5. Microscopic View of Fibrin Blood Clots

Mechanical interactions of cells with their tissue environment through cellular adhesions have been shown to regulate many cell processes, including cell migration and cell division. On the scale of the cell, local mechanical properties of the fibrous extracellular matrix at the micron scale are likely to be more important than the global mechanical properties. In this chapter, we develop a customized optical tweezer microrheology setup that allows us to probe the local mechanical properties of fibrin networks at the cellular scale by measuring the thermally driven fluctuations of micron-sized probe beads embedded in the material of interest. We first validate the technique by comparing microrheological measurements on viscous fluids of varying viscosities and polyacrylamide gels with macroscopic shear rheometry. Next, we test the technique on fibrin networks whose architecture is varied by tuning the fibrin concentration and self-assembly conditions. We show that the surface chemistry of the probe beads crucially affects the micromechanical properties of fine fibrin networks: Without surface passivation, beads accumulate fibrin on their surface and overestimate the shear modulus of the network, while a covalent Pluronic coating creates a depletion layer of about 3 µm thick and leads to an underestimation of the network modulus. Using the high-frequency response of the passivated beads, we determine the persistence length of protofibrils to be 203 ± 75 nm, which is in excellent agreement with prior findings. Finally, we study fibrin networks with a large pore size (∼10 µm), for which we recover the bulk modulus within a factor 3. We conclude that optical tweezer microrheology is a powerful tool to obtain information about the local micromechanical properties of extracellular matrix networks at the scale relevant to cells, as well as the network and fiber response.

Karin A. Jansen, Andre Scholich, Gijsje H. Koenderink
5.1 Introduction

Mechanical interactions between cells and the extracellular matrix play a vital role in the regulation of cell behavior and tissue morphogenesis in health and disease [3, 4, 499]. Cells probe their mechanical environment by exerting traction forces [206]. Studies of cells on top of 2D substrates showed that the stiffness of the substrate has a strong influence on cell spreading, differentiation, migration, gene expression and cell-cell signaling [41, 43–48] (see also Chapter 1 section 1.3.1). Recently, the viscous properties of the substrate were shown to also influence cell behavior [72, 500]. In 3D environments that more closely mimic the extracellular matrix in tissues, it was shown that local mechanical interactions at the micron-scale regulate cell migration [310] as well as cell division [501]. Cells can also locally change the architecture and mechanics of the ECM, as shown in Chapter 3. Cells probe the mechanical properties of their environments on the scale of the size of the protrusions that interact with the matrix fibers, so at the scale of several microns [99, 423, 502, 503].

The mechanics of ECM gels are highly complex: They tend to stiffen under the influence of stress in complex ways [39, 66, 272] (see also section 1.8.1). This is very different from most synthetic materials, like polyacrylamide (PAA), which have a constant stiffness out to very large strains [39]. Cellular traction forces have been measured on top of two dimensional PAA substrates [504, 505] and inside three dimensional hydrogels [257] by applying linear continuum elastic theories to calculate forces from measured bead displacements (see also section 1.7). In the context of 3D extracellular matrices, this method is far from trivial, since the pore size is typically on the order of microns (instead of nanometers) [271], the ECM network typically strain-stiffens [39], and viscous dissipation of the network occurs on cellular timescales [72]. Therefore, quantitative information is needed about the local micromechanical properties of ECM networks to interpret cellular traction forces [247, 249, 503, 506].

One way to measure local mechanical properties is to use nanoindentation [55, 507]. However, this is a surface technique that does not give information about the mechanics inside a 3D gel. Others have used microrheology techniques, such as multiple particle tracking using time-lapse video microscopy [508–511], optical tweezer microrheology [512] or magnetic microrheology [513]. However, most of these microrheology studies focused on the mechanical properties of cells [511, 514, 515] and isolated intracellular networks like actin [333, 373, 427, 516–522]. Only a few studies so far addressed the microrheological properties of extracellular matrices using either optical tweezers [523–528], magnetic tweezers [403, 529–532] or video particle tracking [533]. Most of these studies focused on the low frequency regime though, and did not investigate the influence of the bead’s surface chemistry.

There are two main classes of microrheological techniques: Active microrheology, using optical or magnetic tweezers, and passive microrheology, using
video particle tracking. These techniques are often used to determine the mechanical properties in the low frequency regime. However, when optical tweezers are combined with interferometric detection using quadrant photodiodes, one can obtain also high frequency information over a range up to 100 kHz [515,534]. The advantage of such a high bandwidth is that one can obtain in a single measurement both the network response (at low frequencies) and the dynamics of the individual polymers (at high frequencies) [427,535,536].

Microrheology of fibrous protein networks brings several complications, however. The bead size is an important parameter to consider. If the bead is smaller than the mesh size, then the passive fluctuations will give information about the mesh size and not the mechanical properties of the network [523]. Also, the bead surface chemistry is an important consideration. This parameter has mainly been studied in the context of actin networks [516,537], where it was shown that inert beads report a lower modulus and a higher frequency dependence compared to bulk rheology. In contrast, sticky beads tend to overestimate the modulus, but give the correct frequency dependence. One study claims that sticky beads are necessary to report the correct moduli, even when the bead size is \( \sim \)10 times larger than the average mesh size [538].

Here, we develop an optical tweezer based setup to perform high bandwidth microrheology on fibrous protein networks. We validate the method using viscous fluids of calibrated viscosity, as well as small pore size PAA gels, which have a significant elastic component. We then show that we can probe the local viscoelastic moduli of fibrin networks. For this we employ two different bead types: One set of beads that stick to the network (plain polystyrene beads) and one set of passivated beads that are inert (polystyrene beads covalently coated with Pluronic). We show that both bead types provide the correct concentration dependence of the elastic modulus \( G' \), but the sticky beads overestimate \( G' \), while the inert beads underestimate \( G' \). We can explain this over- and under estimation in terms of a change in local fibrin concentration at the bead’s surface. At high frequencies for the inert beads, we observe clear evidence of entropic elasticity for \( G'' \), with a characteristic \( \omega^{3/4} \) frequency dependence, while at low frequency the data agree with macrorheology. This observation confirms the model proposed in chapter 2, where we concluded based on macrorheometry data that the linear elastic modulus of fibrin networks must be entropic in origin. We can now apply the microrheology technique to probe the influence of cells on their local tissue environment, for instance during cell-mediated stiffening [67,524,525,528]. Also, it could even be combined with simultaneous intracellular microrheology to probe the response of the cell [539]. Optical microrheology is also highly suited for on-line monitoring of blood clotting and fibrinolysis (nano-thromboelastography [540]).
5.2 Materials and Methods

5.2.1 Sample Preparation

So-called ‘fine’ and ‘coarse’ fibrin gels crosslinked by fibrinoligase (FXIIIa) were made as described in Chapter 2, using reagents from Sigma and human fibrinogen and thrombin from Enzyme Research Laboratories (Swansea, UK). Coarse clots were made using fibrinogen dialyzed into the fibrin buffer (without CaCl$_2$, see Chapter 2) before use. The fibrin concentration was varied between 0.4 up to 2 mg/ml. Fine and coarse fibrin gels were allowed to polymerize for at least 1 or 4 hours at 37°C respectively. For optical tweezer measurements, beads at a (final) volume fraction of less than 1% were added just before the addition of thrombin, and the gels were polymerized inside sealed glass chambers. For confocal microscopy, Alexa488 labelled fibrinogen was purchased from Life Technologies (Bleiswijk, the Netherlands), dissolved in either fine clot buffer or coarse clot buffer (without CaCl$_2$, see Chapter 2) and mixed with unlabeled fibrinogen in a 1:20 molar ratio.

Polyacrylamide (PAA) gels were formed by mixing the following ingredients: BIS-acrylamide (40% w/v) was mixed with acrylamide (30% w/v) and water. The solution was degassed by 10 minutes centrifugation. Then ammonium persulfate (APS, 10% w/v) and Tetramethylethylenediamine (TEMED) were added to induce polymerization. The final conditions were (v/v) 1.1% BIS-acrylamide, 1.1% acrylamide, 0.5% APS and 0.15% TEMED. The PAA gels were polymerized at room temperature (22°C) for ~ 4 hours or, in case of rheology measurements, until a plateau in the linear viscoelastic moduli was reached. For optical tweezer measurements, beads were added before APS and the gels were polymerized inside sealed glass chambers. To ensure a homogeneous bead distribution, the glass chambers were rotated for the first ~ 20 minutes of polymerization. To check that the overall stiffness does not change by the presence of beads, we measured the rheological properties of bead-seeded gels by adding the beads just before APS and TEMED. After 15 min, when the sample was still fluid, the sample was gently transported to the rheometer plate to ensure an homogeneous bead distribution inside the sample.

5.2.2 Bead Preparation

Polystyrene beads (1.5 µm diameter) were purchased from Polysciences (Eppelheim, Germany) and stored at 4°C. Silica beads (1 µm diameter) were purchased from G. Kisker GbR (Steinfurt, Germany) and stored at 4°C. In some cases, home-made silica beads were used. These beads were prepared according to a protocol introduced by Stöber et. al. [541] and modified by Zhang et. al. [542]. In short, tetraethyl orthosilicate (TEOS), ammonia (30%) and absolute ethanol were used. Two solutions were made. Solution 1 contained 46
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ml of ethanol and 10 ml of ammonia, while solution 2 contained 7 ml of TEOS and 28 ml of ethanol. Solution 2 was added drop by drop to solution 1 under rapid mixing to get 1.2 µm diameter beads, as determined by transmission electron microscopy by Dominique Thies-Weesie at the University of Utrecht. The polydispersity was measured to be 3.4% (see Fig. 5.15 in the supplementary information (SI)). The silica beads were stored in 100% ethanol at room temperature. All the three bead types were washed with water by repeated centrifugation and resuspension before use.

In this Chapter, we investigate the effect of bead chemistry on the measured mechanical properties in fibrin networks. For this purpose, we render 1.5 µm polystyrene beads inert by coating them covalently with Pluronic using a protocol introduced by Kim et. al. [543]. In short, 15 µl bead suspension was added in a LoBind Eppendorf tube (VWR, Amsterdam, the Netherlands) and sonicated for 5 min. Beads were pelleted by centrifugation for 5 min at 16.1×1000 g to remove the supernatant. The beads were resuspended in 100 µl F-127 Pluronic solution (1% w/w). After 10 min incubation, 40 µl Toluene is added and incubated for 3 hours. This allowed the beads to swell such that the Pluronic chains are inserted. During this time, the tubes are in a rotating wheel (20 rpm). The Toluene is removed by heating the tubes to 98°C in a water bath in the fume hood for 10 min. The excess Pluronic was removed by centrifuging at 16.1×1000 g for 5 min. The final bead solution was stored in fine fibrin buffer (50 mM TRIS-HCL, 400 mM NaCL, pH 8.5) or water at room temperature. The beads were stable for at least two years.

5.2.3 Macrorheology

Rheology measurements were performed with a stress-controlled rheometer (Physica MCR 501; Anton Paar, Graz, Austria). Directly after adding thrombin, the fibrinogen solutions were quickly transferred to the rheometer plate, which was equipped with a steel cone and plate (40 mm diameter, 1° cone angle). The rheometer was preheated to 37°C for experiments with fibrin, or kept at 22°C in the case of PAA. Solvent evaporation was prevented by coating the fibrin sample edges with mineral oil. For PAA, water was added in the wells. The time evolution of the linear shear modulus, $G^*$, was monitored during fibrin polymerization by applying a small-amplitude oscillatory strain with amplitude $\gamma = 0.5\%$ and frequency $\omega = 3.14$ rad/s and measuring the stress response, $\sigma(\omega) = G^*\gamma(\omega)$. The shear modulus is a complex quantity, $G^* = G' + iG''$, having an in-phase elastic component, $G'$, and an out-of-phase viscous component, $G''$. Networks of fine fibrin clots and also PAA reached a constant shear modulus ($G_0$) after about 1 hour. After polymerization, a frequency test was performed by applying 0.5% strain and varying the frequency from 10 to about 0.05 Hz. These frequency tests were compared with microrheology measurements obtained with the optical tweezer setup.
5.2.4 Imaging

To check the local environment around the beads, we perform confocal microscopy on fibrin networks with (coated or not coated) beads and without beads (control). For this we use a confocal fluorescence microscopy on a Nikon Eclipse Ti inverted microscope equipped with a 100x oil 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). Alexa488 labeled fibrinogen was purchased from Life Technologies (Bleiswijk, the Netherlands), dissolved in fine clot buffer (without CaCl$_2$) and mixed with unlabeled fibrinogen in a 1:20 molar ratio. Samples were prepared in sealed glass chambers and polymerized at 37°C for 1 hour (fine clots) or 4 hours (coarse clots) before imaging. The images are summations over stacks of 5–10 µm thick in z, taken 10 µm away from the surface with 0.125 µm step size.

5.2.5 Optical Tweezer Measurements

Setup

The optical tweezer is build in-house on a Nikon Eclipse Ti inverted microscope, which is depicted schematically in Fig. 5.1A. A 1064 nm wavelength continuous laser (YLM-5-1064-LP, IPG Photonics, Germany) is guided via an optical fiber to the back of the microscope, which is placed on a vibration-isolating optical table. The beam goes through a beam expander (ELQ-25-2.5x-1064, Thor Labs) before it is guided through two acousto-optical deflectors (AODs) (DTSXY-400-1064, AA Opto Electronics). The first reflection exiting from the AODs is selected with a pinhole. The pair of AODs gives us control over both the $x$ and $y$ direction of the laser beam and move the laser trap in the sample via a computer via two computer-controlled variable frequency drivers (AAMCS, France). The laser power within the sample can be varied by either changing the laser power directly at the laser driver, or by changing the transmission efficiencies of the AODs.

Probe beads within the sample are optically trapped using a 100x oil objective (NA 1.4, Nikon). The laser light exiting the sample is collected using an oil condenser (NA 1.4, Nikon, Japan, HNA-oil) and projected onto the back-focal plane of the condenser on a quadrant photodiode (QPD) (YAG-44-4AH Excelitas Technologies). The QPD gain is controlled using a field-programmable gate array (FPGA board, NI PXIe- 1073, National Instruments, Netherlands) via a custom written program termed ‘MicroRheology’. The gain was varied between 1 and 7 depending on the laser power and bead size used. The QPD electronics calculates (analogue) the displacements in $x(t)$ and $y(t)$ from the differences in voltages in the four quadrants of the QPD, as shown in Fig. 5.1B. This interferometric particle position detection method results in nanometer spatial and $\sim 10 \mu$s temporal resolution [544]. The $x(t)$, $y(t)$ and
Figure 5.1: (A) Schematic diagram of the optical tweezer measurement setup as described in section 5.2.5. (B) Schematic diagram of the quadrant photo diode (QPD) detector and its operations to calculate fluctuations in $x$ and $y$ in the sample plane. The $x$ and $y$ signals are normalized by the sum-signal $S$.

sum signal is filtered by a low-pass filter (LTC1564, Linear Technology) with cutoff 150 kHz. The signal of the QPD is normalized to the sum signal before analysis to account for noise in the laser intensity. The recording of the position signal was typically 60 sec in fibrin gels, oversampling by recording at a sampling frequency of 200 kHz. Such oversampling avoids aliasing artifacts in data acquisition [545]. The data is analyzed with an home-made program written by Marco Seynen termed ‘MicroRheology’, which is written in C# using Microsoft Visual Studio 2010.

Data Analysis

We developed a customized optical tweezer setup for determining the microrheological properties of biopolymer networks. For this purpose, we sparsely seed our networks with beads and measure the constrained thermal fluctuations in the $x$ and $y$ direction. If a Brownian particle embedded in a material is in thermal equilibrium and there are no other forces acting on it, then the fluctuation-dissipation theorem holds [546] and the position fluctuations $u$ of this particle are related to the response function of the material [547]:

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\[ A''(\omega)_\alpha = \frac{\omega}{2k_BT}S_\alpha(\omega) \]  

(5.1)

In this formula, \( \alpha \) is either the direction \( x \) or \( y \), and \( S_\alpha \) is the integral over the equilibrium fluctuation spectrum:

\[ S_\alpha(\omega) = \int <u_\alpha(t)u_\alpha(0)>e^{i\omega t}dt \]  

(5.2)

\( A'' \) is the imaginary part of the response function. The real part \( A' \) is related to \( A'' \) via the Krames-Kronig integral [547]:

\[ A'(\omega)_\alpha = \frac{2}{\pi} \int_0^\infty \frac{\xi A''(\xi)_\alpha}{\xi^2 - \omega^2} d\xi \]  

(5.3)

We refer to \( A^* \) as the apparent complex response function (\( A^* = A' + iA'' \)), since the fluctuations are recorded in the presence of an harmonic confining potential, namely the laser trap. The rheological complex response function, \( B^* \), is determined by correcting the apparent response function for the contribution of the laser trap with trapstiffness \( k_\alpha \) [534,547]:

\[ B^*(\omega)_\alpha = \frac{A^*(\omega)_\alpha}{1 - k_\alpha A^*(\omega)_\alpha} \]  

(5.4)

If we assume the material is an isotropic and incompressible medium, then the complex shear modulus \( G^* \) is related to \( B^* \) via the generalized Stokes equation [547–549]:

\[ G^*(\omega) = \frac{1}{6\pi RB^*(\omega)} \]  

(5.5)

were \( R \) is the bead radius. This equation is in principle only valid for incompressible media (i.e. Poisson ratio \( \nu = 1/2 \)). However, if the medium is compressible, \( G^* \) can be at most 25% off, given that \( \nu \) is still between 0 and 1/2 as typical for biopolymer networks [548]. For fibrin, values close to 0.5 have been suggested for \( \nu \) in the linear elastic regime relevant for passive microrheology [366]. Interestingly, at high deformations, fibrin gels show evidence of negative compressibility [389]. The importance of compressibility effects is frequency-dependent, since at high frequencies, the network is viscously coupled to the fluid, whereas at low frequency, the system becomes compressible [548]. The crossover frequency \( f_{cros} \), above which viscous coupling dominates, can be estimated from a two-fluid model as [548]:

\[ f_{cros} \approx \frac{G}{\mu R^2} \approx 200 \text{ Hz} \]  

(5.6)
Using parameter values typical for fine fibrin gels, with $G = 10$ Pa, $\mu = 0.001$ Pa·s, $\xi = 100$ nm and $R = 0.75$ µm, we find $f_{cros} \sim 200$ Hz. This means that, above $\sim 200$ Hz, we can consider fine fibrin gels as incompressible.

To determine the linear viscoelastic properties of fibrin and PAA gels, at least 10 beads per condition are measured at a fixed distance of 10 µm above the glass surface, unless stated otherwise. The geometric mean is shown per sample, since the average is more sensitive to outliers with high modulus (see Fig. 5.16 in SI). To allow a comparison of $G'$ and $G''$ from micro- and macrorheology measurements, we averaged the modulus between 5 and 6 Hz. The number of samples per condition for the fibrin gels are summarized in Table 5.2 in the SI. For fibrin, the reported $G''$ are corrected for the solvent contribution ($G'' = \mu 2\pi f$, with viscosity $\mu = 0.001$ Pa·s).

To validate our home-made setup and analysis, we measured the apparent and rheological response functions for viscous fluids of known viscosities (water and water-glycerol mixtures), while varying the trap stiffness by varying laser power. For a viscous fluid, the apparent complex response function $A^*$ reported by an immersed bead held by a trap of stiffness $k$ is calculated as [534]:

$$A^*(\omega) = \frac{1}{k - i\omega\gamma(\omega)} \tag{5.7}$$

where $\gamma$ is the drag of the particle. For a spherical particle, this is Stokes drag $\gamma = 6\pi \mu R$, where $\mu$ is the viscosity of the surrounding medium. The rheological response function is then calculated using eq. 5.4.

**Calibration**

For a trapped particle in an viscous fluid, the Fourier transform of the thermal fluctuations takes the shape of an Lorentzian [545, 547, 550, 551]:

$$S(f) = \frac{k_B T}{\gamma \pi^2 (f_c^2 + f^2)} \tag{5.8}$$

Here $k_B T$ is the thermal energy and $f_c$ is termed the corner frequency [545, 547, 550]. The low frequency response is governed by the laser trap, according to $S(f) = 4\gamma k_B T/\kappa$, while at high frequency the response is governed by the thermal fluctuations of the bead. The corner frequency is the boundary between these two regimes and is proportional to the strength of the trap:

$$k = 2\pi f_c \gamma \tag{5.9}$$

For calibration, 10 up to 15 spherical particles in buffer solution (in case of fibrin) or water (in case of PAA) were measured and the average $k$-values in both $x$ and $y$ was used to analyze data obtained in fibrin and PAA networks.
5.3 Results

5.3.1 Setup Validation

Calibration and Viscous Fluids

We developed an optical tweezer setup to measure local mechanical properties of fibrous networks. To validate this setup, we first measured 1 \( \mu \)m diameter silica beads in water. The beads were sparsely seeded inside a flow chamber with a height of \( \sim 300 \) \( \mu \)m. The beads were trapped 10 \( \mu \)m away from the bottom surface. The Brownian displacement fluctuations of a single bead inside the laser trap were recorded via the QPD. The Fourier transform of these fluctuations gives the power spectral density function \( (S) \), which shows a plateau at low frequencies and a -2 slope at high frequencies (Fig. 5.2A). This result is consistent with eq. 5.8 and earlier reports [547, 552]. The transition from a plateau to a -2 slope is characterized by the corner frequency \( f_c \). Using this corner frequency, the trap stiffness of the laser can be determined with eq. 5.9.

The trap stiffness was linearly dependent on laser power (Fig. 5.2B). This linear dependence is indeed expected and is consistent with earlier reports [547, 552]. In addition, we expect that the trap stiffness shows a depth dependence due to the high numerical aperture (NA) of the used objective and the refractive index mismatch between the immersion oil and the aqueous
Figure 5.3: The dependence of the trap stiffness (A) and the QPD deflec-
tion (B) on focus depth in the sample. Crosses refer to the x-direction,
while open circles denote the y-direction. The symbols are shifted along
the x-axis for clarity in both (A) and (B), the sample depth $z$ is 10, 15, 20
and 25 $\mu$m. For $z = 25$ $\mu$m only 2 beads were measured, while for the other
heights 5–11 beads were measured (see Table 5.1 in SI for statistics).

samples [553,554]. Indeed, the trap stiffness decreased linearly with increasing
focus depth for depths between 10 and 25 $\mu$m, as shown in Fig. 5.3A, consist-
ent with earlier reports [553,554]. The trap stiffness in the $x$ and $y$ direction
have the same magnitude for all sample depths $z$, showing that the laser spot
is symmetric.

The QPD deflection in $x$ and $y$ was calibrated using the plateau level at
low frequencies in Fig. 5.2 using eq. 5.8 and the fit in Fig. 5.2A [550]:

$$S_{\text{fit}} = \frac{C}{f_c^2 + f^2}$$  \hspace{1cm} (5.10)

$$\text{deflection} = \sqrt{\frac{S}{S_{\text{fit}}} = \sqrt{\frac{k_bT}{\gamma\pi^2C}}}$$  \hspace{1cm} (5.11)

Here $S_{\text{fit}}$ is the fitting function with $C$ and $f_c$ as fitting variables. The de-
flexion converts the voltage outputs from the QPD to $\mu$m s and the values
reported here are in the same range as previous reports [555]. The deflection
shows a slight increase with sample depth, as shown in Fig. 5.3B.

The depth dependence of the trap stiffness and deflection shown in Fig. 5.3
emphasize the importance of using a consistent focus depths for optical tweezer
microrheology. In the following controls, we make sure we are always at a depth
of 10 $\mu$m away from the bottom surface. This height is sufficiently far away
Figure 5.4: The response functions of 1μm silica beads in water, for varying laser powers. (A) The real part and (B) imaginary part of the apparent response function. (C) The imaginary rheological response function. (D) The viscous modulus. The trap stiffness is varied from $5.67 \times 10^{-6}$ (green), to $1.20 \times 10^{-5}$ (blue) and $2.63 \times 10^{-5}$ N/m (purple) by tuning the laser power. The dotted lines are the theoretical predictions.

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ness over the whole frequency range, except at the lowest frequencies (≤5 Hz). Low frequency results from optical tweezer microrheology indeed commonly suffer from noise due to sample and laser drift. Note that the $A'$ data are cut-off about one decade earlier than the $A''$ data; this is a consequence of the Kramers-Kronig transformation needed to infer $A'$ from the measured $A''$ and the finite frequency range over which $A''$ is known.

The rheological response function can be calculated from the apparent response function by correcting for the contribution of the trap trap, using eq. 5.4. As shown in Fig. 5.4C, the imaginary part of the rheological response function for beads in water is now independent of trap stiffness and are in close agreement with the theoretical prediction (shown in black) except again at low frequencies. As shown in Fig. 5.4D, the measured $G''$-values for water closely agree with the theoretical prediction of $G'' = 2\pi \mu f$ for all trap stiffnesses. Only at frequencies below 5 Hz, the experiments overestimate $G''$. We obtain similar results for a 50% v/v glycerol-water mixture ($\mu = 5.9$ mPa·s at room temperature [558,559]) (see Fig. 5.17 in SI). We can thus accurately capture the viscous properties of fluids of varying viscosities at frequencies higher than 5 Hz.

Polyacrylamide (PAA) gels

We established that we can accurately measure the viscous properties of fluids of varying viscosities at frequencies above 5 Hz. Next, we investigated a model system with both an elastic and viscous component, namely PAA. We prepared PAA gels with low stiffness to approach the stiffness of fine fibrin gels (i.e. $G'$ on the order of 10 Pa or less). The pore size of these PAA gels should be on the order of a hundred nanometers or less [560,561] and is thus much smaller compared to the bead size used here (1.5 μm). Macrorheology measurements showed that the viscous and elastic moduli of the PAA gels were of the order of several Pascals, as shown in Fig. 5.5 (red and blue symbols). The moduli determined by optical tweezer microrheology (gray lines) are very similar to the values obtained by macrorheology in the overlapping frequency range (0.5−10 Hz). For instance, macrorheology gives $G' = 5.4 \pm 1$ Pa and $G'' = 2.17 \pm 0.26$ Pa at frequency 5 Hz, while optical tweezer microrheology gives $G' = 8.3 \pm 4.4$ Pa and $G'' = 2.5 \pm 1.3$ Pa in the range of 5−6 Hz. We note that noise peaks at frequencies of ~100 Hz reflect vibrations originating from the laser driver. These noise peaks are more pronounced in elastic samples, like PAA gels, than in viscous samples like water and water-glycerol fluids.

The macroscopic rheology measurements can only access the elastic plateau regime for $G'$. Optical tweezer microrheology, in contrast, reveal a slight frequency dependence of $G'$ for low frequencies (i.e. $f \lesssim 50$ Hz) (Fig. 5.5A, solid line with a power law exponent of 0.2) and a steeper frequency dependence at higher frequencies (solid line with a power law exponent of 0.5). This frequency dependence is consistent with a previous report [548] which showed
that dilute PAA gels can be described by the Rouse model \[562,563\]. We note that for long linear polymer chains with narrow molecular weight distribution, the low frequency response is governed by a flow regime and an entanglement regime, while the high frequency response describes the transition towards the glass state as well as the glass state with modulus \(G_g^*\) \[564\]. More concentrated PAA gels \(\sim 2\%\) w/w showed exponents close to 1 \[548,565\] and could be described by a rheological model that combines an continuous relaxation spectrum model and fractional derivatives for the higher frequencies \[565\]:

\[
G^*(\omega) = G_f^* + G_g^* = \int_0^\infty H(\lambda) \frac{i\omega\lambda}{1 + i\omega\lambda} d\lambda + G_1 \left(\frac{i\omega\lambda_1}{1 + (i\omega\lambda_1)^a}\right)
\]

\(\text{(5.12)}\)

where \(H(\lambda)\) is the continuous relaxation spectrum and \(\lambda\) is the relaxation time. \(\lambda_{max}\) is the maximum relaxation time, below which the sample flows. \(H = n_f G_N^0 \left(\frac{\lambda}{\lambda_{max}}\right)^n f\) when \(\lambda < \lambda_{max}\) and 0 otherwise, where \(-n_f\) is the slope of \(G''\) at very low frequencies (< \(10^{-2}\) Hz, not accessible in our setup) and \(G_N^0\) is the linear storage modulus at the plateau. \(G_f^*\) describes the low frequency response from flow to the glass transition state. \(1/\lambda_1\) is the typical crossover frequency between glass and solid state in the mechanical response (at high frequencies) and is related to the microstructure of the PAA \[565\]. \(G_1\) is the high frequency modulus at \(f = 1/\lambda_1\). The exponent \(a\) is below 1 for PAA and was reported to decrease with acrylamide concentration. This model was shown to work up to frequencies of 500 Hz \[565\].

Both the model presented in \[565\] (eq. 5.12) and the Rouse model state that, for intermediate to high frequencies, both \(G'\) and \(G''\) should scale with the same power law exponent \(a\). In particular, the Rouse model states that this exponent should be 0.5. Since we see a power law exponent of 0.5 for \(G'\), we expect to see a power law exponent close to 0.5 for \(G''\) as well. We find a power law exponent of 0.62 ± 0.02 at low frequencies \((f \lesssim 10\) Hz) for \(G''\), and a smaller exponent of 0.45 ± 0.03 by microrheology at higher frequencies (fitted between 10 Hz < \(f < 50\) Hz). The onset of the regime where \(G'\) and \(G''\) have a similar slope is dependent on the gel concentration \[565,566\]. We estimated this onset by using the model presented in \[565\] using:

\[
f_T = \frac{1}{2\pi \lambda_1} \left(\frac{G_N^0}{G_1 \cos(\pi a/2)}\right)^{1/a}
\]

\(\text{(5.13)}\)

Using values typical for our PAA gel \((a = 0.5, G_1 = 100\) Pa, \(G_N^0 = 10\) Pa and \(\lambda_1 \sim 10^{-4}\) s), we estimated \(f_T\) to be around 10 Hz. This estimate is in line with prior measurements \[565\], where \(f_T\) increases with increasing acrylamide concentration \((f_T \sim c^{1.6})\) for gels with a similar BIS-acrylamide content as our gels. This cross-over frequency is also consistent with the power law dependences of \(G'\) and \(G''\) shown in Fig. 5.5.
Overall, the microrheology experiments provide reliable measurements of the viscoelastic moduli of PAA, including the $f^{0.5}$ frequency dependence of $G'$ and $G''$ at intermediate to high frequencies expected on the basis of the Rouse model \cite{548,562,563}. Next, we aimed to investigate the local mechanical properties of fine fibrin gels.

### 5.3.2 Bead Surface Properties Affect Stiffness Measurements inside Fibrin Gels

Having validated our microrheology setup using Newtonian fluids and PAA gels, we investigated the effect of the bead surface properties on the micromechanical properties of fine fibrin networks. For this purpose, 1.5 $\mu$m polystyrene beads were either covalently coated with Pluronic to render them inert, or left uncoated so fibrin adhered to the bead surface. The average pore size of these networks decreased from 350 to 115 nm when the concentration was raised from 0.4 to 2 mg/ml, as estimated using $\xi \simeq 1/\sqrt{\rho}$ (see section 2.4.2 in Chapter 2). Thus, depending on the fibrin concentration, the beads were between 4 and 13 times larger compared to the average mesh size. As a first step, the mechanical properties of 1 mg/ml fine fibrin networks were determined using optical tweezer microrheology with the coated and uncoated beads and compared with macrorheology measurements on corresponding networks prepared without beads.

The bead surface properties play a major role in the determination of the...
mechanical properties, as shown in Fig. 5.6A and B. The most striking observation is that the noncoated beads (red curves) report higher $G'$ and $G''$ values compared to the beads coated with Pluronic (gray curves). The frequency dependence of $G''$ also differs between the two bead types: $G''$ reported by the noncoated beads show a weaker frequency dependence compared to the coated beads. Note that similar to the PAA gels, noise peaks originating from vibrations are present for $G'$ and $G''$ curves at frequencies around $\sim 100$ Hz. Compared to macrorheology data (blue squares), the uncoated beads report larger values for $G'$ and $G''$, whereas the coated beads report smaller values for $G'$ and on average comparable values for $G''$.

To determine the origin for the different micromechanics reported by coated and uncoated beads, we investigated the local fibrin structure. As shown in Fig. 5.6C, in the absence of a surface coating, the density of fibrin close to the beads is much higher than the fibrin density between beads. Apparently, beads accumulate fibrin and possibly act as nucleation centers. In the case of coated beads, the network microstructure as observed by confocal microscopy (Fig. 5.6D right) is qualitatively similar to the control network structure without beads (Fig. 5.6E). However, beads often coincide with regions of lower

Figure 5.6 (facing page): The effect of bead surface properties on the mechanical properties of 1 mg/ml fine fibrin gels measured by optical tweezer microrheology (A,B), and on network structure (C-E). The gray and red curves in (A) and (B) denote the $G'$ and $G''$ measured by beads that are covalently coated with Pluronic or uncoated, respectively. The blue squares denote corresponding macrorheology measurements on networks without beads. Each line represent the geometric average of an independent measurement (see also Table 5.2 in the SI for statistics). The dashed black lines in (A) and (B) are power laws with exponent $3/4$. (C) Noncoated bead inside a fine fibrin network causes a locally enhanced fibrin density. (D) Coated beads do not seem to perturb the fibrin network. (E) 1 mg/ml fine fibrin network without beads (control). The radial intensity distribution for (F) noncoated beads ($n=5$) and (G) beads covalently coated with Pluronic ($n=15$). For (F) and (G) the intensity distributions are centered on the bead ($r=0$) and the intensity is normalized by the average intensity $I_{bkg}$, 5 $\mu$m away from the bead’s center. Red is the average intensity distribution, gray are single bead measurements, and the vertical dashed line indicates the location of the bead surface ($r_{bead} = 0.75$ $\mu$m). The insets are zoom-ins (log-log scale) of $I/I_{bkg}$ in the range $r[r_{bead}, 4$ $\mu$m]. For (C) and (D), the left image are DIC images showing the bead positions, while the right panels are sum images of confocal fluorescence image stacks over 5 $\mu$m or 10 $\mu$m depth respectively. The control confocal image in (E) is a summation over 10 $\mu$m depth. Scale bar denotes 5 $\mu$m for all images.
fibrin density (Fig. 5.6D). This qualitative observation is consistent with prior measurements on networks of filamentous actin, where passivated beads were shown to settle in weaker parts of the gel and to be surrounded by a thin layer depleted of actin [518,522]. We quantified the density distribution of fibrin around the uncoated and coated beads by determining the average radial fluorescence intensity distribution, as shown in Fig. 5.6F and G respectively. The intensity was normalized by the background level, $I_{bkg}$, which was defined as the average intensity of the fibrin network in-between beads (>5 µm away from bead centers). Noncoated beads show a 4 times increase in normalized intensity close to their surface, which decays back to 1 over a range of about 2.5 µm (best seen in inset Fig. 5.6F). Thus, a fibrin layer of about 1.8 µm thick is accumulated on the bead surface. In contrast, in the coated beads case there is a depleted region, where the normalized intensity is about 80% near the bead’s surface and decays to 100% over a range of 3.4 µm (best seen in inset Fig. 5.6G). The depletion layer is thus about 2.6 µm.

The observation of a depletion layer surrounding inert beads immersed in a semiflexible polymer network is in line with publications on microrheology of actin gels [521,522], where coated beads also showed a lower modulus [517–519] and higher frequency dependence [521,537] compared to sticky beads. It was proposed that the thickness of this depletion layer can be estimated by considering a bead in a composite system, as sketched in Fig. 5.7A [546]. Close to the bead, we assume a local incompressible medium with a local modulus $G_{loc}$, while further away from the bead we recover the bulk modulus $G_{bulk}$. In this simplified system, the modulus measured by the bead, $G_{bead}$, is [521,546,567]:

$$G_{bead}(\omega) = \frac{2G_{bulk}(\omega)[\kappa'' - 2\beta^5 \kappa']} {4\beta^6 \kappa'^2 - 9\beta^5 \kappa \kappa' + 10\beta^3 \kappa \kappa' - 9\beta \kappa' - 15\beta^2 \kappa' + 2 \kappa' + 8\beta^3 \kappa''} \tag{5.14}$$

Here $\beta = a/b < 1$, $\kappa = G_{bulk}/G_{loc}$, $\kappa' = \kappa - 1$ and $\kappa'' = 3 + 2\kappa$. We used eq. 5.14 to estimate the depletion layer for coated beads, taking for $G_{bulk}^*$ the modulus reported by macrorheology, and assuming that the local modulus $G_{loc}$ in the depletion layer corresponds to the modulus expected for a fine fibrin network with a concentration equal to 90% of the bulk concentration, $c_p$. Based on the macrorheology data shown in Fig. 5.8B and D, we assume that $G_{loc}' = (0.9)^{11/5}G_{bulk}'$ and $G_{loc}'' = (0.9)^{0.85}G_{bulk}''$, since $G' \sim c_p^{11/5}$ and $G'' \sim c_p^{0.85}$. Taking the moduli reported by microrheology at ~5 Hz as $G_{bead}$ (see Fig. 5.8), we get values of ~3 µm for the depletion layer (see Fig. 5.7B). This estimation is in agreement with the estimation based on the fibrin fluorescence intensity profiles measured by confocal microscopy (Fig. 5.6G), though there is a large uncertainty. This can be attributed, in part, to sample-to-sample variations in $G_{bulk}$. In the future this variability may be circumvented by estimating $G_{bulk}$ by two particle microrheology on the same sample [521,567].
Figure 5.7: (A) Schematic of the local depletion layer around the Pluronic coated beads. The bead radius is $a$, the depletion layer is $b - a$ and $r$ denotes the radial coordinate. In the depletion layer, we assume a local modulus $G_{\text{loc}}$, which is lower than the bulk modulus, while further away we recover the bulk modulus $G_{\text{bulk}}$. The bead measures a superposition of $G_{\text{loc}}$ and $G_{\text{bulk}}$ according to eq. 5.14. (B) The estimated depletion layer thickness for beads in fine fibrin networks as a function of fibrin concentration, estimated assuming the fibrin density in the depletion layer is 90% of the bulk density.

However, we note that there is also quite a large variation in the intensity distribution of the fibrin network near the beads, as evident in Fig. 5.6G. To get a more accurate estimate of the depletion layer of surrounding each bead, one should ideally correlate the micro-environment as visualized by confocal microscopy directly with the measured mechanical properties reported for that bead. Also, we note that we made the simplifying assumption that the local reduction in fibrin concentration near the bead surface is 10% for all fibrin concentrations. In any case, the estimated depletion layer is in the same order of magnitude as values reported for actin networks [521,568,569], which ranged between 0.8 and 2.5 $\mu$m, depending on bead size and average filament length. The depletion layer in fine fibrin networks is larger than reported for beads in semidilute solutions of $\lambda$-DNA ($\leq 0.6$ $\mu$m) [567], which is reasonable, since DNA has a shorter persistence length ($\sim 50$ nm [570,571]) than fine fibrin fibers ($\sim 150$ nm, see Chapter 2).

The confocal microscopy results indicate that noncoated beads are confined by a layer of fibrin with an enhanced fibrin density compared to the bulk, whereas coated beads tend to probe regions that on average have a (locally) reduced fibrin concentration. Consequently, the elastic and viscous moduli measured by sticky beads are about a factor 5 to 6 higher compared to those reported by the inert beads (see also Fig. 5.8). This observation is again
consistent with prior measurements on actin networks, where the modulus reported by beads was correlated with the number of actin molecules bound on the particle surface [518]. Since the fibrin networks are formed in the presence of the beads, it is conceivable that the beads alter the network structure by acting as nucleation or crosslinking points. It would be interesting to test this hypothesis by imaging the networks with scanning electron microscopy and also by following fibrin polymerization with confocal microscopy in time in the presence of beads.

Both the coated and uncoated beads show systematic differences in $G'$ and $G''$ compared to the macrorheology control measurements (blue squares in Fig. 5.6A and B). The noncoated beads overestimate both $G'$ and $G''$, while the coated beads show a very good agreement with macrorheology for $G''$, but systematically report a lower $G'$. To facilitate a direct comparison, we compared the values of for $G'$ and $G''$ obtained by macrorheology at a frequency of 5 Hz with corresponding values determined by microrheology in Fig. 5.8. For the optical tweezer data, we averaged over 5 data points in the frequency domain between 5 and 6 Hz to limit the contribution of noise.

In the case where the beads stick to the fibrin network, $G'$ is about 3 times higher than the macrorheology control (Fig. 5.8B), though showing a similar concentration dependence. Both micro- and macrorheology reveal a power law increase of $G'$ with concentration, with an exponent of 2.3±0.3 in case of microrheology and 2.0±0.1 in case of macrorheology. Both exponents are close to the expected exponent of 11/5 indicated by the red dotted line (see Chapter 2 section 2.4.2). This correspondence is consistent with prior measurements on actin [516], where noncoated (sticky) beads also showed a similar concentration dependence of $G'$ as the bulk modulus measured in that case by two particle microrheology. Interestingly, beads that stick to biopolymer networks that have a more open mesh work, like collagen, were found to underestimate the network stiffness [538], though still reporting the same concentration dependence as macrorheology experiments. Thus, the magnitude of the moduli reported by the probe beads is determined by a combination of surface chemistry and the ratio between bead size and mesh size. The concentration dependence of $G''$ reported by the noncoated beads was steeper for microrheology (with a power law exponent of 2.0±0.2) compared to macrorheology (which showed an exponent of 0.85±0.09), as shown in Fig. 5.8D.

In case where the beads are inert, $G'$ is about 3 time lower than the macrorheology control (Fig. 5.8A), but showing a similar concentration dependence that is consistent with the expected 11/5 power law (red dotted line). The values for $G''$ for the inert beads nicely agree with the corresponding values reported by macrorheology (Fig. 5.8C). Previous measurements in actin also showed an underestimation of the elastic modulus when beads were inert [517, 519]. This underestimation ranged between a factor 2 up to 10
Figure 5.8: The effect of bead surface properties on the measured mechanical properties of fine fibrin gels measured with optical tweezer microrheology (closed squares, averaged between 5-6 Hz) and compared with macrorheology (open squares, at 5 Hz). (A) and (C) are for beads covalently coated with Pluronic, while (B) and (D) are for noncoated beads. The dotted red lines are power law fits with exponent 2.1±1.8, 2.3±0.3 and 2.0±0.2 for (A), (B) and (D) respectively. The solid line in (B) denotes the expected 11/5 power law dependence based on the thermal bundle model (see Chapter 2), while the solid line in (D) is a power law fit with exponent 0.85±0.09.

depending on actin concentration.

It has been suggested by Van Citters et. al. [572] that the stiffness dependence on frequency reported by one particle microrheology will not report the bulk modulus dependence if the beads are not (somewhat) linked to the network. Indeed, prior measurements in actin show that the typical $G'' \sim \omega^{3/4}$ dependence [427,521,573] (expected for semiflexible polymer networks [574–576]) is replaced for inert beads by a steeper, $\sim \omega^1$ dependence [521]. To check this behavior also occurs in the fibrin networks, we determined the dependence of $G''$ on frequency in the high frequency regime ($f > 500$ Hz), where $G''$ is expected to be sensitive to the bending and contour length fluctuations of the fibrin filaments.

As shown in Fig. 5.9, we find that $G''$ reported by inert beads (solid black
Figure 5.9: The power law exponent of $G''$ at high frequency (>500 Hz) for coated (black squares) and noncoated, i.e. sticky (gray open circles) beads in fine fibrin networks of varying concentration. The expected 3/4 dependence for semiflexible polymers is depicted in a dashed black line.

squares) follows a power law in frequency with an exponent that systematically decreases with fibrin concentration, from 0.85 at 0.4 mg/ml, down to 0.65 for 2 mg/ml. Sticky (noncoated) beads report an even steeper dependence of the frequency exponent of $G''$ on fibrin concentration, decreasing from about 0.85 at 0.4 mg/ml to about 0.5 at 1 mg/ml. For the same fibrin concentration, the power law exponent is higher in the case of inert beads (black symbols), and closer to the expected 3/4 dependence for semiflexible polymers than the sticky beads. It could be that the fibrin networks in the bead surrounding is prestressed at increasing fibrinogen concentration, especially when formed in the presence of sticky beads, thus lowering the frequency dependence of $G''$ [372, 575, 576]. In the limit of a highly prestressed network, we expect a 1/2 power law dependence of $G''$ on frequency [372], which corresponds to the smallest exponent we observe for sticky beads in 1 mg/ml fibrin networks.

To summarize, we find that both sticky and inert beads reproduce the correct concentration dependence of $G'$, which is expected on the basis of macrorheology measurements and theoretical models of semiflexible polymer gels. The two bead types, however, report different values for the low frequency modulus compared to the macrorheology control measurements: Sticky beads overestimate both $G'$ and $G''$, while inert beads underestimate $G'$, but not $G''$. Overall, beads passivated with Pluronic capture the macroscopic modulus more faithfully than uncoated beads, in terms of magnitude as well as frequency dependence. The small discrepancy between the magnitude of $G'$
**Figure 5.10**: The elastic $G'$ (A) and viscous $G''$ (B) moduli for 0.5 mg/ml coarse fibrin networks measured using macrorheology (blue squares) or optical tweezer microrheology (solid gray lines). For optical tweezer measurements, 1.5 µm polystyrene beads were used. The dashed black line in (B) denotes a power law with exponent 3/4, as expected for semiflexible polymer networks [574–576], while the dashed line in (A) denotes a power law with exponent 0.35. (C-E) Microscopy images of 1 mg/ml coarse fibrin gels with (C,D) and without (control, E) beads. The networks contain 1.5 µm polystyrene (C) or 1 µm silica (D) beads. For (C) and (D), the left images correspond to summations of confocal images recorded over a 5 µm depth, while right images are single plane differential interference contrast (DIC) images showing the beads. White arrows denote the positions of the beads. Scale bar denotes 5 µm for all images.

and $G''$ reported by micro- and macrorheology can be quantitatively explained by accounting for the presence of a thin layer depleted of fibrin surrounding the beads.

### 5.3.3 Sticky Beads Inside Fibrin Networks with Large Pores

We have shown that the surface properties of the probe beads inside fibrin networks with small pore size ($\xi \sim 200$ nm) play an important role in the reported mechanical properties. In order to extend the microrheology measurements to fibrin networks prepared under more physiologically relevant conditions, we also investigated coarse fibrin networks. These networks form an open mesh
work of \( \sim 100 \) nm diameter fibers with an average pore size on the order of 10 \( \mu \)m at 0.5 mg/ml when prepared at 37\( ^\circ \)C and at pH 7.4 [66] (see also Fig. 2.1 in Chapter 2). Since the 1.5 \( \mu \)m passivated polystyrene beads will diffuse freely within the water-filled pores and will not be confined in these networks [523], we selected uncoated 1.5 \( \mu \)m polystyrene beads that adhered to the fibrin network, as shown in Fig. 5.10C.

\( G' \) as measured with macrorheology shows virtually no dependence on frequency for low frequencies, with an extremely weak power law dependence with exponent of 0.04, as shown in Fig. 5.10A (blue squares). In the microrheology measurements, \( G' \) also becomes nearly flat at low frequencies (\(<50 \) Hz, solid gray curves), while it increases at higher frequencies with a power law dependence on frequency with exponent 0.35 (dashed line in panel (A)). The viscous modulus, \( G'' \), shows a slow increase with frequency in the macrorheology case and overlaps with data from microrheology. At high frequencies (\(>50 \) Hz), \( G'' \) determined by microrheology follows a power law with exponent close to the expected 3/4 dependence for semiflexible polymers (dashed line in (B)) [574–576].

We note that untreated silica beads also spontaneously adhere to the fibrin network and can therefore also be used for microrheology, as shown in Fig. 5.10D.

Interestingly, we see from Fig. 5.10 that both the macroscopic \( G' \) and \( G'' \) are well approximated by microrheology measurements in the overlapping frequency range. To better quantify this, we again compare \( G' \) and \( G'' \) from micro- and macrorheology at a fixed frequency range (5–6 Hz) in Fig. 5.11. Even though statistics is low (with a total of 5 samples, see also Table 5.2 in SI), \( G' \) falls in the same range as reported by macrorheology, while \( G'' \) is a
Figure 5.12: The low strain viscoelastic moduli of fine (red) and coarse (gray) fibrin networks, as measured by optical tweezer microrheology using sticky (noncoated) 1.5 µm polystyrene beads. Panels (A) and (C) depict \( G' \) and \( G'' \) for 0.5 mg/ml fibrin gels respectively, while (B) and (D) are for 0.6 mg/ml gels. The dashed line in (C) and (D) depict a 3/4 dependence predicted for semiflexible polymer networks [574–576].

bit overestimated. These results are surprising, since coarse fibrin networks are far from the incompressible continuum assumption that our data analysis assumes. Interestingly, a recent video particle tracking microrheology study of collagen networks, which have a similar pore size as fibrin, also reported agreement between micro- and macrorheometry [577], although another study reported an underestimation of the moduli by microrheology [538].

Next we compared the frequency dependence of the moduli for the two fibrin network limits of small mesh size ('fine clots', \( \xi \sim 200 \text{ nm} \)) and large mesh size ('coarse clots', \( \xi \sim 10 \mu \text{m} \)), as shown in Fig. 5.12. The storage modulus, \( G' \), shows a steeper frequency dependence for the coarse clots compared to the fine clots, both at 0.5 mg/ml (Fig. 5.12A) and 0.6 mg/ml (Fig. 5.12B). Since the frequency range probed is intermediate between the low-frequency (plateau)
regime and the high-frequency ($\omega^{3/4}$ scaling) regime, it is difficult to interpret this finding. At 0.5 mg/ml fibrin, the viscous modulus, $G''$, shows an identical frequency dependence for fine and coarse clots (Fig. 5.12C). In both cases, $G''$ scales approximately as a power law with exponent 3/4 (see dashed line). This scaling is consistent with the model of coarse clots that we presented in Chapter 2, where we showed that coarse fibrin fibers can be modeled as wormlike bundles of semiflexible protofibrils. At 0.6 mg/ml (Fig. 5.12D), $G''$ for the coarse clots again shows 3/4 power law scaling with frequency, but $G''$ for the fine clots shows a somewhat weaker frequency dependence. As discussed in section 5.3.2, we suspect that the deviation from a 3/4 scaling in case of fine clots is caused by the strong accumulation of fibrin on the noncoated bead’s surface. Confocal imaging suggests that in case of the coarse clots, the sticky beads have a lesser tendency to recruit fibrin (compare Fig. 5.8C and Fig. 5.10C), which may explain why we observe 3/4 scaling for $G''$. A summary of the scaling exponents observed for the high-frequency scaling of $G''$ in coarse fibrin clots at three different concentrations is shown in Fig. 5.13. In each case, the exponents are close to, but somewhat larger, than the theoretical value of 3/4 expected in case of semiflexible polymers.

### 5.3.4 Calculation of Fibrin Fiber Persistence Length

So far, we have extensively investigated the effects of bead surface properties (section 5.3.2) and network pore size (section 5.3.3) on the apparent micromechanical properties of fibrin networks at low frequencies. In this section, we focus on the high frequency response. In particular, we will estimate a fundamental length scale of fibrin fibers, namely the persistence length $l_p$,
from the high frequency response of $G''$.

At high frequencies, the complex shear modulus is governed by the stress relaxation via bending fluctuations of the individual fibers within the network. For semiflexible polymers, we expect [574,578]:

$$G^*(\omega) = \frac{1}{15}\rho\kappa l_p(-i2\gamma_L/\kappa)^{3/4} \omega^{3/4} - i\mu\omega$$

(5.15)

where $\kappa = l_p k_BT$ is the bending modulus of the fibers, $\gamma_L = 4\pi\mu/\ln(0.6\lambda/d)$ is the lateral drag coefficient and $\mu$ is the viscosity of the solvent (here $\mu = 0.001$ Pa·s); $\lambda$ denotes the maximum relaxation length of the fiber, for which we take the mesh size. We estimate for the fiber diameter $d = 20$ nm for fine fibrin networks (as determined by electron microscopy, see Fig. 2.12 in Chapter 2) and $d = 120$ nm for coarse fibrin networks (as determined by turbidimetry [66,297]). We base the $l_p$ calculations only on microrheology measurements where $G''$ exhibits a power law dependence on frequency with an exponent close to $7.5 \pm 1$.

The apparent persistence length for fine fibrin networks determined from microrheology data obtained with passivated beads does not change with fibrin concentration, as shown in Fig. 5.14A (closed black squares) and is on average $547 \pm 330$ nm. It should be noted, however, that eq. 5.15 is valid for $\omega > \omega_1$, where $\omega_1 = \frac{\kappa}{l_{drag}} (\frac{T}{\kappa})^4$ [578]. This limit holds for the most dilute networks ($< 1$ mg/ml), where we estimate $\omega_1$ to be about 300 Hz. However, for the more concentrated gels, $\omega_1$ is expected to shift up to the kHz range ($\sim 5000$ Hz for the 2 mg/ml case). If we only consider measurements within the range of the
assumption $\omega > \omega_1$, then the average $l_p$ is lowered to 406±150 nm. In Chapter 2, we have shown that we can consider fine fibrin clots as networks of loose semiflexible bundles of on average two protofibrils. The persistence length of one protofibril is thus related to $l_p$ by: $l^F_p = l^{pf}_p N_p$ where $N_p = 2$ [320] (see section 2.3 eq. 2.4). The apparent persistence length of one protofibril is thus 203±75 nm. This value is in line with previous reports based on light scattering (120–200 nm for human fibrinogen [353,355]) and electron and atomic force microscopy (500 nm for fish fibrinogen [39,354]). However, the apparent value obtained by microrheology is larger than the value that we derived for the same fine fibrin clots from macrorheology experiments (75 nm, see section 2.5 in Chapter 2). It should be noted that both estimates of the persistence length, from micro- and macrorheology, are model-dependent, and represent average numbers from measurements that ensemble averages over many fibers. Moreover, as discussed in section 5.3.2, the microrheology data are influenced by the presence of a depletion layer surrounding the beads.

The apparent persistence length for fine fibrin networks determined from microrheology data obtained with sticky beads is consistently larger than the values determined with passivated beads (Fig. 5.14A open gray circles). Furthermore, the persistence length shows an apparent increase with increasing fibrin concentration. A likely explanation for these two observations is the evident accumulation of fibrin on the bead surface (Fig. 5.6C and F), giving rise to a significant increase in local fibrin concentration. Due to this accumulation of fibrin, it seems unlikely that the sticky beads in fine fibrin networks reflect single fiber motion, even at high frequencies.

The apparent persistence length for coarse fibrin networks determined from microrheology data obtained with sticky beads is around 40 µm, as shown in Fig. 5.14B (triangles), taking into account that $G''$ is overestimated by a factor $\sim 3$ [579]. This number for the persistence length is in the same range as reported in a previous study that also used optical tweezer microrheology ($\sim 60$ µm [66]). However, it is significantly smaller than values reported for similar coarse clot systems using video microrheology ($\sim 40$ cm [533]). In Chapter 2 we have shown that coarse fibrin fibers can be considered as tight bundles of $N_p$ semiflexible protofibrils, for which the persistence length can be calculated as $l^F_p = l^{pf}_p N_p^2$. Given a bundle size $N_p$ of about 65 measured for similar clots in earlier work [66], this relation suggests a persistence length of only $\sim 10$ nm for an individual protofibril. This number seems unphysically low, given that it even lower than the persistence length reported for DNA ($\sim 50$ nm [570,571]), which can be considered as a flexible polymer. A complementary way to interpret the rigidity measurement is to estimate the Young’s modulus, assuming that the fibers can be modeled as a uniform rigid rod with radius 60 nm and persistence length 40 µm. This assumption gives a Young’s modulus of 16 kPa, which is much lower than earlier reports for ligated fibrin fibers based
on micromanipulation (∼MPa [281–283]). Together with the observation that the coarse clot case is far from the continuum assumption made for the optical tweezer microrheology data analysis, we consider it likely that the microrheology measurements underestimate the persistence length for coarse clots.

5.4 Discussion

Both the elastic and viscous properties of the ECM have shown to play a vital role in the regulation of cell behavior [3, 4, 72, 499, 500]. Here we focus on the local mechanical properties of the ECM, which have been shown to influence cell migration [310] as well as cell division [501]. In particular, we aimed to measure both the low and high frequency response to get simultaneously information about the network and single fiber level.

We developed an optical tweezer setup in-house to investigate the local mechanical properties of biopolymer networks by passive one particle microrheology. The setup was validated using water and a 50% water-glycerol mixture, where we showed that the viscous properties were accurately captured above frequencies of 5 Hz. We showed that the mechanics of PAA hydrogels, which have a significant elastic component, can also be accurately measured with our setup.

In fibrin networks, non-passivated probe beads tend to accumulate fibrin on their surface, as shown in Fig. 5.6C for fine networks and in Fig. 5.10C and D for coarse networks. This observation is consistent with previous studies of microrheology in fibrin, showing that fibrin sticks to beads without any special treatment [523, 533]. Fibrin is thus very sticky, which is important for its purpose in vivo to serve as a plug during wound healing.

We showed that fibrin adhesion to the uncoated 1.5 μm polystyrene beads led to an overestimation of the elastic and viscous shear moduli (\(G'\) and \(G''\)) compared to the bulk rheological properties. In contrast, beads covalently coated with Pluronic (rendering them inert) reported smaller values for \(G'\) and on average comparable values for \(G''\) as macrorheology. These observations are consistent with measurements of actin networks, where the shear modulus reported by (partially) inert beads was lower compared to the modulus reported by beads that stuck to the network [517–519]. Confocal microscopy revealed a depletion layer with a reduced fibrin density around the inert beads with a thickness of ∼2.6 μm. This depletion layer can account for the reported micromechanical modulus, using a model that assumes that the beads sense a superposition of the bulk modulus and a lower local modulus, as shown in eq. 5.14 and in Fig. 5.7A. However, the estimate from eq. 5.14 can be further optimized by measuring the bulk modulus for each sample individually, to remove sample-to-sample variation, for instance with two particle microrheology [521, 567]. Also, the reduction in fibrin concentration in the depletion layer might not be the same for all fibrin concentrations, as assumed in Fig. 5.7B.
This assumption needs to be checked by confocal microscopy in the future.

We measured the thermal persistence length of the fibrin fibers from the high frequency response of $G''$ using eq. 5.15. This equation assumes that the high frequency response reports bending fluctuations of single filaments [574,578]. We showed that the estimates for $l_p$ are reasonable for inert beads, where the apparent persistence length of the fine fibrin fibers was 432 nm. Given that we can consider the fine fibrin fibers as bundles of 2 protofibrils (see Chapter 2), we estimate the persistence length of protofibrils to be 215 nm, which is in line with previous reports based on light scattering (120-200 nm for human fibrinogen [353,355]) and electron and atomic force microscopy (500 nm for fish fibrinogen [39,354]). However, for sticky beads, the apparent persistence length increased with increasing fibrin concentration. It is likely that in this case the beads do not reflect single fiber relaxation, given the accumulation of a dense fibrin layer around the beads and the localization of beads at fiber junctions.

Surprisingly, the modulus reported by sticky beads in coarse fibrin networks reported similar values for $G'$ as reported by macrorheology, but overestimated $G''$ by about a factor 3 (see Fig. 5.11), though statistics for these measurements is low (total of 5 samples). Coarse fibrin networks have a pore size that is larger than the size of the beads (as shown in Fig. 5.10E). Thus, the situation is far from the elastic continuum assumption that we make to calculate $G'$ and $G''$ from the thermal position fluctuations of the beads. Rather, the beads likely sample the fluctuations of the fiber that they are adhered to. The apparent persistence length of the fibers according to the microrheology measurements is on the order of 40 µm, which is in line with previous optical tweezer microrheology results [66], but lower than video microrheology results (∼40 cm [533]). The reported persistence length for coarse clots is likely an underestimate, given that it corresponds to an apparent Young’s modulus of only 16 kPa assuming a cylindrical and uniform fiber, which is much lower than earlier reports for ligated fibrin fibers based on micromanipulation (∼MPa [281–283]). In future it will be interesting to use our microrheology setup to perform similar active micromanipulation of the fibers by using the AOD’s to move the trap. Alternatively, single fibrin fibers may be bent or stretched by AFM [281].

5.5 Conclusion

The local mechanical properties of the ECM were shown to be important parameters for cell behavior [310,501]. In this chapter, we aimed to measure the elastic and viscous micromechanical properties of fibrin networks over a wide range of frequencies, to obtain simultaneous information about stress relaxation at both the network and single fiber level.

For this purpose, we developed an optical tweezer based setup to perform
high bandwidth microrheology on fibrous protein networks. The setup was validated using viscous fluids of known viscosity and PAA gels. Next, we showed that we can measure the low and high frequency response of fibrin networks. For this we used two different bead types: One set of beads that stick to the network (plain polystyrene beads) and one set of passivated beads that are inert (polystyrene beads covalently coated with Pluronic). Both bead types report the macroscopic concentration dependence for $G'$, but the sticky beads overestimate $G'$, while the inert beads underestimate $G'$. We can explain this over- and under estimation in terms of a change in local fibrin concentration at the bead’s surface. At high frequencies for the inert beads, we observe clear evidence of entropic elasticity for $G''$, with a characteristic $\omega^{3/4}$ frequency dependence, which strongly supports the entropic network model presented in Chapter 2. We estimated the persistence length of protofibrils to be $203 \pm 75$ nm, which is in line with previous publications.

We can now apply optical tweezer microrheology to probe the influence of cells on their local tissue environment, for instance during cell-mediated stiffening [67,524,525,528]. The method could also be combined with intracellular microrheology to probe the response of the cell [539].

### 5.6 Acknowledgments

I would like to thank Dominique Thies-Weesie from the University of Utrecht with help for growing the 1.2 $\mu$m silica beads and size quantification; Bjorn Stuhrmann, Marko Kamp, Marco Seynen and Erik Claij for help with building and testing the optical tweezer setup, and the design and mechanical workshop at AMOLF for realizing the different mechanical components. I also like to thank Fred MacKintosh for helpful discussions and Andre Scholich for performing some of the experiments. This work is part of the research programme of the Foundation for Fundamental Research on Matter (FOM), which is financially supported by the Netherlands Organisation for Scientific Research (NWO). This work is further supported by NanoNextNL, a micro- and nanotechnology programme of the Dutch Government and 130 partners.
5.7 Supporting Information

Supplementary Figures and Tables

Figure 5.15: TEM image of the home-made silica microspheres. The bead diameter was 1.2 µm with 3.4% polydispersity. The imaging and analysis of the bead polydispersity is done by Dominique Thies-Weesie at the University Utrecht.

Figure 5.16: Optical tweezer microrheology in 2 mg/ml fibrin fibrin with coated polystyrene beads (n=15). (A) and (B) denote the elastic and viscous modulus $G'$ and $G''$ respectively of individual bead measurements in gray. The dashed black lines denote the average over these measurements, while the red line is the geometric average. The average is sensitive to outliers with high modulus. The dotted blue line denotes the median, which is often close to the geometric average.
Figure 5.17: The response function of a 50% glycerol mixture with water. (A) The real part of the apparent response function. (B) The imaginary part of the apparent response function. The trap stiffness was varied from $6.39 \cdot 10^{-6}$ (green), to $9.08 \cdot 10^{-5}$ (blue) and $1.33 \cdot 10^{-5}$ N/m (purple) by modifying the laser power.

<table>
<thead>
<tr>
<th>$z$ ($\mu$m)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
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</table>

Table 5.1: Statistics for the optical tweezer microrheology data with 1.2 $\mu$m silica beads in water, at varying sample depths.
Figure 5.18: The viscous modulus of water as measured by 1.5 µm PS beads covalently coated with Pluronic. (A) The viscous modulus of water can be reproduced when taking the individual trap stiffness for each bead. (B) The measured viscous modulus gives a large spread in $G''$ when assuming one trap stiffness for all beads. In total 28 beads were measured. Red dotted lines are the theoretical predictions for water. Gray lines are the measurements.

<table>
<thead>
<tr>
<th>$c_p$ (mg/ml)</th>
<th>f, coated</th>
<th>f, noncoated</th>
<th>c, noncoated</th>
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<tbody>
<tr>
<td>0.4</td>
<td>2</td>
<td>3</td>
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</tr>
<tr>
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<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0.6</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
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</tr>
<tr>
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<td>-</td>
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<tr>
<td>2</td>
<td>3</td>
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</tr>
</tbody>
</table>

Table 5.2: Statistics for the optical tweezer microrheology measurements in coarse (‘c’) and fine (‘f’) fibrin networks with 1.5 µm polystyrene beads, with a covalent Pluronic coating (‘coated’) or no coating (‘noncoated’). The typical sample size was more than 10 beads per sample, except for the 0.4 mg/ml for the coated bead case. In this case, the typical sample size was 5 beads per sample. In the case of 1 mg/ml coarse clots, the beads were 1 µm silica instead of 1.5 µm polystyrene.