample was protected from light to prevent photobleaching. The fixed samples were cut into smaller pieces with a sharp surgery knife and transported by a tweezer to a glass-bottom petridish (Mattek Corporation, USA). Buffer was added to prevent drying. The fibrin network was imaged on a confocal fluorescence microscope using a Nikon Eclipse Ti inverted microscope equipped with a 100xoil immersion objective (NA 1.49), a 488-nm laser (Coherent, Utrecht, The Netherlands) for illumination, and a
photomultiplier tube detector (A1; Nikon, Amsterdam, the Netherlands). Z-stacks of 10 µm thick, taking an image every 0.125 µm, were collected 10 µ into the sample. Confocal reflectance microscopy on unstretched (and unlabeled) fibrin samples polymerized inside sealed glass flowchambers was performed on the same setup, using a 488-nm laser for illumination.

We quantified the alignment by using the ImageJ plugin OrientationJ [439] on filtered maximum intensity projections of varying strain levels (using 10 µm thick z-stacks and a bandpass filter to reduce noise). Briefly, OrientationJ evaluates the local orientation and coherency of every image pixel and computes a distribution of angles. A Gaussian window of 2 or 3 pixels and a Gaussian gradient was used to determine the local intensity derivative in x and y [439]. Using these orientation images, we calculated the nematic order parameter. Given a collection of orientation measurements, \( \psi \), in the range \((-90^\circ, 90^\circ]\), the nematic order parameter is computed from the second-order tensor order-parameter \( S_2 \) [440]:

\[
S_2 = \begin{bmatrix}
\langle \cos 2\psi \rangle & \langle \sin 2\psi \rangle \\
\langle \sin 2\psi \rangle & -\langle \cos 2\psi \rangle
\end{bmatrix}
\]

(from equation 4.1)

Angle brackets \( \langle \cdot \rangle \) denote averages over all orientation measurements. The tensor \( S_2 \) is symmetric and traceless. Solving the eigenvalue problem for \( S_2 \) yields two eigenvalues,

\[
\lambda_{1,2} = \pm \sqrt{\langle \cos 2\psi \rangle^2 + \langle \sin 2\psi \rangle^2} = \pm S
\]

(from equation 4.2)

which yield the (two-dimensional) scalar order-parameter \( S \) familiar for liquid crystals. This order parameter quantifies the width of the distribution of orientation measurements. It is zero for a uniform distribution of orientations, and approaches one for a sharply-peaked distribution. We find that in practice \( S \) is between 0.1 and 0.2 for isotropic networks, taking into account the coherency of every image pixel.

4.2.4 Modeling

The structure of fibrin protofibrils

To interpret the SAXS spectra, we will compare our data to an in silico model of fibrin oligomers and protofibrils developed by our collaborators, Artem Zhmurov (Moscow Institute of Physics and Technology), and Valeri Barsegov (University of Massachusetts Lowell). The modeling procedure is described in full detail elsewhere [299, 469], and will be briefly recapitulated here. The starting point was the crystal structure of full-length human fibrinogen (PDB code 3GHG) [329]). The \( \alpha \)C-region (Aα221-610), which does not appear in the crystal structure and is unlikely to contribute to the SAXS scattering, was not included in the structure, unless stated otherwise. There are several unresolved portions of the molecule in the crystal structure data: residues 1-26, 1-57, and
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1-13 at the N-termini of the Aα, Bβ and γ chains, respectively; and residues 201-610, 459-461, and 395-411 at the C-termini of the Aα, Bβ and γ-chains, respectively. These regions do not possess stable secondary/tertiary structures detectable by X-ray crystallography. Hence, they were reconstructed computationally using the VMD software package [470]. The complete structure was energy-minimized by a steepest descent algorithm to remove steric clashes.

A computational procedure was developed to reconstruct double-stranded oligomers denoted as FOn-m, where n and m are the number of fibrin monomers in the upper and lower strands, respectively. Fibrin oligomers from FO2-1 up to FO4-4 were constructed by this procedure. The procedure entailed the following steps:

i) Fibrin monomers were first connected head-to-tail through the D-D interface to form single-stranded oligomers. The monomers were placed such that the D-D interface resembled the PDB structure of the double-D region 1N86 [471] and 1FZG [472];

ii) The Kabsch algorithm for structure alignment [473] was used to position the double-D fragment such that the globular parts overlapped with the corresponding regions in the two abutting fibrin molecules;

iii) The C-terminal parts of the γ-chains in the aligned fibrin monomers were crosslinked in silico to mimic FXIII-mediated crosslinking. Specifically, two covalent bonds were created, one linking residues γGln398 and γLys406 in one γ chain with residues γLys406 and γGln398, respectively, in the other γ chain;

v) A double-stranded oligomer was created by adding a lower strand with a third monomer to the upper strand containing the crosslinked dimer in a half-staggered manner. Initial placement was achieved by Monte-Carlo docking simulations, employing translational and rotational moves;

(vi) The knobs ‘A’ and ‘B’ were inserted into the respective holes ‘a’ and ‘b’ and an energy minimization simulation run was performed. Next, 100 ns-long equilibration in full-atomic resolution was performed using the solvent accessible surface area (SASA) implicit solvent model [474]. In these simulations, the knob-hole bonds were stabilized by using a weak harmonic potential.

(vii) The thus generated oligomer FO2-1 was energy-minimized and equilibrated.

(viii) Oligomers up to FO4-4 were generated by repeating steps (i) through (vii).

To investigate the structural changes in fibrin protofibrils upon stretching, all-atom Molecular Dynamics (MD) simulations were performed in implicit solvent. The FO4-4 oligomer was selected as the model system for these pulling simulations. The geometry of the in silico pulling experiments is shown in Fig 4.2. The oligomer was truncated in the two coiled-coil regions to mimic the long oligomer structure. Since there are six polypeptide chains cut at both
Figure 4.2: Dynamic force spectroscopy \textit{in silico}. To mimic AFM pulling experiments, the left end of the fibrin oligomer FO4-4 was constrained (fixed plane) and a ramped force $f = v_t t$ (where $v_t$ is the pulling speed) was applied to the right end (pulled plane). The oligomer was truncated in the two coiled-coil regions to mimic the long oligomer structure and to ensure that the force was applied evenly to all six polypeptide chains within each monomer.

Besides, the force is thus applied evenly to all chains. Earlier simulations of our collaborators of forced unfolding of single fibrin molecules showed that, in the course of their forced unfolding, the fibrin monomers also rotate around the longitudinal axis [299,335]. For this reason, the amino acid residues tagged by the external pulling force should be allowed to move freely. To ensure this condition, the following simulation approach was developed. Two parallel planes were defined, perpendicular to the axis of the protofibril. The plane at one end of the molecule was constrained (fixed plane), while the other plane (pulled plane) was assumed to be connected to a cantilever tip (virtual bead) by a harmonic spring, mimicking a typical AFM pulling experiments. The end of this spring moved with a constant (cantilever) velocity, thus applying a dynamic force-ramp with a pulling force $f = v_t t$ that increased with the force-loading rate $v_t$. In each fibrin oligomer, residues $\alpha$Asp114, $\beta$Ser144, and $\gamma$Ser86 in the upper strand and residues $\alpha$Gln131, $\beta$Asn164, and $\gamma$Ser105 in the lower strand in the leftmost monomers were constrained. The mechanical force was applied to residues $\alpha$Arg118, $\beta$Lys148, and $\gamma$Leu90 in the upper strand and residues $\alpha$Ile127, $\beta$Asn160 and $\gamma$Leu101 in the lower strand in the rightmost monomer.

**Reconstruction of long protofibrils**

The oligomers constructed \textit{in silico} are much shorter than the (coarse) fibrin fibers in the experiments, where the length between branch points is typically of order $\sim 1 \, \mu m$ or $\sim 23$ fibrin monomers based on the typical mesh size of coarse fibrin networks. To enable a direct comparison of the measured SAXS profiles with computational predictions, our collaborators therefore computationally reconstructed protofibrils with a length of $\sim 1 \, \mu m$. Since the fibrin protofibrils twist around their long axis, simple translation and replication of
Figure 4.3: *In silico* reconstruction of a long fibrin protofibril from short oligomers for computational predictions of SAXS spectra. (a) First, two short oligomer constructs are brought close together by translation and rotation around the protofibril axis until the amino acid residues of the left construct (green dots and arrows) match with the residues on the right construct (yellow dots and arrows). (b) Next, the structures are combined. (c) A third oligomer is added in a similar manner. This is an iterative step that can be repeated until the protofibril is of the required length.

the oligomer structure would result in gaps between repeated structural fragments (FO4-4). Instead, the following procedure was developed. First, the oligomers were truncated so that the amino acid residues at one end were the same as the amino acids at the other end (green and yellow dots in Fig. 4.3). Two copies of the fragment were brought together so that the Cα-atoms of matching amino acids were as close as possible. To this end, the replicated fragment was translated and rotated around its long axis (Fig. 4.3), leading to a protofibril fragment that was twice the length of the initial fragment, FO4-4. This procedure was repeated until the protofibril was ∼23 fibrin monomers long.

**Prediction of SAXS spectra based on simulated structures**

Consider a system of $N$ independent scattering particles forming a fibrin fiber, where each $i$-th scatterer has a scattering strength $f_i$. The total scattering intensity $I$ as a function of the wavevector $q$ is given by:

$$I_f(q) = I_e(q) \sum_i \sum_j f_i f_j \cos(qr_{ij})$$

(4.3)

where $\vec{r}_{ij}^2$ is the vector connecting the $i$-th and $j$-th scatterers and $r_{ij}$ is the projection of $\vec{r}_{ij}^2$ on the direction perpendicular to the primary wave. $I_e = (I_0 e^2/mc^2)(1 + \cos^2(2\theta))/(2d^2)$ is the scattered intensity by a single electron
at a distance $d$ from the detector when the incoming wave has an intensity $I_0$ [457, 475]. $e^2/mc^2$ is the classical electron radius [457]. The wavevector is related to the scattering angle $2\theta$ according to:

$$q = |q| = 4\pi \sin(\theta)/\lambda$$

(4.4)

where $\lambda$ is the wavelength of the incoming light. In case of identical scatterers (such that $f_i = f$ for all $i = 1, 2, ..., N$), we can write a scalar version of eq. 4.3:

$$I_f(q) = I_e^*(q) \sum_i \sum_j \cos(qr_{ij})$$

(4.5)

where $I_e^* = f^2 I_e$. To simplify the calculations, the distribution of inter-atomic distances, $p(r)$, was used, using as input the atomic structure of the oligomers obtained from the MD simulations. The scattering pattern of a single protofibril can then be calculated (in the large $N$ limit) as:

$$I_f(q) = I_e^*(q) \int_0^{r_{\text{max}}} p(r) \sin(qr) \frac{dr}{qr}$$

(4.6)

where $r_{\text{max}}$ is the maximum inter-atomic distance. For a network of protofibrils we need to integrate the scattered intensity over all fiber orientations. In case of an isotropic 3D fibrin network, we obtain:

$$I^{3D}(q) = \langle I(q) \rangle^{3D} = I_e^*(q)$$

(4.7)

Our confocal microscopy experiments demonstrate that a small percentage ($\sim 11\%$) of fibrin fibers was already aligned even at zero strain (Fig. 4.17), consistent with prior observations [389]. We thus assumed that the scattered intensity of unstretched gels is a superposition of intensities for aligned fibers, $I_f(q)$, and unaligned fibers, $I^{3D}(q)$, according to:

$$I_{\text{tot}}(q) = p_a I_f(q) + p_u I(q)^{3D}$$

(4.8)

where $p_a = 0.11$ and $p_u = 1 - p_a = 0.89$ according to the confocal data and previous publications [389]. To compute spectra of strained gels, we used previously published SEM data on strain-dependent fibrin fiber alignment ($p_a(\varepsilon) = \langle \cos(2\theta) \rangle$ [389], where $\varepsilon$ is strain) shown in [389], as input. Upon stretching, two processes occur in parallel: (i) unaligned fibrin fibers will start to align, i.e. $p_a$ increases and $p_u$ decreases; and (ii) already aligned fibers will be stretched and they may exhibit forced unfolding. Hence, stretched gels can be decomposed into 3 fiber populations: (a) a fraction $p_u$ of still unaligned fibers, (b) a fraction of aligned but not stretched fibers, and (c) aligned and stretched fibers. Thus, the expression for $I_{\text{tot}}$ of strained gels becomes:
Chapter 4

section 4.3

\[ I_{tot}(q, \varepsilon) = p_u(\varepsilon)I^{3D}(q) + p_a(0)I_a(q, \varepsilon) + \int_0^\varepsilon \frac{dp_a(\varepsilon - s)}{ds}I_a(q, s)ds \quad (4.9) \]

Or in discrete form:

\[ I_{tot}(q, \varepsilon) = p_u(\varepsilon)I^{3D}(q) + p_a(0)I_a(q, \varepsilon) + \sum_{s=0, \delta\varepsilon, 2\delta\varepsilon,...}^{\varepsilon-\delta\varepsilon} [p_a(\varepsilon - s) - p_a(\varepsilon - s - \delta\varepsilon)]I_a(q, \varepsilon) \quad (4.10) \]

where \( I_a(q, \varepsilon) = I_f(q, \varepsilon), I_u(q) = < I(q) >_{3D} \), and \( \delta\varepsilon \) is the strain increment.

The total scattering intensity at strain \( \varepsilon \) is a combination of scattering from fibers that are unaligned \( (p_u(\varepsilon)I^{3D}(q)) \), fibers that are aligned and stretched \( p_a(0)I_a(q, \varepsilon) \), and a group of fibers that are in varying degrees stretched and oriented with the strain direction \( (\sum_{s=0, \delta\varepsilon, 2\delta\varepsilon,...}^{\varepsilon-\delta\varepsilon} [p_a(\varepsilon - s) - p_a(\varepsilon - s - \delta\varepsilon)]I_a(q, \varepsilon)) \).

In this third term, the summation is performed over aligned and stretched fibers that differ in the extent of their stretching/unfolding and the \( p(r, \varepsilon) \) distribution was taken from the simulated atomic structures of stretched fibrin protofibrils. To calculate the spectra for different strains, say for \( \varepsilon = 0\%, 5\%, 10\%, ..., 150\% \) with strain increment \( \delta\varepsilon = 5\% \), eq. 4.10 is used iteratively. For example, to compute the spectrum for a gel at 15\% strain, we need the spectrum for 0\% strain, etc. Given that the pulling simulations are limited to short fibrin protofibrils of \( \sim 10-50 \) fibrin monomers, the structural models were replicated along the protofibril axis, which coincides with the uniaxial direction of force application. Finally, to account for spectral line-broadening as a consequence of a widening distribution of stretched and unfolded fibrin monomers, we convolved the calculated spectra with a Gaussian function, \( G \), using the following convolution integral:

\[ \int_0^{q_{max}} I_{tot}(q', \varepsilon)G(q - q')dq' \quad (4.11) \]

The width of \( G(x) \) was estimated from the distribution of molecular elongations and a variety of stretched and unfolded structures from multiple forced unfolding runs and was fixed at 0.03 nm\(^{-1}\).

4.3 Interpretation of Scattering of Fibrin Networks

X-ray and neutron scattering can provide information about the periodic molecular packing of ordered materials, as illustrated by measurements on a powdered crystal in Fig. 4.4. The length scales that one can probe are related to the wavelength \( \lambda \) of the incoming X-rays by \( q = 4\pi \sin(\theta)/\lambda \) [457, 476], where
2θ is the angle between the incident and scattered X-rays. In small-angle X-ray scattering (SAXS), the X-rays scattered at low angles (typically at angles smaller than ~2 degrees) are collected using a specimen-to-detector separation of several meters. The scattering vector \( q \) is directly related to distances \( d_s \) in the sample by \( |q| = 2\pi/d_s \). Thus, by setting the angle range, the length scale region of interest can be selected. In practice, this is achieved by changing the sample-to-detector distance. For our SAXS experiments, we used silver behenate powder to check the \( q \)-range. This powder contains crystals with a triclinic unit cell, as shown in Fig. 4.4A [477]. Since the crystals are isotropically oriented in the powder, the scattering pattern consists of concentric rings, which correspond to Bragg reflections from the typical distances of the unit cell (Fig. 4.4B).

In Chapter 2, we showed that fibrin fibers can be considered as hierarchically structured bundles of protofibrils, which themselves are composed of half-staggered fibrin monomers. The fibers branch to form a three dimensional connected network. This network is homogeneous at large scales, but inhomogeneous at scales approaching the mesh size. To model the scattering intensity of such networks, the mesh work can be considered as a collection of fractal
blobs of size $\xi_{blob}$ with fractal dimension $D_m$, where each blob contains $n$ fiber segments (where $n \gg 1$) (see top schematic in Fig. 4.5). At the micron scale, the segments are approximately cylinders of average length $l$ and diameter $d$. While $d$ is equal to the fiber diameter, $l$ is related to the fiber persistence length rather than the network mesh size. Based on these assumptions, a model was proposed to predict the light scattering intensity of fibrous networks [478,479]:

$$I = K_{op} c_p M \cdot S(q) \cdot A(q) \cdot B(q)$$

(4.12)

where $c_p$ is the fibrinogen weight concentration, $M$ is the blob molecular weight, and $K_{op}$ is an optical constant: $K_{op} = (4\pi^2/N_A \lambda_0^2)n^2(\partial n/\partial c)^2$ and is equal to $2.68 \cdot 10^{-7}$ cm$^2$/g$^2$ [479]. Although this model was originally developed to describe light scattering data, the general form of this expression also applies to neutron and X-ray scattering data and has been validated by small angle measurements for wave vectors down to $q \sim 10^{-3}$ nm$^{-1}$ and for fibrin concentrations up to 40 mg/ml [480]. The $q$-dependent quantities in 4.12 are $S(q)$, which is the structure factor describing the spatial correlation between blobs, $A(q)$, which is the structure factor of the fiber segments, and $B(q)$, which is the form factor of a single segment. These quantities can be calculated using the following equations [478,479]:

$$S(q) = 1 - \beta e^{-\gamma \xi_{blob} q^2}$$

(4.13)

$$A(q) = \frac{1}{\left(1 + (q \xi_{blob} / \pi)^{D_m/2}\right)} + \frac{l}{\xi_{blob}}$$

(4.14)

$$B(q) = \frac{1}{\left(1 + q^2 d^2 \sqrt{\frac{l}{32 d}}\right)^{\alpha_s/2}}$$

(4.15)

$D_m$ is the mass fractal dimension of the blobs, $\alpha_s$ is related to the surface fractal dimension of the fiber segments by $D_s = 6 - \alpha_s \simeq 2$, and the parameters $\beta \simeq 1$ and $\gamma \simeq 0.28$ [478]. Here, the form factor of the fiber segments, $B(q)$, only accounts for the overall cylindrical shape. In case of coarse clots, the fiber segments have a complex molecular packing structure, being a bundle of protofibrils that themselves are double-stranded filaments of fibrin monomers. To account for this internal molecular packing structure, the factor $B(q)$ in Eq. 4.13 should be replaced by a structure factor to describe the lateral packing arrangement of the protofibrils multiplied with the form factor of a protofibril (either a cylinder approximation or the scattering intensity $I_f(q)$ that we calculate here from full atom MD simulations of protofibrils).

Fig. 4.5 shows the expected scattering intensity as a function of wavevector (black line) for a coarse clot using typical parameters ($\xi_{blob} = 10$ µm, $l = 0.5$
Figure 4.5: Typical length scales in fibrin networks visible by light, X-ray and neutron scattering. Light scattering probes length scales ranging from the fiber to the network scale (black curve in graph), where the fibrin network (confocal image on the top left, 100x100 µm) can be considered as a collection of blobs (green in top schematic) of fiber segments (blue in top schematic) with fractal dimension $D_m$ [478,479]. X-ray and neutron scattering can probe higher $q$-values (blue curve in graph), where the molecular length scales inside the fibers are probed, such as the axial half-staggering distance among the fibrin fibers (22.5 nm), and the typical spacing between the protofibrils in a fiber cross-section (~19 nm [74], purple in top schematic). The black scattering curve has been calculated using the model presented in eq. 4.12 using parameters expected for coarse fibrin networks ($\xi_{blob} = 10 \mu m$, $l = 0.5 \mu m$, $d = 0.2 \mu m$, $D_m = 1.3$ and $\alpha_s = 4$). The blue curve represents an actual SAXS measurement on a 4 mg/ml coarse fibrin network ($N_p \sim 366$). Curves are shifted along the y-axis (note arbitrary units).
\[ \mu m, \ d = 0.2 \ \mu m, \ D_m = 1.3 \ \text{and} \ \alpha_s = 4 \]. For small wave-numbers, \( q < q_1 \), the network can be considered as a homogeneous system. The scattering intensity exhibits a peak at a \( q \)-value \( q_1 \), which is related to the long-range order in the network [481] and is proportional to the average size of the blobs according to \( q_1 \approx 4.4/\xi_{blob} \) [478]. In the wavevector range where \( q_1 < q < q_2 \), the scattering intensity is determined by the fractal structure within the blobs, provided that \( q \) is larger than \( \xi_{blob}^{-1} \) but smaller than \( d^{-1} \) or \( l^{-1} \). In this case \( I \propto q^{-D_m} \). Prior experiments have shown that \( D_m \) depends on fibrinogen concentration: for dilute fibrin networks (\( \leq 1.5 \) mg/ml), \( D_m \) is close to 1.3 [478], while it can increase to 2.8 for high fibrin concentration (\( \sim 40 \) mg/ml) [480]. Additionally, \( D_m \) was shown to be dependent on the network architecture, varying between 1 and 2.2 for a range of assembly conditions [482–487]. For larger wave numbers, where \( q > q_2 \), we enter a Porod regime, which reflects scattering from the interface between the fibers and the solvent. Here, the surface fractal dimension of the fiber segments dominates the scattering intensity. Depending on the surface roughness, the slope \( \alpha_s \) is either 4 (completely smooth or sharp interface) or -3 (completely rough). Previous studies revealed \( \alpha_s \) values close to -4 for coarse clots [478,479]. The onset of this Porod regime is set by the diameter of the fiber segments according to \( q_2 \approx 2.2/d \) [295,478,479].

The \( q \)-range we are focusing on in this Chapter ranges from \( q \sim 6 \cdot 10^{-2} \) nm\(^{-1} \) to \( q \sim 3 \) nm\(^{-1} \), which corresponds to length scales ranging from \( \sim 105 \) nm down to \( \sim 2 \) nm. In this regime, we probe length scales corresponding to the molecular packing inside the fibrin fibers. When a fiber is oriented vertically in the X-ray beam, the X-rays scatter parallel to the fiber axis, giving rise to a meridional scattering pattern [458] that is sensitive to the half-staggered packing along the fibril axis. In contrast, the X-rays scattered at right angles to the fiber axis produce an equatorial pattern, which reveals the radial packing order of protofibrils within the fiber. If the fiber is rotated with respect to the detector, the pattern on the detector will be rotated accordingly. For an isotropic network of fibers, the meridional and equatorial pattern will therefore be superimposed. Since the patterns represent an average from all fibers encountered by the X-ray beam, both SAXS patterns will appear as a series of concentric circles.

The axial molecular packing structure of fibrin fibers is known to be highly ordered. Electron microscopy and AFM images have clearly revealed a half-staggered arrangement with a periodicity corresponding to half the monomer size, i.e. 22.5 nm [289,290,443]. Previous SAXS studies revealed sharp Bragg peaks, indicative of a large coherence length, at the expected first order \( q \)-value [298,389,455,460,463,480] and higher-order reflections of this same distance [298,455,460,462,463]. Strikingly, the second order was either very faint or absent. It is unknown why this is the case.

The cross-sectional packing of protofibrils inside the fibrin fiber is thought
Figure 4.6: There are two opposing models for the radial packing structure of the protofibrils within a fibrin fiber. One model describes fibrin fibers as fractals with fractal dimension $D_f = 1.3$ [364], while the other claims fibrin molecules are packed in a crystalline array with unit cell $19 \times 19 \times 46$ nm [74]. Recently, Yeromanahos et al [295] unified these models, describing fibrin fibers as crystalline but with pores where protofibrils are missing, leading to a superposition of the crystalline structure and the disordered, fractal structure.

To be less ordered than the axial packing, but there is disagreement about the degree of disorder. One EM study showed that even within a given preparation, the lateral packing order may vary from fiber to fiber [289]. As shown in Fig. 4.6, there are two rather different structural models proposed for the cross-sectional packing. The first model [74] is based on prior SAXS data [298] and proposes that protofibrils are packed in an ordered lattice with a tetragonal unit cell measuring $a \times c \times c = 18.4 \times 18.4 \times 46$ nm, where $18.4$ nm corresponds to twice the distance between protofibrils and $46$ nm to the length of one fibrinogen monomer. Thus, this model predicts the presence of a Bragg peak at a $q$-vector corresponding to a repeat distance of $18.4$ nm, with a width that corresponds to the fiber diameter if the packing is perfectly crystalline. Indeed some measurements by SAXS [461, 464], neutron diffraction [480, 488] and energy-dispersive X-ray diffraction [463, 464] revealed broad Bragg peaks, indicating a small crystal size and partial disorder. However, other SAXS studies found no evidence for lateral crystalline order [462]. The second model for protofibril packing instead treats fibrin fibers as disordered, fractal assemblies of protofibrils (Fig. 4.6). This model was inspired by AFM bending experiments and fluorescence intensity measurements, showing that fibrin fibers can be considered as mass fractals [364] with fractal dimension of 1.3. This means
that with increasing fiber diameter \(d\), the number of molecules per cross-section increases as \(d^{D_f}\) with \(D_f = 1.3\) instead of the quadratic increase expected for an ordered fiber. In other words, thinner fibers are predicted to be less dense than thicker fibers. This view is qualitatively consistent with light scattering studies, showing that fibrin fibers are highly porous and contain more than 70% water [295, 462]. This model predicts that the scattering intensity will scale as \(q^{-D_f}\) [489]. Recently, a reconciliation of these two models has been proposed by considering the lateral packing as crystalline, but with a large number of holes where protofibrils are missing (right most model in Fig. 4.6). This arrangement results in a superposition of a fractal-like scattering and a broad peak due to locally crystalline regions [295, 490].

At the highest \(q\)-values shown in Fig 4.5, when \(q \gtrsim 1\) nm\(^{-1}\), we enter a second Porod regime related to the intra-fiber surfaces [480] (Fig. 4.5), where we expect to see a \(q^{-4}\) dependence. The onset of this regime is related to the radius of one protofibril (\(q = 2\pi/r_{pf} \sim 1\) nm\(^{-1}\), using \(r_{pf} = 5\) nm [294]).

4.4 Results

4.4.1 Internal Structure of Fibrin at Zero Strain

We aimed to study the strain-induced changes of the molecular packing structure of fibrin fibers in gels subjected to a macroscopic uniaxial stretch. As a first step, we compared unstrained fibrin networks prepared with varying fibrin bundle size, expressed in terms of the number of protofibrils in a fibrin bundle, \(N_p\). We varied the bundle size over a wide range of \(N_p = 2\) up to \(\sim 360\). The 2D SAXS patterns were isotropic in all cases, as expected for a random fibrous network, as shown in Fig. 4.7. The patterns show concentric rings whose intensity increases with increasing fibrin bundle size. This effect is more clearly, and quantitatively, shown in Fig. 4.8A, in the form of the 1D SAXS spectra obtained by a radial integration of the 2D scattering patterns, taking the beamstop as center. Note that the curves are shifted along the y-axis for clarity.

The spectrum for the sample containing the thickest fibers (\(N_p \sim 360\)) exhibits a clear peak at \(q = 0.285\) nm\(^{-1}\) (top blue curve labeled ‘1’ in Fig. 4.8A). This peak corresponds to a repeat distance of 22.2 nm and can thus be assigned as the first order reflection of the half-staggered axial packing distance [389]. When the bundle size is decreased to \(\sim 100\) protofibrils per bundle, this same peak is still present, but with a reduced intensity (black line labeled ‘2’ in Fig. 4.8A). For networks of 8 mg/ml fibrin, where the average bundle size is \(\sim 20\), the first order reflection no longer visible (gray curve labeled ‘4’ in Fig. 4.8A)). Interestingly, a small peak re-appears when the fibrin concentration is reduced to 4 mg/ml (gray curve labeled ‘3’ in Fig. 4.8A)). When the bundle size is dropped to 2, corresponding to the fine clot limit, the first order reflection is not visible at all (red bottom curve labeled ‘5’ in Fig. 4.8A).
conclude that the intensity and width of the peak corresponding to the first order reflection of the axial repeat distance is dependent on the bundle size. This result is in agreement with earlier work, where the first order reflection was observed for coarse fibrin clots \[295,298,389,456,463,464,488\], but not for fine clots \[456\]. The peak position corresponds to a repeat distance of 22.2 nm for all bundle sizes, consistent with SEM and AFM studies showing a 22.5 nm repeat \[289,290\] and earlier SAXS studies showing 22 nm \[389\].

Prior SAXS studies of coarse clots revealed, in addition to the first order reflection, also several higher order reflections \[295,298,488\]. This observation is indicative of a long-ranged half-staggered packing order along the fibril axis. Given that the first order peak is observed at \(q = 0.28 \text{ nm}^{-1}\), the second, third and fourth order reflections are expected at \(q = 0.57\), \(q = 0.855\), \(q = 1.14 \text{ nm}^{-1}\), respectively (see vertical dashed lines in Fig. 4.8A). Interestingly, the third and fourth order reflections are evident in all samples, even for those samples where the first order reflection is absent. However, the third and fourth order peaks do get more pronounced with increased bundle size. Intriguingly, the second order reflection is absent for all fibrin samples. The absence of a second order reflection was also noted in prior SAXS studies \[295,460,461\], but the origin of peak suppression has remained unclear.

Figure 4.7: The dependence of 2D SAXS patterns on fibrin fiber thickness. (A) SAXS pattern (background subtracted) of a 4 mg/ml fibrin gel with thick fibers (bundle size \(N_p \approx 366\)), (B) 8 mg/ml fibrin gel with \(N_p \sim 100\) and (C) 8 mg/ml fine fibrin gel with \(N_p = 2\). (A) and (B) share the same y-axis, while (C) is taken with a slightly different q-range by translating the detector in \(x\) and \(y\), but keeping the same distance between the detector and sample. In (A) and (B), the first and third order reflections of the half-staggered axial packing periodicity of fibrin (22.5 nm) are highlighted by white arrows. In (C) these peaks are not distinguishable from the background. Intensity is in arbitrary units (see color bar).
Figure 4.8: The dependence of 1D SAXS spectra on fibrin fiber thickness obtained by radial integration of the 2D SAXS patterns. (A) SAXS spectra of fibrin gels prepared under different conditions: 1) 4 mg/ml fibrin gel with bundle size of $N_p \sim 366$ (blue), 2) 8 mg/ml coarse fibrin gel with $N_p \sim 100$ (black), 3) 4 mg/ml coarse fibrin gel with bundle size $N_p = 44$ (gray), 4) 8 mg/ml coarse fibrin gel with bundle size $N_p = 23$ (gray), and 5) 8 mg/ml fine fibrin gel with bundle size $N_p = 2$ (red). The intensity curves are shifted along the y-axis for clarity. The black dotted lines are power laws with exponents -4 (expected in the Porod regime), -1.3 (expected from the fractal radial packing structure of the fibrin fibers) and -1.5 (observed exponent for fine clots at low $q$). Vertical dotted gray lines indicate the expected positions of the first, second, third and fourth order reflection of the axial half-staggered packing distance (22.5 nm). (B) and (C) are confocal reflectance images of 1 mg/ml fibrin clots with (B) thick fibrin fibers of $N_p = 366$ and (C) fibrin fibers of $N_p = 86$. (C) Confocal fluorescence micrograph of a 1 mg/ml fine fibrin gel, where $N_p = 2$. For all images, a maximum intensity projection is shown over a total depth of 20 $\mu$m. The scale bar is 20 $\mu$m for all images.
To understand why the second order reflection is suppressed, we compared the measured spectra with predicted spectra based on full atom MD simulations of fibrin protofibrils (FO10-9). The expected scattering intensity for a dilute solution of protofibrils is shown in Fig. 4.9 (curve 2), where the gray dashed lines indicate the expected positions of the first, second, third and fourth order reflections of the half-staggering distance. Just like in the experiments, the second order reflection is absent in the simulated protofibril spectrum. To track down the origin of this peak suppression, we systemati-
cally remove certain parts of the protofibril to investigate their contribution to the scattering pattern. The second order reflection is suppressed both with (spectrum nr. 3) and without (spectrum nr. 2) the αC domains. The presence of the αC domains does influence the other reflections corresponding to the half-staggering distance, causing a reduced peak intensity and increased width. Interestingly, when we remove either the β or the γ-nodules, the second order reflection appears. As an extra control, we also looked at single-stranded protofibrils, composed of a string of monomers adhered end-to-end through the D:D interface (nr. 1 in Fig. 4.9). In this case, a new peak appears at $q = 0.157$ nm$^{-1}$, corresponding to a typical distance of 40 nm. This distance corresponds to the distance between the centers of mass of the two end regions: A fibrinogen monomer has a length of 46 nm and two end-regions each with size of 6 nm [491,492]. The distance between the centers of mass of the two end regions is thus 40 nm. For the double stranded protofibrils, this peak is suppressed. We conclude that the second order reflection is not present in SAXS spectra of fibrin gels due to destructive interference originating from the symmetric nature of the fibrinogen molecule. When this symmetry is perturbed by the removal of the β or γ nodule, the second order reflection appears. As we will show later on in this chapter, this feature provides an unambiguous way to detect the onset of forced unfolding of the β or γ nodules.

The overall decrease of the simulated scattering intensity of the protofibril solutions with $q$ exhibits two distinct power regimes, one with an exponent close to -1 at low $q$, and one with an exponent close to -4 at high $q$. We
can rationalize this $q$-dependence by calculating the expected form factor for a protofibril approximated as a cylinder [476]:

$$P(q) = \int_0^{\pi/2} \left[ \frac{2J_1(qR\sin x) \sin(qL\cos x/2)}{qR\sin x} \frac{qL\cos x/2}{qL}\right]^2 \sin x \, dx$$  (4.16)

Here $R$ and $L$ are the radius and length of the cylinder respectively, and $J_1$ is the first order Bessel function. For protofibrils, $R = r_{pf} = 5\text{ nm}$ [294] and we use $L = 5 \cdot 46\text{ nm}$ to compare with the simulations of protofibrils with a length of $\sim 5$ monomers. As shown in Fig. 4.10, the simulated SAXS spectra indeed have a similar dependence on $q$ as expected for cylinders of the radius and length of the protofibrils, with a $q^{-1}$ decrease of $I(q)$ at low $q$ and a Porod regime where $I(q)$ decreases as $q^{-4}$ at high $q$. However, we do not see oscillations in intensity at high $q$ for the simulations. These oscillations are only expected for dilute solutions of monodisperse cylinders and when the cylinders have an internally uniform electron density. Protofibrils are not homogeneous, however, in electron density (one of the reasons why we see Bragg peaks in the first place) and they contain water pockets [74].

The $q$-dependence at low $q$ scales as a power law with an exponent of -1 in case of the simulations, as well as in the case of the form factor of a cylinder (Fig. 4.10).

In the measurements for fine fibrin clots, we observe a stronger $q$-dependence (power exponent of $\sim-1.5$) at low $q$ than the $q^{-1}$ dependence expected from single protofibrils (and cylinders) in solution. This apparent discrepancy is due to the fact that in the experiments we have fibrous networks, whereas the simulations are performed on (single) protofibrils. Light scattering on coarse fibrin networks showed a dependence of $I(q)$ on bundle size and on the mass fractal dimension $D_m$ characterizing the network structure [478,479] (see Fig. 4.5). For fine clots, the limit where the scattering intensity is dependent on the network fractal dimension $D_m$ is $q_2 = 0.11$, which is based on the average diameter of the fine clot fibers of 20 nm (see section 4.3 and Fig. 4.5). Thus, for $q < q_2$, we expect that the scattering intensity scales as $q^{-D_m}$. Experimentally, we find $D_m$ close to 1.5. This interpretation should be tested independently with light scattering experiments. Note that we do not expect to see the Fiber-Solvent Porod regime for fine clots, since for $q > q_2$ we are already in the regime where we expect to see the internal features of the fibers. However, we still do expect a Porod regime at higher $q$-values ($q \gtrsim 1\text{ nm}^{-1}$), which are experimentally not accessible for our fine clot system due to the low scattering intensity of these samples.

We see a clear influence of bundle size on our SAXS measurements of fibrin gels (see Fig. 4.8). Specifically, we observe a change in the power law exponent of $I(q)$ at low $q$-values ($q \lesssim 0.2\text{ nm}^{-1}$). For coarse networks with bundle sizes $N_p$ of 100 or more (curves 1 up to 4), the intensity decreases steeply with $q$.
Figure 4.11: Power law exponents characterizing the $q$-dependence of the SAXS scattering intensity of coarse clots at low and high $q$. (A) Power law exponent of $I(q)$ in the low $q$ regime ($q < 0.2$ nm$^{-1}$) as a function of bundle size. The exponent is close to -1.5 for fine clots ($N_p = 2$) and closer to the Porod limit of -4 for coarse clots. (B) The fractal dimension $D_f$ characterizing the radial (cross-sectional) molecular packing structure of fibers in coarse fibrin clots of 4 mg/ml, obtained by scaling out the overall power-law decrease of $I(q)$ with $q$ in the range of $0.2 \lesssim q \lesssim 1$ nm$^{-1}$ (see 4.12A). The bundle sizes $N_p$ on the x-axis were measured at 4 mg/ml for the $N_p \geq 40$ sample, and estimated based on values measured at $c_p = 1$ mg/ml for the other two samples.

According to a power law with an exponent close to -4. In contrast, for the fine clots (curve 5), the intensity decreases much more weakly according to a power law with an exponent close to -1.5. The difference in power law slopes is statistically significant ($p<0.02$). Figure 4.11A presents a summary of these exponents. We can rationalize this bundle size dependence in the context of the different scattering regimes expected for fibrous networks as sketched in Fig. 4.5. For the coarse clots, the low $q$ regime corresponds with the 'Fiber-Solvent Porod Region', which is the first regime above $q_2$ in Fig. 4.5. In this Porod regime, scattering is dominated by the interface between fibers and solvent [295,347,480]. Since the average fiber diameter is on the order of 100 nm, we expect the Porod regime to set in at $q_2 \sim 0.02$ nm$^{-1}$). In the Porod regime, we expect a power law exponent close to -4, consistent with our SAXS measurements (curves 1 to 4 in Fig. 4.8). As discussed above, for the fine clots, we expect to be in a different scattering regime, dominated the mass fractal dimension of the blobs. This prediction should be tested with light scattering experiments.

At higher $q$-values (in the range of $0.2 \lesssim q \lesssim 1$ nm$^{-1}$), we probe length scales corresponding to the periodicity of the axial packing (discussed above)
Figure 4.12: The scattering intensity measured for coarse fibrin networks with thick fibers multiplied by the fractal dimension \( D_f \) of the mass distribution within the fibers to better reveal the Bragg peaks originating from axial and lateral molecular ordering. (A) Rescaled SAXS curves for coarse gels of varying bundle size, shifted along the y-axis for clarity. (B) Magnified view of a small region of the rescaled SAXS curves, where the background level \( I_{\text{background}} \) (horizontal striped lines in panel A) was subtracted. In (B), the gray curve was multiplied by a factor 10, while black curve was multiplied by a factor 4. For (A) and (B), the blue line corresponds to a 4 mg/ml fibrin gel with bundle size of \( N_p \approx 366 \), the black line corresponds to a 8 mg/ml coarse fibrin gel with \( N_p \approx 100 \), and the gray line corresponds to a 4 mg/ml coarse fibrin gel with \( N_p = 44 \).

but also the radial packing. As discussed in section 4.3, the radial packing structure of fibrin fibers is poorly understood. Two opposing models have been proposed, one modeling the mass distribution inside fibrin fibers as a fractal [364], while the other models fibers as crystalline packings of protofibrils [74] (see Fig. 4.6). If fibrin fibers can be considered as fractals, we expect that the scattering intensity should scale with \( q \) according to a power law with an exponent given by the fractal dimension [489]. In contrast, if fibrin fibers can be considered as crystalline, we expect a peak at a \( q \)-value that is inversely proportional to the average spacing between neighboring protofibrils. One earlier SAXS study reported an average radial repeat distance of 18.4 nm, which corresponds to twice the distance between protofibrils. As shown in Fig. 4.8A, the measured scattering curves for coarse fibrin samples can be considered as a superposition of an overall power-law decrease with superimposed peaks. The exponent characterizing the overall power-law decrease of \( I(q) \) is close to -1.3 (indicated by the black dashed line between 0.2 and 1 nm\(^{-1}\)). This observation is consistent with several prior sets of SAXS measurements [295, 461, 490] and
also with the proposal made by Yeramonahos and co-workers [295, 490] that fibrin fibers are partially crystalline.

To better test whether the SAXS curves can be described by a superposition of a power law and Bragg peaks, we multiply the measured scattering intensity with \(q^{D_f}\), adjusting \(D_f\) such that the curves are flat between 0.2 and 1 \(\text{nm}^{-1}\). As shown in Fig. 4.12, we can indeed find values for \(D_f\) for which the curves are flat in this \(q\)-region. The best-fit values for \(D_f\) systematically decrease with increasing bundle size, as shown in Fig. 4.11B: we find \(D_f = 1.7 \pm 0.05\) for \(N_p = 44\), \(D_f = 1.5 \pm 0.4\) for \(N_p \sim 100\), and \(D_f = 1.3 \pm 0.4\) for \(N_p \sim 360\). The fractal dimensions we find by SAXS are consistent with previously reported \(D_f\) values of 1.7 and 1.3 [295, 364].

Multiplication of the scattering curves by \(D_f\) clearly reveals the Bragg peak at 0.29 \(\text{nm}^{-1}\) corresponding to the axial half-staggered order, but it also clearly shows a second, much broader peak centered around 0.47 \(\text{nm}^{-1}\). This peak is only observed for coarse fibrin systems, with bundle sizes \(N_p > 40\). The position of the peak is independent of the bundle size, as shown in 4.11A (open squares) (See Fig. 4.26 in the SI for fitting of the peaks). The peak position corresponds to a typical distance of about 13 nm. This distance does not correspond to the half-staggered axial order. Instead, it likely reflects the lateral repeat distance between protofibrils. Previous studies in which lateral order was examined reported somewhat larger repeat distances of around 19 nm [298, 461, 463, 464, 488]. However, it has been shown in light scattering studies that the packing density, and therefore the average spacing between protofibrils, is rather variable, depending on assembly conditions such as fibrinogen concentration, FXIII concentration, and buffer conditions [295, 332, 490]. Therefore, we believe that the peak does originate from lateral order. The large width of the peak suggests that this ordering is only short-ranged.

Indeed, the fiber diameter poses a strict upper limit on the range of order that can be expected. In a perfectly crystalline system, the size of the crystal is quantified by the Scherrer length, which sets the full width at half maximum of a Bragg’s peak, \(\Delta q\), according to: 

\[
L_{\text{scherrer}} = \frac{2\pi}{\Delta q} \quad [493].
\]

We use this expression for the Scherrer length to estimate an apparent crystal size. When we plot the Scherrer length obtained by performing Gaussian peak fits to the broad peak corresponding to the lateral ordering, we find that it is independent of \(N_p\) (open squares in 4.12B). This suggests that the extent of (crystalline) cross-sectional ordering is not limited by the fiber diameter, but rather by intrinsic disorder. The presence of intrinsic disorder is consistent with the observation of a fractal dimension, and also with prior quantitative electron microscopy data [289].

For comparison, we also analyze the Scherrer length as extracted from the width of the peak corresponding to the first order reflection of the half-staggering axial repeat. As shown in Fig. 4.12B (black solid circles), in this
case $L_{\text{scherrer}}$ does show an increase with increasing bundle size, meaning that the axial order becomes higher as the fibers become thicker. We expect that the range of axial ordering is limited by the crystal size, which should in turn be related to the length of fiber segments between two branch points [494]. For a fixed fibrin concentration, we expect that the mesh size increases with increasing degree of bundling, as illustrated by the confocal images in Fig. 4.8B, C and D. Assuming that the networks are homogeneous and isotropic, the average mesh size $\xi$ can be estimated from the concentration using, $\xi \simeq \sqrt{1/\rho}$, where $\rho$ is the total fiber length per volume [66]. For a bundle size $N_p = 366$ at 8 mg/ml fibrin, we expect an average mesh size of 1.5 $\mu$m. For the $N_p = 100$, $c_p = 8$ mg/ml and the $N_p = 44$, $c_p = 4$ mg/ml samples, the average mesh size should be about 0.5 $\mu$m. The increase of $L_{\text{scherrer}}$ with increasing $N_p$ seen in Fig. 4.12B can thus be explained by a corresponding increase of the mesh size.

The effect of branching on the peak width is also nicely illustrated by simulations of the scattering pattern from protofibrils of varying length (see Fig. 4.14). When the length is decreased, the overall scattering curve does not change, but the peak corresponding to the first order reflection broadens. As an alternative test, we compared simulated SAXS spectra of single protofibrils (curve nr. 1 in Fig. 4.15 with spectra for mini-networks of 2 or 3 crossed protofibrils (curves nr. 2 and 3). Strikingly, the peak corresponding to the first order reflection shifts from $q \simeq 0.28$ nm$^{-1}$, corresponding to 22.5 nm, to $q \simeq 0.306$ nm$^{-1}$, corresponding to 20.5 nm when a branch is added. At the same
Figure 4.14: The dependence of simulated SAXS patterns for single protofibrils on protofibril length. For the curves in increasing shades of gray, the length increases by half a monomer length going from: FO4-3 to FO5-4, FO6-5, FO7-6, FO8-7, FO9-8 and finally FO10-9. The expected locations of the first, second, third and fourth order reflections of the half-staggering axial repeat distance are depicted by vertical dashed gray lines. The dashed black lines are power laws with an exponent of -1 in the Guinier regime at low $q$ and an exponent of -4 in the Porod regime at high $q$.

In summary, we have shown that the SAXS spectra of fibrin networks are sensitive to the bundle size of the fibers. The half-staggered axial arrangement of fibrin monomers within the fibers gives rise to a sharp reflection at $q = 0.285 \text{ nm}^{-1}$ and several higher-order reflections. However, this reflection is only visible when the bundle size $N_p$ is larger than 40. The larger the bundle size, the more intense and narrow the first order peak. We argue that the peak width is related to the typical length of fiber segments between branch points, which increases as fibrin assembles into thicker fibers. Strikingly, the...
second order reflection of the axial packing order is absent for all samples that we tested. With the help of simulations of protofibrils by Artem Zhumerov and Valeri Barsegov (Fig. 4.9), we can rationalize this finding based on the symmetric structure of the fibrin monomers. Furthermore, the simulations show that the disordered αC-regions emanating from the protofibrils should broaden the peaks. Regarding the radial packing structure of the (coarse) fibers, our SAXS measurements support a recent model proposing a partially ordered arrangement of protofibrils within the fiber [295,490]. We find evidence of a disordered arrangement characterized by a fractal dimension $D_f$ in the range of 1.1 up to 1.7 (depending on bundle size) together with a broad peak stemming from a characteristic spacing between protofibrils of 13 nm.
Figure 4.16: Alignment of a coarse fibrin network ($N_p \sim 100$, 8 mg/ml) under the influence of an increasing uniaxial tensile strain. Maximum intensity projections of 10 μm deep confocal z-stacks for (A) 0%, (B) 15%, (C) 20%, (D) 40% and (E) 65% strain. Scale bar denotes 10 μm for (A-E) and images are rotated to have approximately the same strain direction. Photographs of a fibrin gel in the Linkam tensile tester used for SAXS measurements at applied strains of (F) 0%, (G) 85% and (H) 120%. The black bands are graphite marks used to monitor the strain. The black structures at the two ends of the gel are pieces of Velcro that are embedded in the gel so that the gel ends can be clamped.

4.4.2 SAXS of Stretched Fibrin Networks

Alignment

In section 4.4.1 we have shown that SAXS spectra of fibrin networks exhibit a series of distinct peaks that can be explained in terms of the characteristic half-staggering axial repeat distance of the molecular packing structure of the fibers. We also showed that the intensity and width of these peaks as well as the slope of the overall decrease of $I(q)$ at low $q$ are dependent on fiber thickness. Next we aimed to investigate potential changes in the internal structure of fibrin under elongation.

Fibrin samples were polymerized in a bone-shaped Teflon mold with Velcro as anchor points. The Velcro pieces on each end of the gel were clamped in a Linkam Tensile tester. We were able to stretch the samples up to typically about 120% strain before the gel ruptured, often near one of the anchoring points (Fig. 4.16(F-H)). We observed that under influence of extension, the sample thinned and expelled water, consistent with a previous study [389] (panel F-H). Moreover, the applied tensile strain caused the fibrin network to align.

The degree of fiber alignment was quantified by imaging fixated stretched
Figure 4.17: The 2D order parameter, $S$, for coarse fibrin gels ($N_p \sim 100, 8$ mg/ml) under increasing extensional strain determined by image analysis of maximum intensity projections of 3D confocal z-stacks (Fig. 4.16(A-E)). Each data point represents an individual measurement on a different fibrin gel that has been stretched and fixated at a given strain.

fibrin samples under varying strain levels (Fig. 4.16(A-E)). At 0% strain, the fibers showed no obvious preferential alignment (Fig. 4.16A), while at increasing levels of strain, the fibers progressively aligned more. To quantify this effect, we performed image analysis using the OrientationJ plugin [439] for ImageJ on maximum intensity projections of 10 µm thick z-stacks to determine the 2D order parameter $S$ (defined in eq. 4.2). $S$ is 0 for perfectly isotropic systems and 1 for completely aligned networks. In practice, we typically find $S \sim 0.1 - 0.2$ for isotropic systems, as shown in Chapter 3 and in Fig. 4.17 (0% strain). $S$ rapidly increases from $\sim 0.15$ at 0% strain to 0.7 at 20% strain. At 65% strain, almost the entire network is aligned as seen in the maximum intensity projection (Fig. 4.16E), which results in a high $S$-value of 0.89 (Fig. 4.17). The increase in order parameter with extensional strain is steeper than determined in an earlier study, albeit using a different definition for the order parameter, where the order parameter increases over a range of $\sim 300\%$ strain [389]. There are several caveats with the alignment analysis by confocal microscopy. First we note that from the confocal images the fibers seem to get thinner and come close together with strain, which makes it difficult to track individual fibers. Second, the 2D maximum intensity projection likely increases the order parameter artificially, if fibers that are still in the process of re-orientating in the $z$-direction are projected to the same plane. Therefore, we expect quantitatively more accurate results from a three-dimensional
Figure 4.18: Scattering patterns of 8 mg/ml coarse fibrin gels ($N_p \sim 100$) for (A) 0%, (B) 50%, (C) 90% and (D) 110% strain. The stretching speed was 7 $\mu$m/s between intervals. The peaks are pointed out by white arrows. The color bar on the right displays the intensity axis (in arbitrary units). (A-C) were recorded directly (~2 min) after stretch, while (D) was recorded after ~6 minutes of stress relaxation.

Analysis of the angle distribution of the fibers, which we have started to do using the image toolbox SOAX [45]. In any case, our 2D analysis predicts significant fiber alignment with strain. Next we investigated how the SAXS pattern changes for networks under varying degrees of extensional strain.
The 2D SAXS patterns radically changed when fibrin samples were stretched, as shown in Fig. 4.18. At 0% strain, the scattering pattern showed the same features as patterns measured for fibrin samples in capillaries, being isotropic and showing peaks originating from the half-staggered axial packing order. This observation indicates that the network structure is initially isotropic and that the gels were not perturbed much during sample handling, consistent with the confocal images. When the strain was increased however, the scattering pattern became more and more anisotropic, indicating progressive alignment of the fibrin network with increasing strain. Also, certain peaks initially appeared in the meridional (strain) direction at intermediate levels of strain (Fig. 4.18B and C) and disappeared (Fig. 4.18D) when the strain was further increased. To quantify this behavior in detail, we performed a partial radial integration over a ±15° (total 30°) angle in the strain direction. We thus obtain the meridional scattering pattern originating from X-rays scattered parallel to the axis of the fibers aligned along the strain direction. The meridional scattering is thus sensitive to strain-induced changes in the half-staggered packing along the fibril axis.

The SAXS spectra obtained at 0% strain look identical to the spectra we measured for fibrin networks in capillaries (section 4.4.1). Upon stretching, we observe marked changes in the spectra. Strikingly, these changes depend on the pulling rate, which was either 50 µm/s (Fig. 4.19), or 10 µm/s (Fig. 4.20), or 7 µm/s (Fig. 4.21). At the fastest pulling rate of 50 µm/s (Fig. 4.19), we initially see peaks appearing with increasing strain (light gray to black). The first order peak corresponding to the half-staggered molecular packing distance is not visible at 0% strain, but appears when the strain reaches ~60%, though only after several minutes (compare panel A measured after 2 minutes with panel B measured after 6 minutes). Apparently, the fibrin networks are not directly in a steady state at this high pulling rate. This is supported by the force-extension measurements by the Linkam tensile tester, which show significant stress relaxation at this pulling rate (Fig. 4.27 in the SI). The first order peak is accompanied by a new, broad peak around \( q = 0.45 \text{ nm}^{-1} \) and a smaller peak at \( q = 1.06 \text{ nm}^{-1} \). With increasing strain, also the third and fourth order reflections of the axial half-staggering distance get more pronounced (dashed gray lines). At 80% strain, the peak at \( q = 0.45 \) shifts to slightly lower q-values (\( q = 0.436 \text{ nm}^{-1} \)), which means that it shifts to larger distances. Also, two new, smaller peaks appear, one at \( q = 0.535 \) (close to the expected location of the second order reflection of the half-staggering axial distance) and the other at \( q = 1.54 \) (arrows in panel B).

Interestingly, when the pulling speed was decreased to 10 µm/s (Fig. 4.20), peaks appeared at similar q-values as observed at the higher pulling rate, but they appeared at smaller strain levels and/or after a smaller relaxation time.
Figure 4.19: Scattering intensity in the axial (meridional) direction for a coarse fibrin network (8 mg/ml, $N_p = 23$) that is stretched at a pulling rate of 50 $\mu$m/s. (A) Scattering intensity recorded $\sim$2 min after stretching and (B) $\sim$6 minutes after stretching. Vertical dotted lines indicate the locations of the first, second, third and fourth order reflections of the axial half-staggering repeat distance. The strain level, $\varepsilon$, corresponding to each curve is indicated. The black arrows in (B) indicate peaks that appear with increasing strain levels.

For instance, the first order reflection of the half-staggered distance appeared at 60% strain already after 2 minutes instead of 6 min. Also, a new peak appeared at $q \sim 0.52$ nm$^{-1}$ at a strain level of only 30%. Consistent with these findings, force-extension measurements showed significantly less stress relaxation (Fig. 4.27 in the SI). Another marked difference compared to the 50 $\mu$m/s pulling speed case, is that the peaks corresponding to the axial half-staggering distance decrease in height at high strain levels, and eventually even mostly disappear at the highest strain of $\sim$120%.

At an even lower pulling speed of 7 $\mu$m/s, we again observed the appearance of peaks at similar $q$-values as in the 10 $\mu$m/s case: The peak at $q \sim 0.52$ nm$^{-1}$ again appeared already at a strain of 30%. At slightly higher strains, a shoulder was observed in some cases on the low $q$-side of the peak corresponding
Figure 4.20: Scattering intensity in the axial (meridional) direction for a coarse fibrin networks (8 mg/ml, \( N_p = 23 \)) that is stretched at a pulling rate of 10 \( \mu \)m/s. (A) Scattering intensity recorded \(~2\) min after stretching and (B) \(~6\) minutes after stretching. Vertical dotted lines indicate the locations of the first, second, third and fourth order reflections of the axial half-staggering repeat distance. The strain level, \( \varepsilon \), corresponding to each curve is indicated. The scattering curves are shifted along the y-axis for clarity.
Figure 4.21: Scattering intensity in the axial direction for a coarse fibrin network (8 mg/ml, $N_p = 23$) that is stretched at a pulling rate of 7 $\mu$m/s. (A) Scattering intensity recorded $\sim$2 min after stretching and (B) $\sim$6 minutes after stretching. Vertical dotted lines indicate the locations of the first, second, third and fourth order reflections of the axial half-staggering repeat distance. The strain level, $\varepsilon$, corresponding to each curve is indicated. The scattering curves are shifted along the y-axis for clarity.
to the first order reflection of the axial half-staggering distance at \( q \sim 0.247 \text{ nm}^{-1} \) (indicating the presence of a new repeat distance that is larger than 22 nm). Interestingly, at this low pulling speed, the peaks corresponding to the half-staggered axial packing order completely disappeared at a strain of 90\%, whereas at a pulling rate of 10 \( \mu \text{m/s} \) this happened at a significantly higher strain of \( \sim 120\% \). We note that we observed similar strain-dependent changes in the Bragg peaks for networks with larger bundle sizes \( (N_p \sim 100) \) stretched at the same speed of 7 \( \mu \text{m/s} \) (Fig. 4.28 in SI), though in this case the peaks originating from the axial packing order disappeared at a larger strain level \( (\sim 120\%) \), suggesting that structural relaxation may be slower for thicker fibers.

The observation that peaks corresponding to the half-staggered axial packing order appear with increasing tensile strain can be rationalized in terms of the strain-induced alignment. The progressive alignment of the fibers along the vertical direction makes it possible to distinguish the Bragg peaks in the meridional direction. The appearance of new peaks unrelated to the half-staggered packing as well as the disappearance of peaks with increasing strain indicate that straining in addition causes structural changes in the internal axial packing structure. From section 4.4.1 we know that the second order reflection of the axial half-staggering distance only appears when either the \( \beta \)- or \( \gamma \)-nodule unfolds. Interestingly, we never observed the second order reflection of the half-staggering distance at the expected location (second vertical dashed line). Upon straining, we observe the appearance of a new peak at \( q \)-number that is close to the position of the second order reflection (see second vertical dashed line), but at a somewhat lower \( q \)-value. Potentially, this peak may be attributed to the second order reflection of a (partially) unfolded half-staggering axial distance of \( 22 + \delta d \). Here \( \delta d \) varies between 2 and 6 nm to account for the higher order peaks at \( q = 0.45 \text{ nm}^{-1} \), \( q \sim 0.247 \text{ nm}^{-1} \) and \( q = 0.52 \text{ nm}^{-1} \). To test whether this is a reasonable interpretation, we compared our scattering intensity plots with simulated scattering intensity profiles of protofibrils pulled \textit{in silico}. These full atom simulations were done by Artem Zhmurov (Moscow Institute of Physics and Technology) and Valeri Barsegov (University of Massachusetts Lowell).

The simulations show that under the influence of increasing extensional strain, the two strands of the protofibril move closer together as the initial 'slack' in the protofibril is pulled out, as shown in Fig. 4.22A. Already at strains below 30\%, there is some elongation of the protofibril, without significant unfolding. At 30\% strain however, the forces are high enough to partially unfold one of the \( \gamma \)-nodules inside the protofibril (see also the zoom-in in panel B), giving rise to an \( 3-4 \text{ nm} \) extension. This \( \gamma \)-nodule unfolds more and more with increasing strain. At the same time, the alpha-helical coiled coils formed by the \( \alpha \), \( \beta \) and \( \gamma \) chains exhibit varying degrees of unfolding and transfor-
Figure 4.22: Structural changes during protofibril stretching predicted using full atom simulations. (A) Representative images of (part of) a protofibril during stretching. The strain level is indicated. (B) Zoom-in of the unfolding event at 30% elongation, where the γ-nodule of one of the protofibril subunits unfolds, giving rise to a 3–4 nm extension. Red denotes α-helical secondary structure, blue arrows denote β-sheet secondary structure, and gray denotes random coils. These data were kindly provided by Artem Zhmurov.

mation to beta-sheet structure. These pulling experiments were performed for several short protofibrils (FO2-2 up to FO5-5). While the exact unfolding pathway varied, the main features were all shared, namely that unfolding of one of the γ-nodules started at 30% strain, while the alpha-helical coiled coils transformed into β-sheet structures. This picture is consistent with vibrational spectroscopy measurements and staining tests with the β-sheet-specific dye Congo Red on stretched fibrin gels, which also showed an increased β-sheet content when fibrin networks were strained [336,366].

The structures shown in Fig. 4.22 were used to compute the X-ray scattering intensity. Fig. 4.29 in the SI shows the simulated intensities for single (partially) unfolded protofibrils in solution. The first order reflection of the
half-staggering distance migrates to smaller $q$-values (i.e. larger distances), as one might expect, with increasing strain levels, together with the corresponding higher order reflections. However, these scattering intensity graphs cannot be directly compared with our SAXS experiments, since we start from an isotropic sample. With increasing strain, the network aligns more and more to the strain direction, as shown in Fig. 4.16 and Fig. 4.17. Thus, we need to account for fiber alignment and inhomogeneous strain distributions in the network (see section 4.2.4). We achieve this by assuming that the scattered intensity of stretched gels is a superposition of intensities for aligned fibers and unaligned fibers. The aligned fibers include fibers that just got oriented along the strain direction and are still unstrained, as well as fibers under varying levels of strain, depending on the macroscopically applied strain level. The total scattering intensity is calculated according to eq. 4.10. As a last step, to account for spectral line-broadening as a consequence of a widening distribution of stretched and unfolded fibrin monomers, the scattering intensity plots are convolved with a Gaussian function with a width of 0.03 nm$^{-1}$.

Fig. 4.23 shows predicted scattering intensity plots for two stretching simulations of isotropic solutions of protofibrils comprising a mixture of folded and (partially) unfolded protofibrils as a function of extensional strain. Interestingly, the first order reflection does not move much with strain, though it does broaden with increasing strain and decreases in height. The third and fourth order reflections of the half-staggering axial repeat distance are also present for all strain levels, though the height decreases with increasing strain. The scattering intensities below 30% strain show the influence of protofibril stretching and alignment without (significant) unfolding. At 15% strain, a peak appears between the third and fourth order reflections at $q \sim 0.992$ nm$^{-1}$, which coincides with a slight shift of the first order and third order reflections to lower $q$-numbers (arrows in Fig. 4.23). These three peaks are related to an elongation of the 22 nm half-staggering axial repeat distance by 15%. We note that at strains around 15%, the second order reflection of the half-staggering axial repeat distance is not yet apparent and there is no significant unfolding yet.

Once the strain reaches 30%, one of the $\gamma$-nodules in the elongated protofibrils starts to unfold, giving rise to the appearance of the second order reflection in the SAXS spectra due to symmetry breaking (as discussed in section 4.4.1). The location of the second order reflection is notably at smaller $q$-values than expected based on the rest length of the half-staggering axial repeat distance ($q = 0.285$ nm$^{-1}$ for 22 nm), indicating that indeed the $\gamma$-nodules of protofibrils that are already under tension start to unfold first. The second order reflection peak shifts to lower $q$-numbers as the strain is further increased. This observation is consistent with our experimental data obtained at the slowest pulling speed of 7 $\mu$m/s, where a peak appeared when the strain reached 30% strain, at a $q$-value close to, but smaller than, the expected sec-
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Figure 4.23: Predicted scattering intensity obtained from in silico pulling experiments on a mixture of full atom structures at various levels of alignment and unfolding using eq. 4.10 and a Gaussian convolution as described in section 4.2.4. (A) and (B) show the simulated scattering intensity using two different protofibril pulling runs. Vertical dotted lines indicate the locations of the first, second, third and fourth order reflections of the axial half-staggering repeat distance (22 nm). The strain level, $\varepsilon$, is indicated. The data for these scattering intensity plots were kindly provided by Artem Zhmurov.

In summary, the simulations predict that a variety of new peaks should appear once the strain reaches 30%, as a consequence of $\gamma$-nodule unfolding. These peaks are expected to be located at $q$-values in-between the positions of the first and second order reflections originating from the rest length of the half-staggering axial repeat distance (22 nm). These peaks can thus be taken as a clear signature of $\gamma$-nodule unfolding. The peaks that we observed in-between the expected locations of the third and fourth order reflections (from the rest length of the half-staggering axial repeat distance (22 nm)), together
with the shoulder that appears on the low-\(q\) side of the first order peak, are related to an extension of the 22 half-staggering axial packing distance, but should not be directly taken as a signature of protofibril unfolding.

**Lateral Packing Changes at High Strain**

So far, we have focused on the change of the axial molecular packing structure of the fibrin fibers with increasing extensional strain. We have shown that unfolding starts at strains above 30\% at low pulling speeds (<10 \(\mu m/s\)), while at the highest pulling speed the unfolding is delayed (50 \(\mu m/s\)). Next, we investigated how the lateral packing structure of the fibrin fibers changes under influence of extensional strain. To analyze the change in lateral packing order, we averaged the scattering intensity orthogonal to the strain direction by a line of 10 pixels in width to obtain the equatorial scattering pattern originating from X-rays scattered perpendicular to the axis of the fibers aligned along the strain direction.

As discussed in detail in section 4.3, the lateral packing structure of the fibrin fibers is thought to be less ordered than along the axial direction. There are two main models from the literature that describe the lateral packing of protofibrils in a fibrin bundle, as shown in Fig. 4.6. One model describes the fibrin fiber as mass fractals with a fractal dimension of \(D_f \sim 1.3\) [364], with no internal crystal structure. This would give rise to a \(q^{-D_f}\) dependence for the scattering intensity. The other model describes the fibrin fiber as a ordered lattice with a tetragonal unit cell measuring \(a \times c \times c = 18.4\times 18.4\times 46\ nm\), where 18.4 nm corresponds to twice the distance between protofibrils and 46 nm to the length of one fibrinogen monomer [74,298]. This model would give rise to a peak around 18.4 nm. Recently, a combination of these two models has been proposed, where the lateral packing is crystalline, but with a large number of protofibrils missing (‘holes’). This arrangement results in a superposition of a fractal-like scattering and a broad peak due to locally crystalline regions [295,490]. In section 4.4.1, we have confirmed this superposition for coarse fibrin networks, though we observed a broad peak at a lower distance (\(\sim 13\ nm\)) than expected and the fractal dimension varied between 1.3 – 1.7 depending on bundle size.

At 0\% strain, the equatorial scattering curve (light gray curve in Fig. 4.24) looks similar to the isotropic averages for fibrin gels in capillaries presented in section 4.4.1 and to the meridional averages obtained for gels at 0\% shown in section 4.4.2. At high \(q\), we see the third and fourth order reflections of the half-staggering axial packing. When the fibrin network is strained (light gray to black), we observe subtle changes in the scattering curves. We do not see any peak at the predicted 18.4 nm distance predicted from the crystal structure proposed by Yang et al. [74] (dotted vertical lines). However, the data do show a broad peak at \(q \sim 1.0\ nm^{-1}\). At low strains, this broad peak coincides with the expected positions of the third and fourth order reflections of the
Figure 4.24: Scattering intensity in the lateral (equatorial) direction for coarse fibrin networks (8 mg/ml, $N_p = 23$) stretched at a pulling rate of 7 μm/s. (A) Scattering intensity recorded ~2 min after stretching and (B) ~6 minutes after stretching. Vertical dotted lines indicate where first and second order peaks are expected based on the repeat distance of 18.4 nm proposed by Yang et al. [74, 298]. The observed peak attributed to the lateral packing (13 nm), and its second order reflection, are indicated by the vertical gray dashed lines. The strain level, $\varepsilon$, is indicated. The black dashed line at high $q$ in (A) denotes a power law with exponent -4 (Porod). The scattering curves are shifted along the y-axis for clarity.
However, the contribution of the axial packing decreases with increasing strain due to fiber alignment. This means that we expect the third and fourth order reflections from the axial half-staggering repeat distance to disappear with increasing strain. Thus, the peaks observed at higher strains should be attributable to the lateral packing structure of the fibrin fibers. In the capillary measurements we observed a broad peak at $q \sim 0.47 \text{ nm}^{-1}$ that we attributed to a partially crystalline lateral packing structure with a 13 nm repeat distance (dashed vertical lines). For the strained gel, we also observe a broad peak at approximately this distance for some samples, which persists for a broad range of strains (Fig. 4.31). We interpret the broad peak at $q \sim 1.0 \text{ nm}^{-1}$ as the second order reflection of this peak. The peak at 1.0 nm$^{-1}$ does not move with increasing strain values, until the strain reaches values of $\gtrsim 80\%$, where significant unfolding takes place. At these high strain levels, the second order reflection of the lateral repeat distance (13 nm) shifts to higher $q$-numbers. Also, the Porod regime at high $q$ (black dashed line), which is related to the solvent-protofibril interfaces as explained in Fig. 4.5, is moved out to higher $q$-values. This observation indicates that the protofibrils themselves become thinner once significant unfolding occurs. This interpretation is consistent with the predicted structures shown in Fig. 4.22, where protofibrils become significantly thinner at strains of $\sim 80\%$, once a significant portion of the $\gamma$-nodule is unfolded.

We note that we observe similar equatorial scattering curves also for the other pulling rates, as shown in Fig. 4.31 in the SI, where the peaks attributed to lateral order disappear at the same strains ($\sim 120\%$) where the peaks attributed to the axial packing order disappear.

**Plastic Deformation**

So far we have shown that our experiments confirm predictions from simulations that protofibril unfolding starts at 30% strain. A key experimental signature is the appearance of a Bragg peak corresponding to the second order reflection of the (elongated) half-staggering axial repeat distance. We further showed that the lateral packing does not change much with strain, except at high extension where the axial order has mostly disappeared. In this section we investigate how reversible these changes are by stretching coarse fibrin networks to different levels of strain, and returning to a lower strain level, as shown in Fig. 4.25.

At a strain of 45%, we observe the first order reflection of the half-staggering axial distance (22 nm), as well as the third and fourth order reflections (vertical dashed lines). Also, there is a peak close to the second order reflection that we attribute to the second order reflection of the elongated half-staggering axial repeat distance. At this strain level and pulling speed (7 $\mu$m/s), the protofibrils have partially unfolded $\gamma$-nodules.
When the strain is increased further to 60%, the fibrin fibers are more aligned (Fig. 4.16 and Fig. 4.17), leading to an increase of the intensity of the first, third and fourth order reflections of the half-staggering axial distance (Fig. 4.21), and the scattering curve shows more obvious signs of unfolding (peaks in-between the first and second order reflection of the axial half-staggering distance, indicated by the first two vertical lines). Interestingly, when the strain is returned back to 45%, the scattering curve suggests that the fibers remember the strain direction, since the first, third and fourth order reflections have a higher intensity compared to the original state of the network at 45% strain. The second order reflection is still present upon returning to 45%, though it has a large shoulder to lower $q$-values. These observations are supported by the scattering intensity in the equatorial direction, where the first and second order reflections of the lateral packing distance between protofibrils (13 nm) are better visible compared to the original state of the network at 45% strain.

When the strain is increased from 45 to 90%, there is significant protofibril unfolding, since the peak corresponding to the first order of the axial half-staggering distance completely disappears. Interestingly, when the sample is relaxed back to 45%, the first order re-appears together with its higher order reflections (third and fourth order) but the second order is not present. This implies that there is complete refolding, even though the sample is still under 45% strain. One possible explanation is that protofibrils, in combination with unfolding, also slide along each other to relieve tension [413]. The equatorial scattering now shows a broad shoulder at $q$-numbers where we expect to see peaks originating from lateral ordering. We attribute this to significant fiber alignment (as also indicated by the peaks of the axial packing) and a broad disorder in the lateral packing distance. The observation that the first order reflection re-appears after sample relaxation is consistent with prior SAXS observations on fibrin films [455], though in that case the maximum strain levels were lower (~40% strain) and the waiting time was hours instead of minutes. When we strain the sample to 110% and then return back to 45%, the structure does not refold back completely, as indicated by the reduced height of the first, third and fourth order reflections of the axial half-staggering distance compared to the control case (thick gray line). This idea is supported by the equatorial scattering intensity, which shows that the onset of the protofibril Porod regime is delayed, which indicates thinner protofibrils compared to the control (0% strained to 45% at a strain rate of 7 $\mu$m/s).

4.5 Discussion

We have investigated the origin of the remarkable extensibility of fibrin networks by performing in situ SAXS measurements on fibrin gels subject to uniaxial tension in combination with full-atom simulations of the force-extension
Figure 4.25: Scattering intensity in the axial (meridional) (A) and the cross-sectional (equatorial) (B) direction for a coarse fibrin network (8 mg/ml, $N_p = 23$) stretched at a pulling rate of $7 \mu m/s$, recorded $\sim 2$ min after stretching. The fibrin gel is pulled to the indicated strain level (0.45, 0.6, 0.9 or 1.1) before relaxing it back to a strain level of 0.45. The vertical dashed lines in (A) indicate the locations of the first, second, third and fourth order reflections of the axial half-staggering distance. The dotted vertical lines in (B) denote the predicted locations of the peaks originating a crystalline lateral packing of the protofibrils assuming a unit cell measuring $18.4 \times 18.4 \times 46 \text{ nm}$ ($q = 0.341 \text{ nm}^{-1}$ and higher order reflections) [74]. The dashed vertical lines in (B) denote the observed peaks attributed to the lateral packing ($q = 0.483 \text{ nm}^{-1}$ and higher order reflections). The scattering curves are shifted along the $y$-axis for clarity.
behavior of fibrin protofibrils (performed by Artem Zhmurov and Valeri Barsegov). We showed that fibrin network elongation is accompanied by forced $\gamma$-nodule unfolding when the strain exceeds 30%. This process gives rise to a peak in the scattering intensity located in-between the expected location of the first and second order reflections originating from the rest length of the half-staggering axial repeat distance (22 nm). We showed that these peaks can be taken as a clear signature of $\gamma$-nodule unfolding. We also observed peaks in the scattering intensity in-between the expected locations of the third and fourth order reflections (from the rest length of the half-staggering axial repeat distance (22 nm)), together with a shoulder on the low-$q$ side of the first order peak. We showed that these peaks are likely related to an extension of the alpha-helical coiled coil regions of the protofibrils, and should not be taken as a signature of protofibril unfolding.

Depending on strain rate, complete unfolding occurs at strains close to 90% or higher. This is in line with a previous publication, which showed that the first order reflection of the half-staggering distance (22 nm) vanished at 100% elongation [389]. However, this strain is larger than strains reported for fibrin films ($\sim$40%) [456]. We note that for fibrin films the second order reflection of the half-staggering distance was also observed at 0% strain [455,456], which is suggestive of mechanical perturbations during sample preparation. This hampers a quantitative comparison of the strain required for unfolding between our results and the prior SAXS data for fibrin films.

To compare the simulated scattering intensity of stretched protofibrils with our SAXS experiments, we used previous published data on fibrin network alignment [389]. This is not ideal, since fibrin alignment is likely to depend on the pulling rate as well as on the mesh size of the fibrin network. To quantify fiber alignment for our own networks, we have performed confocal microscopy on fibrin networks that were fixed after stretching to various levels of strain (Fig. 4.16) and we analyzed the fiber alignment using maximum intensity projections of 10 $\mu$m thick z-stacks. This analysis is likely to provide artificially high values for the order parameter, since all fibers are forced onto one plane. We expect quantitatively more accurate results from a three-dimensional analysis of the angle distribution of the fibers, which we have started to do using the image toolbox SOAX [495]. In principle, we can also use 3D fiber tracking data directly as input to simulate the scattering intensity. This is still work in progress.

We convolved the scattering intensity calculated from simulated protofibril structures with a Gaussian function with a width of 0.03 nm$^{-1}$. This width was chosen to fit the experiments. To check whether this width is reasonable, we compare this width to the broadening from the experimental setup. In experiments, the scattering intensity data are broadened by misalignment and energy spread of the incident beam. This broadening is estimated to be 2–3
Å [496], corresponding to a broadening of about 0.003–0.004 nm$^{-1}$. This experimental broadening is about 7–10 times smaller than the value used to calculate SAXS curves from simulations. This indicates that the modeled SAXS curves can be further improved in the future. However, qualitatively the simulations do indicate which scattering peaks to expect from protofibril stretching and unfolding, and at what $q$-values, and the simulation predictions are all confirmed experimentally.

In the experiments we used coarse (bundled) fibrin gels ($N_p = 23$ and $N_p \sim 100$), whereas the simulations were performed for protofibrils ($N_p = 1$). Thus, the comparison between experiment and simulations is not entirely fair. To address this issue, we also performed SAXS experiments on stretched fine fibrin gels, which have minimal bundling (see Chapter 2, $N_p = 2$). We observed qualitatively the same features as for the coarse fibrin networks, as shown in Fig. 4.30 in the SI, though the scattering intensity was reduced. We did not observe a peak corresponding to lateral ordering, as one might expect in the absence of significant protofibril bundling (panel B).

### 4.6 Conclusion

In this Chapter, we have shown SAXS data for fibrin gels under elongation, supported by full-atom simulations on stretched protofibrils (from our co-workers, Artem Zhmurov and Valeri Barsegov) to interpret strain-induced changes in molecular structure. First, we investigated the effect of lateral packing on the scattering patterns by varying the bundle size at 0% strain. We showed that the axially symmetric molecular packing structure of fibrin can explain the suppression of the second order reflection of the 22 nm axial repeat. If the symmetry is perturbed by unfolding of the $\gamma$ or $\beta$-nodule, the second order appears. We further showed that the Bragg peaks corresponding to axial order are much more pronounced for thicker fibers and that the peak width is correlated to the mesh size of the network. Further, we found indications of a partially ordered lateral packing structure of the fibers with a characteristic repeat distance of 13 nm, independent of bundle size. When the fibrin gels were subjected to uniaxial stretch, we observed clear evidence for forced molecular unfolding. At an applied strain of 30%, peaks appear between the first and second order reflections of the 22 nm axial repeat distance. Predicted SAXS curves based on simulations of stretched protofibrils showed that these peaks are an unambiguous signature of $\gamma$-nodule unfolding. Furthermore, both simulations and experiments showed that the peaks originating from axial order become broader and less intense with increasing strain, indicative of increased disorder. At strains of $\sim 90–100\%$, the peaks disappeared in the experiments, but only for small pulling rates. This loss of axial order coincided with a loss of lateral order. By combining SAXS experiments with MD simulations, we can now understand the molecular basis of fibrin’s exten-
sibility and strain-stiffening response. Forced unfolding may also help explain intriguing recent findings that mechanical stretch protects fibrin fibers against fibrinolysis [465–467]. Moreover, forced unfolding may potentially unmask cryptic binding sites for cells and extracellular matrix molecules, in a similar manner as in fibronectin [497] and could be exploited to design molecular switches [498].

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4.8 Supplementary Information

Supplementary Figures

Figure 4.26: Three fitting examples of two Gaussians per sample. The fits are depicted in red dashed lines. The blue line depicts a 4 mg/ml fibrin gel with bundle size of $N_p \sim 366$, the black line is a 8 mg/ml coarse fibrin gel with $N_p \sim 100$ and the gray line is a 4 mg/ml coarse fibrin gel with bundle size $N_p = 44$. Gray is enhanced by a factor 10, while black is magnified by a factor 6.
Figure 4.27: Example tensile test curves measured on coarse fibrin gels ($N_p = 23$, 8 mg/ml) for pulling rates of 50 $\mu$m/s (black) and 10 $\mu$m/s (gray). The strain levels for 50 $\mu$m/s were 40% (first peak) and 60% (second peak), while for the 10 $\mu$m/s only the 50% strain level is shown. There is significantly more force relaxation in the 50 $\mu$m/s case compared to the 10 $\mu$m/s case.
Figure 4.28: Scattering patterns of coarse fibrin gels \((N_p \sim 100, 8 \text{ mg/ml})\) for varying strain levels (A) 2 minutes after stretching and (B) 6 minutes after stretching. Vertical dotted lines indicate the locations of the first, second, third and fourth order reflections of the half-staggering distance. The strain levels are indicated. Scattering curves are shifted along the y-axis for clarity.
Figure 4.29: Simulated scattering intensity for dilute solutions of (partially) unfolded protofibrils as depicted in Fig. 4.22, without convolution with a Gaussian function. Black arrows highlight the strain-induced migration of the first order reflection of the half-staggering axial packing distance to lower $q$-values. Vertical dotted lines indicate the locations of the first, second, third and fourth order reflections of the axial half-staggering repeat distance. The strain level is indicated. The simulated scattering curves are shifted along the y-axis for clarity.
Figure 4.30: Scattering intensity in the axial (A) and lateral (B) direction for fine fibrin networks ($N_p = 2, 8 \text{ mg/ml}$) pulled at $20 \mu\text{m/s}$. (A) Scattering intensity directly ($\sim 2 \text{ min}$) after stretching and (B) $\sim 6 \text{ minutes}$ after stretching. Vertical dotted lines indicate the locations of the first, second, third and fourth order reflections of the axial half-staggering repeat distance. The strain level, $\varepsilon$, is indicated. The scattering curves are shifted along the y-axis for clarity.
Figure 4.31: Scattering intensity in the lateral direction for coarse fibrin networks ($N_p = 23$, 8 mg/ml) pulled at 10 µm/s. (A) Scattering intensity directly (∼2 min) after stretching and (B) ∼6 minutes after stretching. Vertical dotted lines indicate to 18.4 nm, and its second order reflection, expected for the model presented by Yang et. al. [74,298]. The observed peak attributed to the lateral packing (13 nm), and its second order, is indicated in vertical gray dashed lines. The strain level, $\varepsilon$, is indicated. The scattering curves are shifted along the y-axis for clarity.