2 Measurement techniques

This chapter describes the main techniques used for experiments throughout this thesis. The microstructure of biopolymer networks was characterized with the use of confocal fluorescence microscopy. Individual filaments were imaged by atomic force microscopy and electron microscopy. Turbidimetry measurements were performed to probe the diameter and mass/length ratio of filaments in their native, hydrated state. Finally, macrorheology and microrheology were used to probe the mechanical properties of the networks.
2.1 Structural Characterization

2.1.1 Network Characterization by Confocal Microscopy

![Confocal microscopy images](image)

**Figure 2.1.** Confocal microscopy images of fluorescently labeled networks of (A) fibrin fibers, (B) collagen fibers and (C) microtubules, each prepared at a protein concentration of 1 mg/ml. Images represent single plane. Scale bars, 10 µm.

The various biopolymer networks studied throughout this thesis were labeled with the fluorescent dye Alexa488 (fibrin), Oregon Green (collagen) and Rhodamine (microtubules). Networks were imaged with a spinning disc confocal microscope (Leica Microsystems, Rijswijk, Netherlands) using a 488 nm laser (Sapphire 488-30 CHRH, Coherent Inc., Utrecht, Netherlands) or a 561 nm laser (85-YCA-015, Melles Griot) for illumination and a back-illuminated cooled EM-CCD camera (C9100, Hamamatsu Photonics, Herrsching am Ammersee, Germany) for detection (Fig. 2.1). Three-dimensional (3D) image stacks were obtained by scanning through the z-direction in steps of 0.1 µm over a range of 10 µm with a piezo-driven 100x/1.3 NA oil immersion objective. Maximum intensity projections were made with ImageJ (http://rsbweb.nih.gov/ij/).

An estimate of the characteristic spacing between filaments (or pore size, ξ) was obtained by image analysis software in MatLab 7.1 (kindly provided by P.M. Bendix) [20]. Confocal images were band-pass filtered and maximum intensity projections of 101 planes separated by 0.1 µm steps were made with ImageJ. Binary images were obtained by thresholding maximum intensity projections with a threshold equal to the average pixel intensity plus one standard deviation [156]. Pixels with intensity above threshold were considered to be part of fibers. The distance between these pixels within each row and column was taken as ξ. The distribution between pixels in the X and Y directions was fitted well to an exponential, $P = P_0 e^{-\xi/\xi_c}$ with $P_0$ and the decay length $\xi_c$ as fitting parameters, indicating that the networks were isotropic.
2.1.2 Filament characterization by electron and atomic force microscopy

**Figure 2.2.** High-resolution images of various protein biopolymers. (A) AFM image of dried 0.2 mg/ml fibrin fibers. Area, 5x5 µm. (B) AFM image of dried 2 mg/ml collagen fibrils. Area, 3x3 µm. (B) EM image of 0.14 mg/ml vimentin filaments. Scale bar, 0.2 µm.

Atomic Force Microscopy (AFM) images of filaments in a dried state (Fig. 2.2 A, B) were obtained in tapping mode with a Dimension V Scanning Probe Microscope (Veeco, Plainview, NY) using a silicon triangle cantilever with 15-30 kHz resonance frequency and 0.01-0.50 N/m nominal spring constant (Veeco). Fibrin and collagen gels were polymerized on mica surfaces in a moist 37°C atmosphere. Since the resulting gels were too dense to allow imaging by AFM, we removed excess material just before imaging by peeling the top of the gel off with filter paper. In this way, only filaments stuck to the mica were exposed and the filament density was sufficiently low to distinguish individual filaments by AFM. The samples were finally dried with nitrogen before imaging. The fibril diameter was analyzed in Nanoscope 6.14r1 software (Veeco) from the height, averaging over 20-30 fibrils per experimental condition. We compared the height with the width (determined from Gaussian fits to line profiles across each fibril), and found that the height was typically 3-5 times larger than the width for both fibrin and collagen filaments. This discrepancy is probably caused in part by a profile broadening effect due to tip-sample convolution. In addition, drying of the fibrils, that typically contain at least 50% water, may cause collapse and a reduction in height.

In order to visualize filaments in their hydrated state, an AFM fluid cell (Veeco) equipped with a tip with 2 nm radius and 0.12 N/m nominal spring constant was used. Samples were polymerized on parafilm in a moist 37°C atmosphere and next deposited on a glass slide coated with Formvar layer by peeling the top of the gel off. Next, the filaments were fixed with 1% glutaraldehyde solution (GA) and placed under the scanning probe of the microscope. 1-fold polymerization buffer was pipetted on top of the sample to keep filaments in a hydrated state. Images were taken in contact mode.

Vimentin filaments (Fig. 2.2 C) were imaged by electron microscopy (EM, Carl Zeiss, Oberkochen, Germany). Samples were polymerized at 22°C followed
by fixation with 1% glutaraldehyde on a glow-discharged, carbon-coated copper electron microscopy grid (Cu Copper grids, 300 mesh, SPI, Germany). The filaments were allowed to bind to the support surface for 15 seconds. The sample was then washed with distilled water and stained for 15 seconds with 2% uranyl acetate dissolved in water. The staining solution was removed by washing the sample with distilled water and the grid was dried in air.

### 2.1.3 Filament characterization by turbidimetry

![Figure 2.3](image)

Figure 2.3. Wavelength dependent turbidity of 2 mg/ml collagen I networks (circles), mixed 80/20 collagen I/V networks (triangles), fibrin coarse clots (squares) and fine clots (diamonds). The data are scaled according to the theoretical prediction in Eq. (2.3) for long (>800 nm) rod-like polymers with a diameter below 200 nm. Pure networks of collagen V (stars, inset figure) do not follow the predicted relation, presumably because the filaments have a typical length of only $\sim 1 \, \mu m$.

The diameter of filaments in their native, hydrated state was determined using turbidimetry. Data were analyzed according to a theoretical model of Yeromonahos et al. [327], which is a corrected version of an earlier model of Carr and Hermans [52] for gels composed of rigid rod-like polymers. Gels were polymerized in 1 cm-path length cuvettes (UV-Cuvette micro, Plastibrand, Germany) and fibril diameters, $d$, and mass-length ratios, $\mu$, were obtained by measuring the light transmission through the samples over a range of incident wavelengths, $\lambda$, between 350-650 nm with a UV1 Spectrophotometer (Thermo Optek). The transmitted light intensity, $I_t$, is lower than the incident intensity, $I_0$, due to light scattering by the turbid gels. The optical density, $D$, of the solution is defined as [157]:

$$\frac{I_t}{I_0} = 10^{-D.L}$$

(2.1)

and the turbidity, $\tau$, is:
\[ \tau = D \ln 10, \]  

(2.2)

where \( L \) is the path length in \( cm \) and \( D \) is in units of \( cm^{-1} \). For a solution of randomly oriented fibers that are long (>800 nm) and thin compared to \( \lambda \) (<200 nm diameter), the turbidity is [327]:

\[ \tau \lambda^5 = A \mu (\lambda^2 - Ba^2), \]  

(2.3)

where \( A = (88/15) c \pi^3 n_s (dn/dc)^2 (1/N_A) \) and \( B = (368/462) \pi^2 n_s^2 \). In these equations, \( N_A \) is Avogadro’s number, \( n_s \) is the refractive index of the solvent \( (n=1.33 \) for water), \( dn/dc \) is the specific refractive index increment \( (dn/dc=0.186 \ cm^3 g^{-1} \) for collagen [42] and 0.17594 \ cm^3 g^{-1} \) for fibrin [327]), and \( c \) is the protein concentration expressed in \( g/ml \) [327]. Plots of \( \tau \lambda^5 \) versus \( \lambda^2 \) should give straight lines whose slope \( (=A\mu) \) yields the mass/length ratio and whose intercept with the \( y \)-axis \( (-A\mu Ba^2) \) gives the square of the average radius, \( a \) (Fig. 2.3). Data for fibrin networks and collagen networks of varying concentration could all be fit well by straight lines. Only pure networks of collagen V did not follow the expected scaling. This was probably due to the shorter length of collagen V fibers (on average 1 \( \mu m \)) compared to collagen I and fibrin fibers, which places these filaments right on the edge of the validity of the light scattering model.

### 2.2 Mechanical Measurements

#### 2.2.1 Macroscopic Rheometry

[Figure 2.4. Schematic representation of linear and nonlinear rheology of a strain-stiffening network probed by LAOS and prestress measurements.]

Rheology was performed with a stress-controlled rheometer (Physica MCR 501, Anton Paar, Graz, Austria) equipped with a steel cone top plate
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(dimensions are specified in each chapter) and flat bottom plate (50 mm diameter). The bottom plate temperature was controlled using a Peltier element. Samples were polymerized between the rheometer plates, in combination with wet tissues or mineral oil applied to the sample edge, and a hood surrounding the geometry was used to maintain a moist atmosphere. Network polymerization was monitored by measuring the increase in the shear moduli with an oscillating shear at a frequency, $\omega$, of 3 rad/s and small strain amplitude, $\gamma$, of 0.5%. We verified that this strain amplitude was small enough not to perturb network polymerization. The ratio of the stress response, $\sigma(\omega)$, to the applied strain equals the complex shear modulus, $G^*(\omega) = G'(\omega) + iG''(\omega)$. The elastic modulus, $G'(\omega)$, represents the in-phase (storage) response, while the viscous modulus, $G''(\omega)$, represents the out-of-phase (loss) response. When the elastic modulus reached a constant value, the linear viscoelastic moduli were measured as a function of frequency between 30-0.03 rad/s at a small strain amplitude of 0.5%. The non-linear moduli were measured by applying a large amplitude oscillatory shear (LAOS) with $\omega = 3$ rad/s and $\sigma$ (stress-control mode) or $\gamma$ (strain-control mode) increasing logarithmically until sample yielding. To characterize the onset of nonlinearity, the critical strain, $\gamma_{\text{crit}}$, or critical stress, $\sigma_{\text{crit}}$ (for strain-stiffening samples) and yield strain, $\gamma_{\text{yield}}$, or yield stress, $\sigma_{\text{yield}}$ (for strain-weakening samples) were defined as the value where $\sigma$ differs from the product $G_0\gamma$ by more than 10%, where $G_0$ is the linear elastic modulus [294].

LAOS stress-strain data collected by the rheometer software were analyzed in the form of Lissajous plots and Fourier Transform spectra (see Chapter 5).

For fibrin gels, LAOS measurements were complemented with prestress measurements. The differential moduli, $K'$ and $K''$, were measured as a function of applied prestress [94, 39, 265]. Samples were pre-stressed with a steady stress $\sigma_0$, and the differential response was measured by superposing a small oscillatory stress, $|\delta\sigma_0| < 0.1 \sigma_0$. This ensures that the measured oscillatory strain response is sinusoidal (Fig. 2.4). The stress-dependent tangent modulus follows from the oscillatory strain response, $K^* = \delta\sigma_0/\delta\gamma_0$. $K'$ was independent of frequency and of the waiting time in the pre-stressed state, demonstrating that the prestress did not cause viscous flow.

2.2.2 Optical Tweezer microrheology

To investigate thermal bending fluctuations of fibrin filaments and their rheological consequences in situ, optical microrheology was used (Fig. 2.5). Fibrin networks were polymerized in the presence of strongly adherent polystyrene particles with a diameter of 1 $\mu$m (Polybead® Polystyrene Microspheres, Polyscience, Inc.). Particles in the gel interior were weakly trapped using a laser with wavelength of 1064 nm [160]. Their thermal position fluctuations were detected by a quadrant photodiode at a sampling rate of 195 kHz.

There are two interpretations of the particle fluctuations inside the network interior. The first interpretation assumes that the bead fluctuations measure the local network rheology. In this case the shear modulus of the gel can be inferred from the particle fluctuations by linear response theory [98]. At high frequencies the shear modulus is controlled by the relaxation of individual polymer chains...
2.2 Measurement techniques

Figure 2.5. Schematic representation of beads embedded inside a polymer network. One bead is trapped in a focused laser beam.

[96, 207]. For a semiflexible polymer with a persistence length, $l_p$, similar to or greater than the contour length, the modulus is given by:

$$G^*(\omega) = \frac{1}{15} \rho \kappa l_p (-2i\zeta/\kappa)^{3/4} \omega^{3/4} - i\omega\eta,$$  \hspace{1cm} (2.4)

where $\rho$ is the filament length per volume, $\kappa = l_p k_BT$ is the bending stiffness, $\zeta = 4\pi\eta/\ln(0.6\lambda/d)$ is the lateral drag coefficient and $\eta$ is the solvent viscosity at $37^\circ C$. Using for $\lambda$ the characteristic mesh size, one can measure the polymer persistence length, $l_p$.

The second interpretation of the particle fluctuations is that the beads directly report on transverse fluctuations of the fibers to which they adhere. In this case we expect the same qualitative dynamics, with the same $3/4$ exponent in either time or frequency dependence [83, 104]:

$$\langle (\Delta h(t))^2 \rangle \simeq 0.082 \left\{ \ln \left[ \frac{\kappa \ln(L/a)}{4\pi\eta a^3} \right] \left( \frac{k_BT}{\kappa} \right)^{1/3} \frac{k_BT}{\eta} t \right\}^{4/3}$$  \hspace{1cm} (2.5)

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