Towards *in situ* visualization of biopolymer networks under shear

This chapter describes the design and first testing of a home-made shear cell device. This setup combined with a confocal microscope creates a tool to visualize and quantify deformations of biopolymer networks subjected to a shear deformation on the micron scale. We illustrate the performance of the shear cell by confocal observations of fluorescently labeled fibrin coarse clots under shear. The possibility to identify network deformation mechanisms under shear will help to understand the origin of the remarkable mechanical properties of biopolymer networks, which include strain-stiffening and high extensibility. Furthermore, the shear will enable a quantitative analysis of the degree of non-affinity of the strain field in sheared gels by tracking displacements of fluorescently labeled beads embedded in the network. Finally, for sufficiently thick and rigid filaments such as fibrin or collagen fibers, it should be possible to directly observe fiber bending or stretching, and the mechanism of network rupture at large shear strain.
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8.1 Introduction

Cell and tissue deformability depend on networks of protein biopolymers. These biopolymers are many orders of magnitude stiffer than typical synthetic polymers because they are built from much larger, macromolecular protein building blocks. Synthetic polymers tend to be highly flexible and act as linear springs. Their rheology is often well described by the classical rubber elastic theory, which assumes that the deformation is uniform, or affine [167, 74]. This is a good assumption for rubber-like materials, since they have a very small mesh size [257]. In case of an affine deformation, the polymers are predominantly stretched, and the network elasticity can be calculated by orientationally averaging over the entropic stretch response of the individual polymer chains. Due to the small persistence length of the chains, the force-extension behavior is linear over a wide range of applied strain, up to hundreds of percents [16]. In contrast, the mechanical response of networks of stiff biopolymers tends to be highly nonlinear, with strain-stiffening setting in already at strains of a few percent [94, 285, 195]. Moreover, simulations of stiff fiber networks in two and three dimensions show that the strain field can be highly nonaffine, and that this nonaffinity may actually cause nonlinear elasticity [118, 311, 67, 144, 311, 40]. The degree of nonaffinity depends on crosslink density and filament bending stiffness. At high crosslink density, the strain field is affine (A) and the polymers are predominantly stretched. However, decreasing either the crosslink density or the bending stiffness of the fibers leads to a shift to nonaffine (NA) deformations, involving fiber bending or reorientation. Recent lattice simulations demonstrated an additional nonaffine regime where filament bending and stretching are coupled [40]. Other recent simulations showed that the crosslink density where fiber networks transition between NA and A behavior sets in is also sensitive to length polydispersity: even a small fraction of longer filaments in a network of shorter ones can shift the NA/A crossover point to lower crosslink densities [11].

While there has been much computational effort on nonaffine phenomena and shear-dependent microstructure in biopolymer networks, there has been little experimental work on real systems [190, 307, 16]. Rheology can give indirect information about the occurrence of nonaffine deformations. For instance, when the elastic modulus of a network is lower than expected on theoretical grounds in the affine limit, it is likely that nonaffinity is present. Such a discrepancy was observed in Chapter 6 for networks of collagen fibrils. However, it is not straightforward to make a quantitative comparison of experiments with theory, since this approach requires that the bending stiffness of the fibers and the network connectivity is well-known. Another indirect indication of nonaffinity is a system-size dependence of the rheology, as reported for collagen networks [8], though it is difficult to distinguish nonaffine effects from other effects such as slippage at the plates or network confinement.

A more direct experimental approach to probe nonaffinity is to map the 3D-strain field inside sheared gels by optical microscopy. The strain field can be obtained by analysis of bright field or fluorescence images of the network itself, either by extracting filament contours [282] or by cross-correlating images [295].
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Figure 8.1. Schematic representation of biopolymer network subjected to an oscillatory shear in a parallel plate shear cell. The 3D strain field can be mapped by measuring displacements of embedded fluorescent tracer beads. Deviations, of the positions from an affine response (displacement in the direction of shear (x-axis), grey to green) are a measure of nonaffinity in the displacement field. $\gamma$ denotes strain. Picture adapted from [16].

Alternatively, fluorescent tracer beads can be embedded in the network and tracked with sub-pixel accuracy [190, 307]. The degree of nonaffinity can be deduced from the deviation of bead displacements from the imposed macroscopic shear, and from the angle and relative position of particle pairs before and after shear (Fig. 8.1). Such a bead-tracking approach was used to show that F-actin networks crosslinked by small rigid crosslink proteins deform increasingly non-affinely when the crosslink density or the length of the filaments is decreased, consistent with numerical predictions [190]. Actin networks crosslinked by the flexible crosslinking protein filamin were shown to display filament alignment under shear [189] while fibrin networks become less nonaffine with increasing applied strain amplitude, reflecting entropic origin of the nonlinear elasticity [307]. In contrast, the degree of nonaffinity in gels of flexible polyacrylamide chains was shown to be independent of the density of polymer chains or crosslinks [16]. The nonaffinity in this system was ascribed to structural inhomogeneities introduced during gel preparation.

Strain field mapping of sheared networks by optical microscopy requires shear cells with at least one glass plate that is transparent for visible light. Two different categories of shear cell geometries have been developed. The first category is that of parallel plate shear cells, where the sample is sheared by translating two parallel plates that are larger than their separation relative to each other. This geometry was used for quantifying nonaffinity in actin networks [190] and for imaging of colloidal suspensions under flow [1, 2, 63, 319, 117, 116, 62, 276, 23, 24]. Planar shear is convenient for oscillatory measurements but is unsuitable for flow (continuous shear) experiments since only a finite strain can
be achieved. Rotational geometries, which are used in traditional rheometers, are more flexible, allowing both oscillatory and continuous shear measurements. Such a system equipped with a counter-rotating cone and plate [70, 319, 318, 34, 217] or plate-plate cell [109, 25, 248] was used for colloidal suspensions. The advantage of counter-rotating plates, which move in opposite directions, is that a zero velocity plane is situated at a gap height that depends on the relative velocities of the two plates. Sheared objects can be conveniently imaged in this stationary plane without moving out of the field of view. Most shear cells to date were custom-built, but recently several groups placed rotational rheometers on top of a microscope, so that simultaneous measurements of the shear stress can be made [263, 307, 23, 16, 24, 31].

We decided to build a parallel plate shear cell with one stationary and one translating plate. The parallel plate design is convenient for crosslinked biopolymer networks, for which we use almost exclusively oscillatory tests. The cell was designed for small sample volumes, which is important since we work with purified proteins that are generally obtained in small yields. Moreover, we implemented temperature and humidity control, in order to maintain physiological \(37^\circ\text{C}\) conditions. The cell was designed to access a large frequency range (<0.1 to 100 Hz), similar to that accessible by standard bulk rheology. Furthermore, the cell can reach, depending on gap size, large strain amplitudes of up to 2000%. This covers the high strain regimes at which stretching of extensible fibers such as fibrin and intermediate filaments can be observed, as well as eventual network rupture.

This chapter describes the design of this new shear cell device, which can be placed on a confocal microscope to image changes in the microstructure of biopolymer networks in response to an applied shear over a large range of frequencies and strain amplitudes. We demonstrate the performance of this setup by test experiments with fluorescently labeled fibrin networks. Combined with rheometry, which provides the macroscopic network stiffness, this new device can provide new insights into the mechanisms that govern the highly nonlinear response of the biopolymer networks that structure living cells and tissues.

### 8.2 Parallel Plate Shear Cell

To visualize changes in the structure of biopolymer networks in response to a macroscopic shear deformation on microscopic length scales, we designed a shear cell (SC) with two glass plates. The cell can be mounted directly on top of a Leica (Leica Microsystems, Rijswijk, Netherlands) inverted spinning (Nipkov) disc confocal microscope (Fig. 8.2 A). The spinning disc permits fast confocal scanning at frame rates of hundreds frames per second [316]. The sample is confined between a moveable top plate, and a stationary bottom plate, through which it can be imaged using a high numerical aperture, 100x oil immersion objective. The two main components of the shear cell device are: (1) a cylindrical fixture with movable shaft that provides the shearing motion and (2) a position sensor (optoNCDT, Micro-Epsilon Messtechnik, Ortenburg, Germany)
that records the displacement of the top plate (Fig. 8.2 B).

The movable shaft consists of a titanium (grade 5) hinge spring system, placed on a core tube and protected by a metal casing (Fig. 8.2 B). The hinge spring system is connected at the bottom with a plastic holder for the top microscope coverslip (Fig. 8.3 C). The movement of the SC is based on the Lorentz force principle. An applied electric current induces a magnetic field between two magnets (Fig. 8.3 A). The home-made voice coil (84 coils separated with 0.4 mm distance) that is wound on one side of the core tube is then pushed back and forth, in and out of the magnetic field produced by the Neodymium magnets (grade N52). Since the hinge spring system is mounted directly on the core tube and is connected with the top coverslip holder, it will move the top plate and produce a shear in the direction parallel to the bottom glass slide.

Both glass plates are microscope coverslips of 24 x 32 mm$^2$ with a thickness of 0.15 mm (♯1.5 coverslips, Marienfeld Laboratory Glassware, Germany). They are held in place by metal bars with screws on the two shorter sides and are easily exchangeable. A micrometer screw mounted on the bottom metal glass holder (Fig. 8.3 D) can be used to adjust the gap distance between the plates over a range of 0 to 800 $\mu$m. Alignment of the slides parallel with each other is achieved by adjusting three screw legs mounted on the bottom part of the hinge spring system casing (Fig. 8.3 C). The distance between the plates at different xy-locations within the focal planes was measured with the z-focus of the microscope objective. Fluorescently labeled particles with a diameter of 1 $\mu$m stuck to the bottom and top coverslip were imaged by confocal microscopy and used as a reference for plate alignment. The shear cell legs were adjusted until the height variation between the two plates over the whole coverslip area was less than 2 $\mu$m.

To enable experiments at physiological temperatures of 37°C, the bottom glass slide holder was equipped with several surface-mounted power resistors
Figure 8.3. Shear cell (SC) design. (A) Schematic representation of the individual SC components: (1) SC bottom plate, (2) micrometer screw for gap adjustment, (3) metal bar with screws to hold the bottom microscope coverslip, (4) hinge spring system, (5) bottom glass coverslip, (6) temperature control sensors, (7) solvent trap filled with water, (8) movable holder with the top microscope glass slide, (9) core tube with voice coil, (10) two magnets, (11) screw leg(s) for parallel alignment of the two glass coverslips. (B) Photograph of the metal core with the hinge spring system surrounded by metal casing. (C) Bottom view of the hinge-spring system, showing the movable top glass holder with a glass slide and three screw legs used to align the plates. (D) Photograph of the shear cell bottom plate with position sensor, micrometer screw and bottom glass slide holder. (E) Photograph of the fully assembled shear cell.

(SMD) and 3 temperature sensors (TSIC506FTB, Zentrum Mikroelektronik Dresden, Germany). Due to thermal conduction along the metal holder, an equal temperature is maintained all around the sample. To minimize evaporation, rectangular wells were cut in the bottom glass holder which could be filled with water. This water reservoir maintains moist conditions and also helps to improve the temperature gradient all around the sample. The fully assembled shear cell is shown in Fig. 8.3 E.
8.3 Electronics and software interface to control shear deformation

Shear cell movement is controlled by a programmable sine-wave generator and a closed loop feedback system, both implemented in digital hardware (National Instruments Reconfigurable I/O, Austin, TX). With this generator we can apply an oscillatory shear, a constant strain, or a superposition of the two, either in a continuous or a step-wise manner. The feedback system is a proportional-integral-derivative regulator (PID) that compares the current position, which is measured with a laser position sensor (optoNCDT, Micro-Epsilon Messtechnik, Ortenburg, Germany), with the desired position from the sine-generator and outputs the required current to move the shaft to the desired position. The current is by definition equivalent in this case to the force based on the Lorentz force equation:

\[
\vec{F} = q \vec{V} \times \vec{B} = q \frac{d\vec{l}}{dt} \times \vec{B} = \vec{T} dl \times \vec{B},
\]

where \( F \) is a sum of the forces necessary to overcome the inertia of the motor and plate, the force to shear the sample, and the force to move the hinge spring system. Further, \( q \) is electrical charge, \( V \) is voltage, \( B \) is magnetic induction and \( I \) is applied current, which is in the presence of spring system linear with \( F \) and with displacement. This is in contrast to a normal voice-coil system, where \( I \) is linear with \( F \) and with acceleration. The force required to move the shaft is much larger (around 9 N) than the force generated by the sample itself, which is expected to be in the pN range assuming that \( F = \sigma A \), where \( \sigma \) denotes stress in units of Pa and \( A \) is the microscopic filament area that is approximately equal to squared network mesh size, \( \xi^2 (\mu m)^2 \). Therefore, we did not attempt to extract shear stresses from the force needed to drive the plate.

The two parameters that can be tuned during an experiment are the frequency and the amplitude of the shear. The maximum relative plate translation along the \( x \) axis is 2 mm, for frequencies below 20 Hz. With gap sizes of 400 \( \mu m \), this means that we can apply strains up to 500\%, which is well within the range that is relevant for biopolymer networks, even including extremely resilient materials such as fibrin and intermediate filaments, which have breakage strains of 200-300\%. The maximum frequency that can be applied is 100 Hz, with a displacement of at most 100 \( \mu m \). The system has a mechanical resonance frequency of 37 Hz.

To control the shear cell and visualize the data we developed software that can control the temperature, PID parameters, program the desired movement, and record the shaft motion over time. The actual shaft position, desired position, and driver current can be measured every 50 \( \mu s \). The program can visualize the data as time-series graphs, power spectra, and Lissajous curves. Higher harmonic terms in the power spectrum and non-ellipsoidal shapes of the Lissajous curves can be indicative of rheological nonlinearities (see Chapter 5). However, the driver output does not correspond directly to the force generated by the sample during shearing, so the nonlinearities cannot be quantified.
The software can also automatically record images of the sheared sample in one focal plane at a time, by interfacing with a black-illuminated cooled EM-CCD camera (C9100, Hamamatsu Photonics, Herrsching an Ammersee, Germany) on the microscope. In future, we plan to extend the software for automated collection of z-stacks over time. This will be necessary to determine the 3D network structure and to measure the strain field with the use of beads embedded inside the network.

8.4 **Fibrin network under shear**

To demonstrate the performance of the shear cell, we polymerized a coarse fibrin network with a protein concentration of 0.5 mg/ml \( (G' = 4.7 \text{ Pa}, \ G'' = 0.58 \text{ Pa}, \ \xi \sim 4 \mu\text{m}) \) in the shear cell, using a gap distance of 70 \( \mu\text{m} \) between the plates. A drop of fibrinogen solution of which 10% was labeled with Alexa488 was confined between the parallel glass plates and polymerized for 2 hours on the microscope.

![Figure 8.4. Polymerized 0.5 mg/ml fibrin network before shear (A) and during oscillatory shear at 0.5 Hz and increasing strain amplitude: 160% (B) and 280% (C). Images represent single planes. Inset in (A) shows maximum intensity projection of a z-stack of 101 planes over a depth of 10 \( \mu\text{m} \). Scale bars, 10 \( \mu\text{m} \).](image)

Directly after polymerization (before shear), the network consisted of long, thermally undulated filaments (Fig. 8.4 A). The network appears to be slightly inhomogeneous when looking at a single plane. This is typical at the rather low protein concentration in this experiment. Three-dimensional image stacks obtained by scanning through the z-direction show that the fibers in fact do form a fully connected network (inset of Fig. 8.4 A). When an oscillatory shear of 160% strain and 0.05 Hz is applied, the fibrin network is visibly stretched (Fig. 8.4 B). Individual filaments are aligned and elongated along the shear direction. Depending on the filament orientation, filaments are compressed (black arrow) or extended (white arrow). Individual fibers oriented out of plane continuously appear and disappear from the imaged focal plane during oscillations, depending on their orientation with respect to the shear direction. At strains...
of 280%, highly stretched filaments were observed (Fig. 8.4 C). At even higher strain amplitudes, filaments detached from the glass surfaces before we were able to observe any fiber rupture. Therefore, in future it is recommended to coat the glass surface in order to prevent network slip at high shear.

8.5 Conclusions and Outlook

In this chapter we presented a new custom-built shear cell device which can be used to visualize changes in the microstructure of biopolymer networks under shear. This tool complements bulk rheological studies and can be used to quantify the degree of nonaffinity in the mechanical response. Further modifications of the system are however needed to optimize its performance. This includes improvement of the setup itself as well as in the software interface.

Regarding improvements to the hardware, there are several issues. Manual alignment of the glass plates with the use of screw legs using fluorescence beads as a reference for the top and bottom plates, which is done in the current design, is not very accurate and rather laborious. In future it would be better to implement automated adjustment of the gap to the desired size and tilt goniometers or pivot system to ensure parallelism [319, 276]. Furthermore, with the current design it is difficult to maintain a constant and homogeneous distance between the glass plates during experiments, due to forces exerted on the plates by the sample during polymerization. These forces pull the thin coverslips towards one another, decreasing the gap and potentially bending the plates. Possible solution here is to exchange the top coverslip with a thicker glass slide, or to use spacers that guarantee a constant distance between the coverslips over the entire sample area. However, spacers may generate additional friction during sample shearing. We observed that the stability of the gap depends on the amount of sample between the plates. A small sample volume compared to the total shear cell capacity may create a liquid meniscus, introducing capillary forces that pull the plates together. Therefore, it is important to fill the entire cell with sample. Another problem with the current design is that is difficult to maintain moist conditions and prevent solvent evaporation for 37°C experiments that take longer than 1-2 hours. Once the sample is loaded and the setup is closed, there is no easy way to refill the water in the solvent trap. This is problematic for assembly of some proteins such as fibrin and collagen, for which polymerization takes longer than 2 hours. Therefore, additional elements to ensure a saturated water atmosphere during measurements are required. Finally, the current device can apply a deformation but not measure the shear stress. In future, we plan to add a force sensor to the bottom plate of the shear cell to measure the shear stress directly. Knowledge of the shear stress is crucial to quantify the nonlinear viscoelastic response of the sample that accompanies the structural changes seen by confocal microscopy.

Regarding the software, it will be useful to synchronize shearing with acquisition of time- and/or z-stacks. This would enable 3D reconstruction of deformed networks under shear, in order to visualize deformations of filaments or quantify the 3D strain field. Strain-field mapping and network visualization are
needed to obtain a full understanding of the origin of the nonlinear mechanics of biopolymer networks.

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