A narrow-gap rotational rheometer for high shear rates and biorheological studies

Ein Dünnspaltrotationsrheometer für hohe Scherraten und biorheologische Untersuchungen

Der Technischen Fakultät
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vorgelegt von
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Abstract

In this thesis, a commercial rotational rheometer has been modified to facilitate rheological measurements at a gap width of a few micrometers. Working at narrow gaps presents many advantages: It enables to extend the range of applicability of the rheometer to measure, for instance, low viscosities, high shear rates and normal stresses at the smallest amount of samples and to carry out experiments directly at biological cells. Although the parallel-disk geometry allows for easy adjustment of the gap width, in commercial rheometers it suffers from errors that restrict the measurement gap width such as squeeze flow of air during zero-gap error setting due to misalignment of the parallel-disks and insufficient flatness of the plates while zeroing the device plates. To minimize the zero-gap error, a procedure has been developed to align the parallel-disks to each other within an uncertainty of ±0.7 µm gap width, i.e. the zero-gap precision is improved by a factor of 30 and more. In oscillation, the precision can be enhanced to about ±0.2 µm. Compared to other narrow-gap devices, our setup offer the advantage to not only allow for oscillatory studies but also to enable unidirectional studies at a narrow-gap width, at the same time, taking full advantages of the versatility of commercial rheometers.

In the first part of this work, a commercial rotational rheometer in the parallel-disk configuration is modified so that the disks are aligned perpendicular to the axis of rotation with a precision in parallelism of about 1 µm independent of the rheometer reading. It enables samples to be studied at gap widths well below the absolute error of commercial rheometers, which is typically in the range between 25 µm to 75 µm. At gap widths of 20 µm, this modification allows the measurement range for shear rate to be extended from typically $10^3$ s$^{-1}$ up to $10^5$ s$^{-1}$. It also enables measurement of low viscosities such as that of solvents or water.

As an application example, the viscosity functions of the polymer solutions are studied at high shear rates up to $10^5$ s$^{-1}$ with the modified parallel-disk geometry in the second part of this work. Shear-rate range enables the study of the second Newtonian branch. Here we found that there is a crossover from disentangled to entangled solutions. It happens, however, at much higher concentrations than for the zero-viscosity. Furthermore, it is possible to measure the normal-stress differences and carrying out birefringence measurements to investigate changes in polymer orientation at these high shear rates.
Finally, within a few micrometers gap width, we determined the average rheological quantities of a biological cell. While cell-to-cell variation is typically very large, the average cell properties within the monolayer can be determined with much higher precision. This method enables quantification of the impact of biochemical treatment on the rheological properties of the cells and may be used as a diagnostic tool to identify variations in the rheological cell behaviour due to diseases. Depending on the chosen gap width, the cells may be compressed or elongated. Finally, the impact of pre-stress on the shear properties of the cells can be studied. While the dynamic moduli strongly increase with compression, the power-law exponent that describes the frequency-dependence of the moduli increases with the gap width.
Zusammenfassung


Im ersten Teil der Arbeit wird die Modifikation der Platte-Platte-Konfiguration kommerzieller Rotationsrheometer vorgestellt. Es wird gezeigt, wie die Platten sukzessive senkrecht zur Drehachse ausgerichtet werden, um einen Genauigkeit in der Spaltweite von etwa 1 µm zu erreichen. Dabei wird die Spaltweite unabhängig vom der Bestimmung mit dem Rheometer bestimmt. Dadurch werden Messungen bei Spaltweiten deutlich unterhalb des Absolutfehlers kommerzieller Rotationsrheometer ermöglicht. Letzterer liegt typischerweise im Bereich zwischen 25 µm und 75 µm. So kann z.B. bei Spaltweiten von 20 µm der Messbereich für Scherraten von typischerweise $10^3$ s⁻¹ auf $10^5$ s⁻¹ erweitert werden. Es ermöglicht darüber hinaus die Messung niedriger Viskositäten wie die von Wasser oder von Lösungsmitteln.

hinaus ist es mit der Modifikation möglich, auch Normalspannungsdifferenzen und optische Eigenschaften bei den hohen Scherraten zu untersuchen.

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# Nomenclature

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<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A</td>
<td>surface area</td>
<td>mm²</td>
</tr>
<tr>
<td>h</td>
<td>distance between cone and lower plate</td>
<td>mm</td>
</tr>
<tr>
<td>c</td>
<td>measured mean cell concentrations</td>
<td>%</td>
</tr>
<tr>
<td>F_N</td>
<td>normal force without the contribution due to centrifugal forces</td>
<td>N</td>
</tr>
<tr>
<td>G</td>
<td>shear modulus</td>
<td>Pa</td>
</tr>
<tr>
<td>G'</td>
<td>storage modulus</td>
<td>Pa</td>
</tr>
<tr>
<td>$\langle G'_\text{cell} \rangle$</td>
<td>average storage modulus of cells</td>
<td>Pa</td>
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<tr>
<td>G''</td>
<td>loss modulus</td>
<td>Pa</td>
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<tr>
<td>$\langle G''_\text{cell} \rangle$</td>
<td>average loss modulus of cells</td>
<td>Pa</td>
</tr>
<tr>
<td>h</td>
<td>gap thickness</td>
<td>µm</td>
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<tr>
<td>$h_{\text{adhered film}}$</td>
<td>adhered film thickness</td>
<td>µm</td>
</tr>
<tr>
<td>$h_R$</td>
<td>real mean gap thickness</td>
<td>µm</td>
</tr>
<tr>
<td>I</td>
<td>light intensity</td>
<td>a·u.</td>
</tr>
<tr>
<td>$I_o$</td>
<td>maximum light intensity</td>
<td>a·u.</td>
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<tr>
<td>k</td>
<td>thermal conductivity</td>
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<tr>
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<tr>
<td>Re</td>
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<tr>
<td>T</td>
<td>temperature</td>
<td>K</td>
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<tr>
<td>u</td>
<td>circumferential velocity</td>
<td>m s(^{-1})</td>
</tr>
<tr>
<td>(V_{\text{Slip}})</td>
<td>slip volume</td>
<td>(\mu)m(^3)</td>
</tr>
<tr>
<td>x</td>
<td>axis normal to y and z</td>
<td>mm</td>
</tr>
<tr>
<td>y</td>
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<td>z</td>
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**Greek symbols**

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<tr>
<td>(\alpha)</td>
<td>cone angle</td>
<td>degree</td>
</tr>
<tr>
<td>(\Delta)</td>
<td>difference</td>
<td>-</td>
</tr>
<tr>
<td>(\Delta H)</td>
<td>zero-gap error thickness</td>
<td>(\mu)m</td>
</tr>
<tr>
<td>(\Delta n)</td>
<td>refractive index</td>
<td>-</td>
</tr>
<tr>
<td>(\Delta \phi)</td>
<td>phase shift</td>
<td>-</td>
</tr>
<tr>
<td>(\delta)</td>
<td>phase angle</td>
<td>degree</td>
</tr>
<tr>
<td>(\Omega)</td>
<td>angular velocity</td>
<td>rad/s</td>
</tr>
<tr>
<td>(\mu)</td>
<td>dynamic viscosity</td>
<td>mPa\cdot s</td>
</tr>
<tr>
<td>(\mu_0)</td>
<td>zero-shear rate viscosity</td>
<td>mPa\cdot s</td>
</tr>
<tr>
<td>(\mu_\infty)</td>
<td>infinity-shear rate viscosity</td>
<td>mPa\cdot s</td>
</tr>
<tr>
<td>(\tau)</td>
<td>shear stress</td>
<td>Pa</td>
</tr>
<tr>
<td>(\tau_0)</td>
<td>shear stress at no wall slip</td>
<td>Pa</td>
</tr>
<tr>
<td>(\tau_R)</td>
<td>shear stress at maximum radius</td>
<td>Pa</td>
</tr>
<tr>
<td>(\dot{\gamma})</td>
<td>shear rate</td>
<td>s(^{-1})</td>
</tr>
<tr>
<td>(\dot{\gamma}_c)</td>
<td>critical shear rate</td>
<td>s(^{-1})</td>
</tr>
<tr>
<td>(\dot{\gamma}_m)</td>
<td>measured shear rate</td>
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<td>s(^{-1})</td>
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<td>$\nu$</td>
<td>kinematic viscosity</td>
<td>m$^{2}\cdot$s$^{-1}$</td>
</tr>
<tr>
<td>$\rho$</td>
<td>density</td>
<td>kg·m$^{-3}$</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>surface tension</td>
<td>N·m$^{-1}$</td>
</tr>
<tr>
<td>$\langle \sigma_{\text{cell}} \rangle$</td>
<td>normal force per cells</td>
<td>N</td>
</tr>
<tr>
<td>$\pi$</td>
<td>$\Pi = 3.1416$</td>
<td>-</td>
</tr>
<tr>
<td>$\omega$</td>
<td>frequency</td>
<td>Hz</td>
</tr>
</tbody>
</table>

**Subscripts**

- $c$: critical
- cells: biological cells
- film: material film
- $R$: radius
- $v$: volume
- $w$: wall
### List of abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSMZ</td>
<td>Deutsche Sammlung von Mikroorganismen und Zellkulturen</td>
</tr>
<tr>
<td>ERK</td>
<td>The Ras/Raf/extracellular signal-regulated kinase</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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1 Introduction and background

The term rheology was first used by Bingham. Rheology is the study of deformation of matter under the influence of stress. Rheological studies enable quantification of material properties such as viscosity, viscoelastic response and normal stress differences [1, 2]. Fluids respond to stress by flow in different ways [3]. Newton identified a simple relation defining the viscosity of a fluid, which measures the internal resistance to flow as proportionality constant between the shear stress and shear rate (velocity gradient). Newtonian fluids show constant rheological properties under stress. Water is an example of a Newtonian fluid. Non-Newtonian fluids show deviation from their simple relationship i.e. the response of those fluids exhibit a nonlinear relation between applied stress and rate of deformation. In addition, they show viscoelastic response. For example, synthetic polymeric materials exhibit a non-Newtonian behaviour. Their behaviour lies between the extremes of elastic and fluid response. However, plastic is a material that deforms like an elastic solid as long as the applied stress is below the yield stress limit. When the applied stress exceeds this limit, the material shows a fluid response. Viscoelastic fluids show combination of elastic and fluid response and their deformation changes with time.

To identify and distinguish the responses of Newtonian and non-Newtonian fluids, several techniques are used for measuring their rheological properties [1, 2, 4]. Capillary viscometer is the oldest used technique to measure the volumetric flow rate, the pressure drop at narrow gaps and at high shear rates. As a result the viscosity of Newtonian fluid can be determined [5, 6]. It is possible to study low viscous samples under unidirectional shear using capillary viscometers [7-9]. The shear is produced between the stationary capillary wall and the fluid, which is driven by a pressure difference over the closed channel [2, 4, 10, 11]. For non-Newtonian fluids the shear rate up to $10^6$ s$^{-1}$ is measured based on pressure-driven slit die design [5, 6]. This method is based on forcing a fluid through a thin rectangular channel or slit. Viscous heating at high shear rate can affect the results and the data should be adjusted [6].

Moreover, measuring the viscosity of a Newtonian and shear-thinning materials can be achieved using Couette flow between concentric cylinders [12]. The shear rate and shear stress can be identified by measuring the separation, rotating speed, and the torque that maintain the rotation. However, normal stresses are not measurable in this system and viscous heat due to frictions may be
a major contribution in such a flow. Another technique is used to measure the rheological behaviour up to moderate shear rate: the cone and plate geometry [3, 13]. The shear rate is constant throughout the gap. The shear stress is independent of the measurement position. It is calculated by measuring the torque required to maintain the rotation of the plate. The shear stress range is limited by the cone angle and viscous heating.

The parallel-disk geometry was first suggested by Mooney [14]. This geometry is usually implemented for higher viscosities at gap widths of about 1 mm and it can reach shear rates up to $10^3 \text{ s}^{-1}$ [15-18]. The advantage of parallel-disk geometry is to select the shear-rate range by adjusting the gap width at small gaps. However, the parallel-disk geometry at gap widths below about 1 mm suffers from errors in determining the zero point. They are caused by viscous resistance to squeeze flow during zeroing and by unevenness and small inclination angles of the plates [15, 19-21]. Effective values for the zero-gap error of about 25-75 $\mu$m have been reported [8, 20, 22]. While the data may be corrected for the zero-gap error [15, 21], plate inclination and unevenness result in a superposition of elongation flow with the shear flow, which is difficult to access [23]. At low torques, apart from the rheometer resolution, precision is further reduced by contact line forces, which result in a constant torque offset [24].

There are different reasons to conduct measurements in narrow gaps. For example, it is possible to determine the behaviour of complex fluids at extremely high shear rates and allow for a measurement of the infinity-shear viscosity plateau and normal stress difference that would otherwise be immeasurable. Moreover, the range of the shear-rate can be increased at the same plate velocity. In a commercial rheometer the range of shear rates can be increased by two orders of magnitude of about $10^3 \text{ s}^{-1}$ to $10^5 \text{ s}^{-1}$ by decreasing the distance between the plates from 1 mm to 20 $\mu$m. Therefore, it is now possible to measure complete flow curves across several orders of magnitude for the shear rate. The sample size decreases significantly, which can be important with expensive or rare samples. Because the same shear rates already appear at much lower of maximum velocities, the occurrence of flow instabilities and draining due to centrifugal force are moved to higher shear rates [15]. Thin gaps, in principle, also allow for better temperature control at high shear rates. Furthermore, it enables to study the effect of geometrical confinement [25] and the rheology of biological cells [26]. Finally, friction heat can be dissipated more efficiently to significantly minimize temperature gradients in the sample.

Different groups studied high shear rates at narrow gaps using parallel-disk geometry. Connelly and Greener [15] used a conventional parallel-disk rotational rheometer to measure a thin
film at a gap width of about 50 µm and high shear rate of about $10^5$ s$^{-1}$. In their setup they used T-loop (thixotropic loop) to detect the errors accomplished the viscosity measurements in parallel-disk geometry such as viscous heating, radial immigration, and surface tension. These errors were easily detected and separated from the actual rheological responses. Binding and Walters [27] used two parallel plates in torsional-balance rheometer with same diameter measuring a thin film of 3.2 µm at a high shear rate of about $10^5$ s$^{-1}$. Streator et al. [17] measured the viscosity of different lubricants films at high shear rate and thin-gaps using magnetic slider/disk interface. This setup did not enable in situ measurement of the thickness of the film between the plates. It was measured via the ellipsometry or electron spectroscopy technique. Jonsson and Bhushan [16] developed a capacitance technique based on a magnetic disk drive configuration for in situ measurement of the parallel plate thickness. They reached $10^7$ s$^{-1}$ shear rate in their rotational experiments.

Micro-rheological techniques have been used to investigate the dynamic behaviour of materials. For instance, diffusing wave spectroscopy, surface force apparatus [28, 29] and atomic force microscope have been used to examine the behaviour related to confinement of molecules at the nano-scale. These techniques are inadequate for measurement scale above micron and hence sliding plate devices are needed [22]. Several groups have built piezoelectric devices to carry out oscillatory studies at gap widths below 100 µm [30-33]. At small amplitudes frequencies up to the kHz range can be explored. Granick and co-workers developed a shear apparatus for oscillatory studies that works at gap widths down to the sub micrometer range [34, 35]. Clasen and McKinley and coworkers developed a flexure-based rheometer to reach a narrow gaps of about 1-10 µm [36-38].

The contribution of the present study is to overcome the significant errors in the gap width of commercial rotational rheometers such as the squeeze flow of air, misalignment of the parallel-disks and insufficient flatness of the plates while zeroing the device plates. To minimize zero-gap error, the rheometer has been modified and the gap width has been measured independently from the rheometer reading. We propose a procedure to align the parallel-disks to each other within uncertainty of ±0.7 µm gap width. This enables to extend the range of applicability of rotational rheometers to measure low viscosities, high shear rates and normal stresses with a parallel-disks configuration comparing with commercial rheometers.

This modified setup has a wide range of applications. To name a few, the viscosity of water is out of the measurement range of commercial rheometer due to the fact that the measured viscosity decreased with gap width at gaps < 100 µm [22] due to the typically gap error of about 25-75 µm. On
the other hand, the viscosity function of strongly shear-thinning polymer solutions such as xanthan gum is measured at gap width of 20 µm that extend the shear rate limits up to $10^5$ s$^{-1}$. Due to using glass plates, it is possible to apply optical methods such as birefringence measurements. Additionally, the rheology of biological cells can be investigated quantitatively. The linear viscoelastic properties range has been reached and average cell dynamic moduli have been measured using this approach. This may pave the way to use monolayer measurements as a diagnostic tool for quantitative study of typical or average rheological properties of the cell and how they are affected by biochemical treatment or by diseases, e.g. in cancer cells. As a first proof of principle, the impact of biochemical modifications on the rheological properties of the cells is quantified. The experimental setup is used to explore the critical shear stress that needs to detach the cells at unidirectional stress controlled test.

The thesis is arranged as follows: Chapter 2 shows how the commercial rotational rheometer is modified to measure in narrow gaps. As an example of its applications to study low viscous samples at high shear rate, chapter 3 presents an experimental study with the modified setup of low-viscous polymer solutions. Chapter 4 focuses on the average rheological properties of biological cells in a monolayer of a width of a few microns. Finally, conclusions and an outlook are presented in Chapter 5.
2 Rotational thin-gap rheometer

This chapter starts with introducing the standard rotational rheometer geometries and their work limits. Then it identifies thin-gap rheometry main features, restrictions, applications and home-made devices. Finally, describe in detail the modified commercial rotational rheometer and show the setup, advantages and low viscosities high shear rate examples of applications.

2.1 Standard rotational rheometers

2.1.1 Geometries and range of applications

There are several methods and geometries for rheological measurement of material properties. The most common and important geometries are cone-and-plate geometry, parallel-disk geometry and concentric cylinders. Figure 2.1 shows cone-and-plate geometry. This geometry has many advantages such as a constant shear rate throughout the sample and direct measurement of both shear and normal forces. The first normal stress differences, \( N_1 \) is calculated from a total thrust measurement. The assumption used to derive the equation of motion for a cone-and-plate are [13, 39]:

- Steady flow
- Laminar flow
- Isothermal system
- Free spherical boundary
- Negligible gravity, wall slip and boundary effects
- Gap width: \( h = r \cdot \tan \alpha \)
- Velocity of the cone surface: \( u = \Omega \cdot r \)
Applying the aforementioned assumptions to the equation of motion and using the boundary condition at the cone and plate surfaces yields the following equation for shear rate [1, 2]:

\[
\dot{\gamma}(r) \approx \frac{u}{h} = \frac{\Omega \cdot r}{r \cdot \tan \alpha} = \frac{\Omega}{\tan \alpha} \approx \frac{\Omega}{\alpha} \quad \text{for} \quad \alpha \ll 1 \quad (\alpha \leq 5^\circ) \tag{1}
\]

The shear rate is constant throughout the sample measurement. The deviation in the shear rate at cone angle of 40 is about 0.3%. The torque \( M \) is calculated via Eq. (2) and the shear stress \( \tau \) can be obtained from torque, where \( R \) is the cone radius:

\[
M = \int_0^A r \cdot \tau dA = 2\pi \tau \int_0^R r^2 dr = \frac{2}{3} \pi R^3 \tau \tag{2}
\]

The shear stress in the measuring sample is constant and independent of position and can be calculated from Eq. (2) since the shear rate \( \dot{\gamma} \) is constant as shown in Eq. (1). From Eq. (1) and (2) the viscosity of the fluids is obtained:

\[
\eta = \frac{\tau}{\dot{\gamma}} = \frac{3\alpha}{2\pi} \frac{M}{\Omega R^3} \tag{3}
\]

The first normal stress difference \( N_1 \) can be measured with the cone-plate configuration when the tension in the streamlines produces an axial force pushing the cone, and the plate apart with a force which can be measured while using the same configuration, the second normal stress difference can be determined by measuring the pressure distribution over the surface of the cone [40]. The normal
Rotational thin-gap rheometer

force $F_N$ which is measured in axial direction by the rheometer is used as a raw data in order to calculate the first normal stress difference as shown in Eq.(4)

$$N_1 = 2 \frac{F_N}{\pi R^2} \quad (4)$$

Along with the geometrical torque and angular velocity, effects such as sample evaporation, mass transfer between the sample and the surroundings, restricts the measurement via this method. Beyond the critical shear rate, the sample is pulled out of the gap and decrease the effective area that caused by centrifugal forces and normal stresses.

The parallel-disk geometry on the other hand, (Figure 2.2) is the other geometry that was used in this study. The parallel-disk configuration is usually employed for higher viscosities at gap widths in the millimeter range. Yet, it has the advantage to select the shear-rate range by adjusting the gap width. Unlike the cone-and-plate configuration, the shear rate in the parallel-disk configuration depends on the radial position and on the gap width under the following assumptions:

- Steady flow
- Laminar flow
- Isothermal system
- Free cylindrical boundary
- Negligible gravity, wall slip and boundary effects
- Velocity of the plate surface: $u = r \cdot \Omega \cdot \frac{y}{h} = u(r, y)$
The maximum shear rate \( \dot{\gamma}_R \) and shear stress \( \tau_R \), which are at the outer rim of the smaller disk, result in [13, 39]:

\[
\dot{\gamma}_R = \Omega \cdot \frac{R}{h} \tag{5}
\]

and

\[
\tau_R = \frac{2 M}{\pi R^3} \tag{6}
\]

here, \( M \) is the torque at outer plate surface, \( R \) is the radius of the plate and \( h \) is the gap width. Eq. (6) is for calculating the maximum shear stress of Newtonian fluids at outer plate surface. The viscosity of Newtonian fluids is then calculated using the right hand side of Eq. (3) as with cone-and-plate geometry.

With the parallel-disk configuration, the difference between the first and second normal stress differences, \( N_1 - N_2 \), can be determined from the normal force on the plates:

\[
N_1 - N_2 = \frac{F_N}{\pi R^2} \left( 2 + \frac{d \ln F_N}{d \ln \Omega} \right) \tag{7}
\]

Figure 2.2: Schematic representation of the parallel-disk geometry [1, 2].
where $F_N$ is the normal force without the contribution due to centrifugal forces.

Another geometry, the concentric cylinders have two known setup: the Searle and the Couette systems where the inner and outer cylinders rotates respectively with respect to the other fixed cylinder. The concentric cylinders in Figure 2.3 are used to reduce solvent evaporation and the sample amount. They are used to test low viscous materials. The assumptions for the concentric cylinders as a rheometric tool are [1, 2]:

- Steady flow
- Laminar flow
- Isothermal system
- Velocity of the cone surface: $u_\theta = \Omega \cdot r$ and $u_r = u_z = 0$
- Negligible gravity, wall slip and end effects
- Symmetry in $\theta$

![Figure 2.3: Schematic representation of the concentric cylinders geometry [1, 2]](image)
The wall-shear stresses at the inner and outer concentric cylinders can be calculated using the equations below:

\[ M = 2\pi R_i^2 L \tau_w (R_i) \]  
\[ M = 2\pi R_o^2 L \tau_w (R_o) \]

where \( M \) is the torque at inner and outer cylinders, \( R_i \) is the inner cylinder radius and \( R_o \) is the outer cylinder radius, \( L \) is the length of wetted cylinder and \( \tau_w \) is the shear stress at the wetted cylinder. For concentric cylinders (Couette system), the shear rate can represented by Eq.\,(10) [1, 2]

\[ \dot{\gamma} = r \frac{d\Omega}{dr} \]  

From Eq.(8) and Eq.(10) and \( \tau_i = \eta \cdot \dot{\gamma}_i \) gives

\[ \eta = \frac{\tau_i}{\dot{\gamma}_i} = \frac{M}{4\pi \Omega LR_i^2} \left(1 - \beta^2 \right) \]  

where \( \beta^2 = \frac{\tau_w (R_o)}{\tau_w (R_i)} = \left(\frac{R_o}{R_i} \right)^2 \) is the radius ratio of the inner and outer cylinders. Eq. (11) is used to calculate the viscosity of Newtonian fluids only. Generally, for very small gaps (\( \beta^2 > 0.99 \)) the curvature can be neglected and stress distribution will be almost the same as that in parallel-disk setup. Normal stresses are not measurable in this system.

Oscillatory experiments investigate the viscoelastic responses of the samples on different time scales [1, 2]. The time scale is varied by changing the oscillation frequency. Oscillatory experiments apply a sinusoidal strain \( \dot{\gamma} \) with angular frequency \( \omega \) and amplitude \( \gamma_0 \) to the sample in simple shear:

\[ \dot{\gamma}(t) = \gamma_0 \sin(\omega t) \]  

If the material being studied is a perfectly elastic solid, then the shear stress \( \tau \) in the sample will be related to strain through Hooke’s law where, \( G \) is the shear modulus.

\[ G = \frac{\tau}{\gamma} \]
In a Hookean solid, the stress is in phase with the applied strain. However, if the material being tested is a Newtonian liquid, the stress in the liquid is related to shear rate $\dot{\gamma}$ through Newton’s law of the first part of Eq. (3). The stress in a Newtonian fluid is out-of-phase with the strain by an angle of $\pi/2$. For a viscoelastic material, the stress response will be some combination of Hookean solid and a Newtonian liquid. The stress will be out-of-phase with the strain quantified by a phase angle $\delta$, where $0 < \delta < \pi/2$.

$$\tau(t) = \tau_o \sin(\omega t + \delta)$$

In the case of oscillatory experiments, the stress is a sinusoidal function having the same frequency as the strain. Thus, the stress can be separated into two orthogonal functions that oscillate with the same frequency, one in-phase with the strain and one out-of-phase with the strain by an angle $\pi/2$.

$$\tau(t) = \gamma_o \left[ G'(\omega)\sin(\omega t) + G''(\omega)\cos(\omega t) \right]$$

Here, $G'$ is the storage modulus and $G''$ is the loss modulus. The relative values of storage and loss moduli provide information regarding to an elastic solid $G'$ and viscous liquid $G''$. As an example of viscoelastic materials, biological cells have been studied in chapter 4 for different oscillatory responses.

2.1.2 Standard rotational rheometer restrictions

One of the most important issues that limit the measurements of shear viscosity is the torque limit of the rheometer. Furthermore, the shear rate measured in the rotating plate of a parallel-disk rheometer suffers from deviations from the expected value in perfect parallel geometries. The relative error becomes larger with both an increase in misalignment between the surfaces or a decrease in the central gap between the surfaces. In addition, at low torques, apart from the rheometer resolution, precision is further reduced by contact line forces, which result in a constant torque offset [24]. While the manufacturer states a torque resolution of 0.5 $\mu$Nm [41] the measured torque at minimum viscosity was 12 $\mu$Nm, which is close to the criterion for a practical low-torque limit [42, 43].

At high angular velocities, centrifugal forces in rotational rheometers may cause axial forces on the plates and cones [44, 45]. The effect of this contribution to axial forces can be corrected [22, 44] according to Eq.17.
Rotational thin-gap rheometer

\[ F_{\text{Neff}} = \frac{3}{40}\pi \rho (\dot{\gamma} H R)^2 \]  

where \( F_{\text{Neff}} \) is the normal stress differences due to centrifugal force.

Another issue that may affect the measurements at high shear rates is radial migration. The critical shear rate \( \dot{\gamma}_c \) for the onset of radial migration due to centrifugal forces is given by [8]:

\[ \dot{\gamma}_c = \sqrt{\frac{20\sigma}{3\rho H^3}} \]  

where \( \sigma \) is the surface tension. Given that \( \dot{\gamma}_c \sim H^{-2/3} \), the onset of radial migration moves to higher shear rates while reducing the gap width.

Viscous heating significantly affects the flow properties of Newtonian fluids at high shear rates. The effects of viscous heating can be identified by the Nahme number \( Na \), which is a dimensionless ratio of the time scales for thermal diffusion to viscous heating [46]:

\[ Na = \frac{d \eta \dot{\gamma}^2 H^2}{dT k} \]  

where \( \eta, \kappa \) and \( T \) are dynamic viscosity, thermal conductivity and temperature, respectively. This shows that the Nahme number diminishes considerably by reducing the gap width.

At high Reynolds numbers, deviations from viscosimetric flow may occur. The Reynolds number \( Re \), which is defined as:

\[ Re = \frac{\Omega R H}{\nu} = \frac{\dot{\gamma} H^2}{\nu} \]  

where \( \nu \) is the kinematic viscosity. Hence, maintaining the shear rate, the Reynolds number decreases quadratically with the gap width.

Sliding along the wall is often encountered in foams, emulsions or concentrated suspensions and insufficient cohesion can lead to dramatic errors [14]. Wall sliding can be avoided by using rough surfaces whilst measuring wide gaps [13], but this is not possible in narrow gaps. Nevertheless, wall-slip can be detected and subsequently corrected in this case. Figure 2.4 illustrates this situation. Wall-slip often exists in layers which are considerably narrower than the gap width itself. In this case the
measured gap can be considered as a three-layer system and the wall shear rate $\dot{\gamma}_{\text{wall}}$ can be calculated by Eq. (21):

$$\dot{\gamma}_R = \left(1 - 2 \frac{h_{\text{film}}}{h}\right) \dot{\gamma}_V + 2 \frac{h_{\text{film}}}{h} \dot{\gamma}_{\text{wall}} = \dot{\gamma}_V + 2 \frac{h_{\text{film}}}{h} \dot{\gamma}_{\text{wall}} = \dot{\gamma}_V + \frac{2}{h} \dot{V}_{\text{Slip}}$$  \hspace{1cm} (21)

In the second step, the assumption has been utilized that the layer thickness of the near-wall films is much smaller than that of the bulk region. Finally, the wall shear rate with the equally unknown thickness of the lubricating film $h_{\text{film}}$ was summarized to wall-slip velocity $V_{\text{Slip}}$ in the last step. Eq. (21) describes a linear relation between measured shear rate, volume shear rate $\dot{\gamma}_V$ and wall-slip velocity $V_{\text{Slip}}$. The unknowns are $\dot{\gamma}_V$ and $V_{\text{Slip}}$ with the measured variable coupled via the film thickness $h$. At a constant shear stress, Figure 2.5 shows plots of $\gamma_R$ vs. $2/h$ to calculate the

Figure 2.4: Sketch for the correction of the wall-slip
unknown variables. The wall-slip velocity is the slope of the curve at a certain shear stress and the intersection represents the volume shear rate.

![Graph of shear rate vs. shear stress](image)

**Figure 2.5**: Correcting the wall-slip in accordance with Eq.(21). With the shear stress $\tau_2$, there is no wall slip.

Sliding along the walls leading the erroneous determination of the shear rate is not the only issue concerning walls. The material adheres to the inner surfaces of the plates, produce insufficient cohesions. For that reason the rheometer does not show the real gap width and the shear rate does not measure at the real gaps. Figure 2.6 (a) illustrates this situation. The correction can be done analogously to wall-slip. The starting point is Eq. (21). For resting near-wall layers ($\dot{\gamma}_{wall} = 0$) as sketched in Figure 2.6 (b), the profile from which the shear rate results in a volume back from the intersection with the y-axis. The thickness of the near-wall film can then be estimated from the intersection with the y-axis. With this gap width, the two near-wall layers would just touch.
Figure 2.6: (a) Sketch for the effect of insufficient cohesion. (b) Correction of adhesive films according to Eq.(21).
2.2 Standard thin-gap rheometry

2.2.1 Advantages and error uncertainties

Commercial rotational rheometers are used to quantify a wide range of viscosities at high shear rates. The concentric cylinders and the parallel disks at gap widths of 1 mm are used for low viscosities and high viscosities respectively. Nevertheless, it presents the advantage to select the shear-rate range by adjusting the gap width. At gap widths below about 100 µm, however, it suffers from errors in determining the zero point. They are caused by viscous resistance to squeeze flow during zeroing and by unevenness and small inclination angles of the plates [15, 19-21].

Thin gaps, in principle, also allow for better temperature control at high shear rates. Furthermore, it enables to study the effect of geometrical confinement [25] and the rheology of biological cells [26].

At high shear rates, the measurement range is restricted due to viscous heating, inertial deviations from viscosimetric flow and radial migration due to centrifugal forces or normal stress differences that may overcome surface tension forces [13, 15, 21, 47-49]. In this thesis, we report a way to reduce the gap width in commercial rheometers well below 100 µm and thereby overcome the significant error in the gap height while zeroing the device plates. Comparing to self-built machines it offers the advantage of using the full capacity and versatility of commercial rheometers. This allows an extension of the measurement range to shear rates of up to $10^5$ s$^{-1}$ and to low viscosities down to about 1 mPa·s. Moreover, lowering the gap below 100 µm make it capable to extend the shear rate range for normal-stresses measurements.

In commercial rotational rheometers, there is a small misalignment between the two surfaces. The shear rate measured in the rotating parallel-disk rheometer suffers from deviations from the expected value in perfect parallel geometries. The deviations become larger with either an increase in misalignment between the surfaces or a decrease in the central gap between the surfaces. The proportionality constantly increases with a relative tilt of the plates and decreases with the fluid film thickness [50]. Figure 2.7 shows the main factor of errors that accomplished with standard rheometer setup. Roughness and variations in the flatness of the surface plates of a few micrometers are insignificant in gap widths of 1 mm: They will add an additional shear flow superimposed at gap widths below 10 µm [15]. Strong oscillations may occur between the rotated plate and the stationary one, the frequency of which is dependent on shear rates due to the relative movement of the inclined
Rotational thin-gap rheometer

plate [21]. Even more significant is usually the limited parallelism of the plates. It is often even in the range of 25-70 µm [22].

Figure 2.7: Sketch showing the different types of error accomplished by standard rheometer setup.
The geometric imperfections limit the minimum gap width and also lead to a zero-gap error. Typically, the zero-gap is detected by the fact that the rotating plate is lowered until it touches the bottom plate after which the upper plate is lifted slowly by increasing of the measured normal force. The two plates are already in contact at a mean measurement gap, comparable to the average geometric deviations. Even with flat, perfectly parallel plates, the gap width suffers from this error. As Figure 2.8 shows, which can lead to squeeze the air in the gap to a systematic error $\Delta H$.

![Diagram of Rotational thin-gap rheometer](image)

**Figure 2.8**: Squeeze flow of the air (a) and zero-point error in the parallel-disk configuration due to the squeeze (b) for a disk of radius $R = 25$ mm and a lowering of the plate $50 \mu m/s$ as a function of the normal force at which the zero gap is identified.

This error can be accurately predicted using Stefan’s equation Eq.(22) [13]:

$$
\Delta H = \frac{3\pi}{2} \eta_0 \frac{R^4 h}{F_N}
$$

(22)

here, $\eta_0$ denotes the viscosity of the air, $R$ the disk radius, $h$ velocity with which the plates are pressed together and $F_N$ is the normal force. As the Stefan equation shows, it is particularly important for large plates, and can be reduced by slow motion of the plate and high normal forces when adjusting the zero-gap [22].

As an example of zero-gap error correction in ordinary parallel-disk geometry, Figure 2.9 shows the viscosity function of silicon oil tested in a gap width range between 1000 $\mu$m and 100 $\mu$m. In this case, the gap error was about 60 $\mu$m.
Figure 2.9: Viscosity function of a silicone oil with different gap widths. Parallel-disk configuration with plate radius $R = 25$ mm. The straight line represents constant shear stress of 32.4 Pa.

The zero-gap error leads to measuring a gap dependent viscosity. The closer the measuring gap, the lower the measured viscosity range measurements (see Figure 2.9) [13, 21]. However, the zero-gap error can be determined and corrected using Newtonian fluids and carrying out measurement at a constant shear stress and different set-up gap widths $h_R$ [22]. Whereas the rheometer detects the gap width $h_R$, the real mean gap width $h$ is greater by the zero-gap error $\Delta H$ [21, 51]:

$$h = h_R + \Delta H$$

(23)

Substitution of Eq. (23) in Eq. (5) gives:

$$\dot{\gamma} = \frac{\Omega R}{h_R + \Delta H}$$

(24)

where the maximum shear rate reads:

$$\dot{\gamma}_R = \frac{\Omega R}{h_R}$$

(25)

where $\Omega$ is the angular velocity. Substituting the constitutive law $\tau = \eta \cdot \dot{\gamma}$, the real shear rate determined by the rheometer results:
Rotational thin-gap rheometer

\[ \tau = \eta \frac{h_R}{h_R + \Delta H} \dot{\gamma}_R \]  

(26)

where \( \tau \) the shear stress and \( \eta \) the viscosity of the Newtonian fluid. Dividing Eq. (26) by \( \dot{\gamma}_R \) and substitute \( \tau / \dot{\gamma}_R \) with \( \eta_R \) the result leads to:

\[ \frac{h_R}{\eta_R} = \frac{h_R}{\eta} + \frac{\Delta H}{\eta} \]  

(27)

Lowering the gap width \( h_R \) in a series of measurements yields different viscosity values \( \eta_R \). The actual viscosity can be deduced from the slope of \( h_R / \eta_R \) vs. \( h_R \), as shown in Figure 2.10. The zero-gap error is obtained by extrapolation to the y-axis.

\[ \text{Figure 2.10: Determining the zero-gap error from the data in Figure 2.9 according to Eq(27)} \]
The raw data shown in Figure 2.9 are corrected for shear rate and viscosity of the silicone oil as shown in Figure 2.11 using the results from Eq.(27). At the gap width of 200µm the viscosity is 0.12 Pa·s. The correction factor does not reduce the error in the gap width and it still higher value at gaps beyond 100 µm.

2.2.2 Home-made devices

To study the rheology of thin samples, several groups have built home-made devices, which generally work in the oscillatory mode. A flexure-based micro-rheometer was developed by Clasen and McKinley [36-38] is used to measure the rheological properties of materials at narrow gaps of about 1-10 µm. Figure 2.12 shows a sketch of the flexure-based rheometer (FMR), which is a shear-rate-controlled device that can be used to measure the shear stress in plane Couette flow. Parallel control of the upper and lower shearing surfaces has been established by using white light interferometry, a three-point nanopositioning stage, by utilizing piezo-stepping motors and plates constructed from glass optical flats. This configuration is suitable for determining the microgap-dependent flow behaviour of complex fluids over 5 decades of shear rate in the oscillatory mode [36]. The FMR’s capabilities are demonstrated through a characterization of the complex stress and gap-dependent flow behaviour of a typical microstructured food product (mayonnaise) over gaps ranges from 8 to 100 µm and stress levels from 10 to 1500 Pa. The gap-dependent rheological response was
Correlated to the microstructure of the emulsion and changes induced in the material by prolonged shearing. It is capable of producing large deformations, whilst also maintaining a constant gap separation with a submicron resolution [38].

Granick and coworkers [34, 35, 52, 53] have designed and built a micrometer-gap rheometer with optical access to study the shear of ultrathin liquids. This model consists of three plates conventionally made of steel or aluminum, as shown in Figure 2.13. The bottom plate has two main functions. First, it serves as mount for three finely polished steel rods along which the middle and top plate slide vertically. Axial ball bearings ensure smooth motion along these rods. A second function of the bottom plate is to support three piezoelectric inchworms. The middle plate encases an optical window (see Figure 2.13). The tilt of this plate is controlled by adjusting the piezoelectric inchworms that serve as a tripod support for this plate. The upper plate holds a second optical window; separated from the middle plate by three spacers that provide coarse adjustment of the spacing between the two windows. This top optical window is not attached directly to the top plate, but hangs as a boat from two piezoelectric bimorphs. The windows are aligned in the following steps. First, by turning coarse adjustment screws, the upper window is brought manually to a position within a millimeter or so of the bottom window. Next the spacing is further reduced using piezoelectric inchworms to push the lower window upward. Parallel alignment is achieved using either capacitance sensors (if the windows are opaque) or interferometry [34].
Figure 2.13: Sketch of the microgap rheometer with the white light interferometry unit [34]

Jonsson and Bhushan [16] developed a capacitance technique based on a magnetic disk drive configuration for in situ measurement of the parallel plate thickness. They achieved shear rate of $10^7$ s$^{-1}$ during their oscillatory experiments. The apparatus consisted of a variable-speed spindle, a tri-axis stage for positioning of the slider, a two-directional load cell for normal and friction force measurements, and a capacitance measurement unit. They measured interface capacitance simultaneously with friction within a head-disk interface. They found that the decreasing friction with increasing sliding speed was attributed to the slider tipping onto its inlet taper section [54].

Figure 2.14: Sketch of the interface capacitance device [54]
Besides planar micro Couette devices, several groups used micro capillary viscometers to study shear-dependent viscosities at shear rates up to \(10^5\) s\(^{-1}\). Pipe et al. [8, 9] detected the viscometric response at high shear rates of a wide range of fluids from constant viscosity mineral oils to strongly shear-thinning polymer solutions. Using microfluidic channels, high shear rates can be reached while the inertial and viscous heating still remain unaffected in the measuring range. In their study, they showed an agreement between results from the parallel-disk rheometry and micro capillary rheometer for Newtonian fluids. Pan and Arratia [7] used a microfluidic rheometer to study the viscosity of liquids at low Reynolds number using in situ pressure sensors. They found that this technique is capable to measure the viscosity curve of Newtonian and non-Newtonian samples for the shear rate range up to \(10^4\) s\(^{-1}\).
2.3 Modified thin-gap rheometer with parallel-disk geometry

While the aforementioned home-made thin-gap devices only permit to determine viscosities of viscoelastic materials, it is desirable to exploit the full capacity and versatility of commercial rotational rheometers. Subsequently, it is described how the improvement of the zero-gap precision by a factor of 30 and more has been achieved. The experiments were implemented with a Physica UDS 200 rotational stress-controlled rheometer at a temperature of (298.16 ± 0.1) K. The measuring device is based on a highly dynamical motor-driven system without gears and without mechanical force transducer [41]. The technical specifications of the rheometer are listed in Table 2.1. Modifying the parallel-disk configuration results in an extension of the range of applicability.

Table 2.1: Selected specifications for the Physica UDS 200 rheometer [41].

<table>
<thead>
<tr>
<th>Variable</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>-80 – 600</td>
</tr>
<tr>
<td>Torque (µN·m)</td>
<td>0.5 – 150,000</td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>10^4 – 100</td>
</tr>
</tbody>
</table>

An Ubbelohde capillary viscometer was used in this study to measure the zero-shear rate viscosity of low viscous solutions and the results were compared with the modified parallel-disk rheometer. It consists of the four sections, capillary, the venting, the filling tube, a reservoir, a reference level vessel with measuring sphere, a pre-run sphere and lastly a cleaning tube. The mean of three measurements at a room temperature (298.16 ± 0.1) K is established.

To work in the parallel-disk configuration at gap widths of the order of 10 µm, zero-point errors have to be considerably reduced. The plates have to be aligned parallel to each other and perpendicular to the axis of rotation. To achieve this, a commercial rheometer has been modified as sketched in Figure 2.15 (a). The glass plates are used from Melles Griot. The stationary plate has a diameter of 75 mm and an evenness of 1λ, the rotating plate has a diameter of 50 mm, its evenness is λ/20, where λ is the testing wavelength (633 nm). The rotating plate is attached to a measurement head of the rheometer. The head has a diameter with 25 mm. To align the stationary plate perpendicular to the rotation axis, it is fixed to a tripod, which is mounted on the rheometer table. The tripod is aligned by three micrometer screws with a fine resolution of 1 µm and fixed to the rheometer with three screws after adjustment; see Figure 2.15 (b).
Rotational thin-gap rheometer

Figure 2.15: (a) Sketch of the setup within the rotational rheometer. The gap width between the rheometer plates is measured with a confocal interferometric sensor; the samples are viewed with the camera. (b) The tripod is aligned with three micrometer screws and fixed to the rheometer with three screws.

The gap width is measured with a customized confocal interferometric sensor (CIS) from STILSA. It has a working distance of 20 mm, a measuring range of 90 µm and allows detecting of the gap width with an axial accuracy of 10 nm according to manufacturer specifications [55]. The sensor is placed underneath the fixed glass plate on a traverse to measure the gap width at different locations especially near the rim of the rotation plate. Figure 2.16 shows the traverse that holds the sensor and the camera placed underneath the rheometer basement.
The following procedure has been implemented to align the glass plates with the desired precision. After careful cleaning, the top glass plate is placed in contact with the top of the bottom glass plate and glued to the rheometer head using the adhesive Vitralit 6129 from Panacol-Elosol GmbH and UV-exposure. This adhesive thick material has a very low thermal expansion coefficient of 36 ppm/K and could be easily detached by using hot air blower at 423.16 K for several minutes. The UV-exposure light from Sylvania has a wavelength of 385 nm and an intensity of about 120 mW/(cm)$^2$.

The upper plate is lifted by about 50 µm. The gap width between the plates is measured with the sensor near the edge of the smaller plate at three different locations, forming a triangle. During these measurements, the upper plate rotates at constant speed of 0.01 s$^{-1}$ to measure the variation of the thickness in time. Due to the inclination of the upper plate with respect to the rotating rheometer axis, the gap width varies periodically during rotation. Since the lower plate is not perfectly perpendicular with respect to the rheometer axis, the mean value of the gap width is different at each position of the sensor. Figure 2.17 (a) shows the measurement at beginning of this procedure.
Rotational thin-gap rheometer

Figure 2.17: Setting up the rheometer disks. The upper disk rotates slowly while the gap width is detected with a sensor at three different locations: before adjustment (a), after adjusting the lower plate (b), after final adjustment of the upper disk (c).

The lower plate is adjusted with micrometer screws that shown in Figure 2.15 (b) to minimize deviations in the mean gap width. After adjusting the lower plate, the sinusoidal undulations of the gap width are of equal amplitudes. Figure 2.17 (b) shows the next step in this approach. The upper disk is dismantled from the rheometer head by leaving it in acetone for several minutes and subsequent exposure to hot air for a short period of time. Thereafter, placed on the levelled lower plate and glued to the rheometer head. Parallelism is checked again with the sensor to achieve undulations at each location with peak-to-peak amplitude of about 1 µm, see Figure 2.17 (c). The setup is now calibrated and ready for rheological measurements. The gap width referred to in this approach is the mean of the undulations. Prior to each experiment, the zero-gap is measured with the CIS sensor as a three point average and then adjusted to make sure the rheometer zero-gap is identical with sensor reading. This reduces the error in zeroing drastically, and the real gap width is validated correctly and independently from the rheometer reading. Figure 2.18 shows a block diagram illustrating the adjustment procedure.
Rotational thin-gap rheometer

Figure 2.18: Block diagram represents the approach procedure that used to check the parallelism.

With the modification, the unidirectional measurements can be carried out at gap widths down to about 10 µm after reducing the zero-gap error to its minimum value. Additionally, the oscillatory measurements can be carried out down to 2 µm gap width if the plates oscillate around a phase that corresponds to the mean gap width. While the maximum shear rate in this configuration (Eq.(25)) is directly proportional to the rotational velocity, the gap width and the shear rate are inversely related.
As an attempt at testing the modified setup, Figure 2.19 depicts the viscosity function of silicon oil independent from the gap width. The measuring data shows a comparison between 20 µm and 1000 µm gap widths. At both gaps there are a broad plateau where the viscosity data is measured between shear rate of 10 s⁻¹ and 3200 s⁻¹. The deviation in the viscosity measurements is less than 3%, which is in line with the relative error in parallel-disks geometry at a gap width of 20 µm. At higher shear rates of about 3200 s⁻¹, the major limitation is that maximum rotational velocity has been reached for a gap with of 1000 µm. Comparing the result with Figure 2.9, at a gap width of 1000 µm there is a difference of about 2.5% in the measured viscosities due to zero-gap error detection.

![Figure 2.19: Viscosity data for silicon oil at temperature of (297.95±0.1). The data shown here are independent of the gap width with maximum deviations less than 3%. The vertical points in 1000 µm represent maximum velocity reached.](image)

As another example, the parallel disk configuration is used to measure low viscous materials and their viscosity functions have been investigated. To name a few low viscosity liquids, distilled water, ethanol with a purity of 99.8% from Roth, toluene with a purity of 99.9% from Merck KGaA. The viscosity data obtained for distilled water at gap widths ranging between 1000 µm and 20 µm shows Figure 2.20 (a). The viscosity data shows a minimum or plateau. At lower shear rates the data values increase due to the low-torque limit of the rheometer [42, 43]. At higher shear rates, the main limitation is inertia for low viscous liquids. Yet, at a gap width of 1000 µm, the Reynolds number (taken with the real viscosity) is already about 170 at the minimal viscosity value, which is more than three times larger than the real viscosity of water. Narrowing the gap, the Reynolds number in Eq.(20)
, is drastically reduced and the minimal values for the viscosity data approach the real value. At a gap width of 20 µm, there is a broad plateau where the viscosity data is equal to the ideal one measured with an Ubbelohde viscometer. Here, the Reynolds number ranges between 1.8 and 28. Although the minimum shear rate due to the low-torque limit does not depend on the gap width, the data scatter at lower shear rates in the narrowest gap is larger than at wider gaps, which is apparently due to dust particles that affect rheological measurements at narrow gaps [37]. Finally, the vertical data points indicate the high shear-rate limit for the respective gap width in these stress-controlled measurements. Figure 2.20 (b) depicts the minimum viscosity values obtained for distilled water, ethanol and toluene as a function of the gap thickness. The straight lines in the diagram indicate the measurements with Ubbelohde viscometers for comparison. As depicted, the viscosity of low viscous liquids such as toluene can be reliably determined at gap widths of about 20 µm. Deviations from Ubbelohde data are less than 4%. While the manufacturer states a torque resolution of 0.5 µNm [41] the measured torque at minimum viscosity was 12 µNm, which is close to the criterion for a practical low-torque limit [42, 43]. Viscous heating as described by Eq. (19) are rendered irrelevant for low viscous liquids. Yet it would result in a drop in viscosity at high shear rates, which is not observed in this case. Measuring the disk-surfaces temperature on the gap side directly after the rheological runs at maximum shear rate did not show any deviation from ambient temperature. For 20 µm gap widths, the Nahme number is below $10^{-4}$ or smaller and hence viscous heating is not relevant in the low viscous liquids. Furthermore, the radial migration effects have been considered at high shear values. The critical shear rate $\gamma_c$ for the onset of radial migration due to centrifugal forces that is given by Eq. (18) is about $7 \cdot 10^2$ s$^{-1}$ at a gap width of 1000 µm. It increases to $2.5 \cdot 10^5$ s$^{-1}$ at a gap width of 20 µm.
To conclude the modification of the parallel-disk configuration by reducing the zero-gap error and detecting the real gap width independently from the rheometer reading offers significant advantages. It permits to extend the shear rates range up to $10^5 \text{ s}^{-1}$ by lowering the gap width down to 20 µm with a precision in the gap width of about ±0.7 µm. At such a lower gap width the modified setup is capable to measure low viscous samples down to about 1 mPa·s. For oscillatory studies, the uncertainty can be reduced to about ±0.2 µm. At the same time, the setup offers the versatility of standard rotational rheometers, like carrying out normal force measurements and step increase in shear rate and stress.
3 Polymer solutions at high shear rate applications

3.1 Introduction and background

Polymer solutions are a paradigmatic class of rheological systems. Besides being of fundamental interest to rheologists, polymer solutions are important in a wide number of industrial and agricultural applications. They are used in coatings, adhesives, drilling fluids, foods, personal care, oil recovery, and agriculture, for instance, as viscosity modifiers, stabilizers, thickeners, drag reducers, drug delivery agents, flocculants, absorbents, dispersants etc. [56-59]. The market for xanthan gum alone, for instance, has been estimated in 500 M$/year in 2009 and continues to grow [59]. Due to the great importance of polymer solutions in many diverse systems and applications, a fundamental understanding of their rheology is crucial.

The rheological properties of polymer solutions at rather low shear rates and their dependence on molecular weight and solvent have been studied extensively for many years. Very dilute solutions have apparently a constant viscosity independent from shear rate [60, 61]. At higher concentrations, the solutions show strong shear thinning [45, 59-64]. The onset of shear thinning depends on the molecular weight [65] and takes place at a shear rate that corresponds to the inverse of the longest relaxation time in the polymer solution [66]. The viscosity functions are usually well described by Carreau-Yasuda, and Cross models [31, 67-69]. Shear thickening due to flow-induced aggregation has been reported recently in solutions of high molecular weight polymers [70]. At very high concentrations, the solutions may also exhibit yield stress.

Polymer solutions under deformation show time dependent intermediate responses between elastic and viscous, therefore, polymers’ viscosity function and normal stress differences are adequate to designate their rheological properties. The rheological properties of polymer solutions in unidirectional shear flows could only be studied with standard rotational rheometer in a shear rate range up to about \(10^3\) s\(^{-1}\) due to restrictions of available apparatuses. At this shear rate, the solutions are usually well within the shear thinning range. At higher shear rates, which are encountered in a number of applications, a second Newtonian branch is expected. The commercial rheometer has been improved to systematically study and characterize the second Newtonian branch of polymer solutions.
Hence, with this setup it is possible to push the measuring limits about the rheological behaviour of polymer solutions considerably.

The first Newtonian branch has been studied intensively to characterize the polymer solutions close to equilibrium. Depending on their concentration and their molecular weight, polymer solutions in good solvents are classified into five states, refer Figure 3.1 [66]: dilute particle solutions, semi-dilute particle solutions, semi-dilute network solutions, concentrated particle solutions, and concentrated network solutions. In $\theta$-solvents, semi-dilute network solutions do not occur. The quantitative details depend on the polymer itself and its structure. Polyelectrolytes show certain abnormalities [59]. In salt-free solvents, the zero-shear viscosity increases considerably in a narrow range of concentrations at the crossover from dilute to semi-dilute. This is attributed to stronger repulsion between the polymers due to electrical charges. In the semi-dilute regime, the zero-shear viscosity follows the Fuoss law [71, 72], i.e. it is proportional to the square root of the polymer concentration. In the entangled regime, the zero-shear viscosity increases with concentration according to a power law with an exponent of 3/2. At higher concentrations, the electrostatic blobs overlap and the polyelectrolytes behave as neutral polymers in good solvents. In the presence of high salt content, the polyelectrolytes behave rather as neutral polymers in a $\theta$-solvent. In the semi-dilute disentangled regime, the zero-shear viscosity show quadratic increases with polymer concentration [73] and in the entangled regime it depends on the concentration according to a power law with an exponent of about 14/3. Figure 3.2 [73] shows experimental data for xanthan with typical scaling laws.
Polymer solutions at high shear rate applications

Figure 3.1: Solution states of polymer solutions [66]. MW: molar mass, c: concentration, [η]: intrinsic viscosity.

Figure 3.2: Zero-shear viscosity of xanthan [73]. Solid symbols: salt-free, open symbols: high salt concentration. c*: crossover concentration from dilute to semi-dilute particle solutions; c_e: from disentangled to entangled; c_D: from behaviour of charged polymers to that of neutral polymers.
The experimental data is well described by scaling laws derived by Dobrynin and colleagues [74, 75]. The scaling laws are based on ideas of electrostatic persistence length proposed by Odijk [76] and by Skolnick and Fixman [77], who assumed a Debye-Hückel potential, and considered that the electrostatic persistence length is proportional to the Debye screening length [78, 79]. In very dilute salt-free solutions, the counter ions are homogeneously distributed, yet the Debye screening length is larger than the distance between chains [80]. Therefore, the charges on the chain interact via the unscreened Coulomb potential and each polymer can be represented as a chain of electrostatic blobs. In semi-dilute solutions, the hydrodynamic interactions between chain sections are screened on length scales larger than the correlation length. Inside the correlation blob, the motion of different chain sections are strongly coupled hydro-dynamically like in dilute solutions [80]. At high salt content, the salt ions control the screening of Coulomb interactions and the Debye screening length becomes smaller than the electrostatic blob size; the polyelectrolyte has then the same structure as a neutral polymer in a good solvent [80]. Nevertheless, at very high polymer concentrations, the contribution of salt ions to the screening may become small again resulting in a salt-free behaviour [74]. At intermediate salt concentrations electrostatic intra-chain interactions induce additional stiffening beyond the Debye screening length [74, 76, 77].

While the above classification and critical concentrations refer to the first Newtonian branch, where the polymers are close to equilibrium, it remains unclear how these are affected in the second Newtonian branch, i.e. far away from equilibrium. At the onset of this regime, the shear rate has surpassed the fastest inverse relaxation times relevant in shear flows. The average polymer deformation in the second Newtonian branch is high and is not supposed to change significantly any more. It is often assumed that in this range the polymers are completely stretched and disentangled [40]. Deoxyribonucleic acid (DNA) studies show, however, that the average polymer extension seems to tend to asymptotic values of less than half of its contour length at high shear rates [81, 82]. As a result of competition between Brownian motion and chain convection, the DNA chains carry out an periodic stretching and tumbling motion [82, 83]. The number of entanglements, in any case, decreases at higher shear rates in the shear-thinning region [66]. Hence, if there are different solution states in the second Newtonian branch, their critical concentrations will be different from the first Newtonian branch.
Although the second Newtonian branch is of interest for the fundamental understanding of polymer systems far from equilibrium as well as for applications like coating, turbulence reduction agents or drilling, there is hardly any data on the second Newtonian branch of polymer solutions. The values for the infinity-shear viscosity of the second Newtonian branch are often obtained without reaching the second Newtonian branch by extrapolating deviations from the power-law behaviour according to Carreau-Yasuda or Cross models and hence are somewhat arbitrary [84, 85]. Yet, there is no systematic study of the second Newtonian branch in polymer solutions. The main reason seems to be the lack of adequate measurement equipment: The shear rates for the second Newtonian branch are usually out of reach for commercial rotational rheometers, which reach shear rates of about $10^3 \text{s}^{-1}$. At the expected low infinity-shear viscosities, furthermore inertia becomes dominant, limiting the applicability to lower shear rates and higher viscosities [86]. Higher shear rates can be reached with high-pressure capillary viscometers. However, the viscometers are usually designed for considerably higher viscosities and do not allow precise measurements of the low infinity-shear viscosities of polymer solutions.

Besides the infinity-shear viscosity and the fastest relevant relaxation times data, normal-stress differences data in polymer solutions is generally rather scarce. There is neither data for the second Newtonian regime nor any for shear rates well beyond $10^3 \text{s}^{-1}$. For an aqueous xanthan solution and for rather concentrated polyacrylamide solutions, normal stress differences could be measured at shear rates up to $10^3 \text{s}^{-1}$ [45, 87]. At high angular velocities, centrifugal forces in rotational rheometers can overcompensate the elastic normal stress differences [44, 45], an effect that nevertheless can be corrected for [22, 44].

Flexible polymers tend to degrade at high shear rates making it impossible to reach a second Newtonian plateau at the initial molecular weight [88]. However, in the absence of entanglements even poly(ethylene oxide) solutions do not seem to be degraded by shear flows [70]. Semi-flexible and rigid polymers like xanthan or guar gum are known to be considerably less susceptible to mechanical scission in shear flows than flexible polymers such as polyacrylamide or poly(ethylene oxide) [89]. The molecular weight of xanthan, for instance, did not change considerably even after long time exposure to turbulent flows [73, 90].
To elucidate the shear-induced change of the sample’s microstructure numerous optical techniques have been applied [91]. For transparent polymer solutions, the most important methods are small angle light scattering, dichroism and birefringence. Dichroism in these samples results from concentration fluctuations and primarily senses structural anisotropy perpendicular to the flow direction [91]. The scattering angle in small angle light scattering depends on the size of the sample components and their orientation. Fluctuations result in butterfly patterns. While small angle light scattering is a single-point technique, shear-induced deformation and alignment of the components in two dimensions is often detected by birefringence [91, 92]. The modified commercial rotational rheometer in parallel-disk configuration described above is employed to measure the flow curve of polymer solution samples such as poly(ethylene oxide) and xanthan gum solution in a wide range of viscosities at shear rates up to about $10^5$ s$^{-1}$. Besides, offering optical access through transparent plates, this configuration enables measurement of normal forces and henceforth normal-stress differences at gap widths of a few µm.
3.2 Materials and methods

3.2.1 Chemicals

In this part, semi-dilute entangled polymer solutions are studied. Since shear degradation is severe in flexible polymers, the main focus is on semi-flexible polymers like xanthan gum.

Poly (ethylene oxide) (PEO), is a non-ionic, synthetic polyether that is readily available in a range of molecular weights. This polymer is commonly used as a drag reduction agent. It is amphiphilic and soluble in water [93]. PEO has been used for several years as an electrolyte solvent in lithium polymer cells [94]. The shear rheology of PEO solutions has been extensively studied. Comprehensive overview of its rheological behaviour reported by Molyneaux [95]. Gauri and Koelling [96] showed the concentration and temperature dependencies of the shear rate and extensional rheology. PEO rheological properties were investigated using controlled stress-rheometer. It is inferred that PEO solutions at different molecular weights do not exhibit a yield stress and the data fits well with the Cross-model [97].

Xanthan gum is a high molecular weight, anionic, extracellular polysaccharide produced by the bacterium Xanthomonas campestris [98]. Xanthan gum solution is used as viscosity modifiers, stabilizers, thickeners, drag reducers [45, 99-106]. The xanthan gum polymer structure as shown in Figure 3.3 consists of a β-1,4-linked glucan backbone with charged trisaccharide side chains (β-D-mannopyranosyl-(1,4)-α-D-glucopyranosyl-(1,2) β-D-mannopyranosyl-6-O-acetate) on alternating residues [99, 107, 108]. The influence of the mechanical polymer properties have been studied for different degrees of the side chain substitutions [109, 110]. Xanthan shows both anisotropic and isotropic phases in solution [111-113]. For isotropic xanthan solutions viscosities, the temperature dependency can be described with the Arrhenius law. The polymer molecular weight as well as the concentration and the ionic strength dominate the relative amount of each phase. Xanthan gum as a polyelectrolyte solution has complex rheological properties due to the polymer’s sensitivity according to presence of ions in its solution [75, 114-116]. The xanthan gum solution shows a Newtonian plateau at low shear rates followed by a shear thinning regime at higher shear rate. The shear viscosity of xanthan solution is well described by the Carreau-Yasuda and the Cross model. In the dilute regime (i.e. at concentrations below 0.01 wt.%), aqueous xanthan solutions are Newtonian [107].
Figure 3.3: Xanthan polymer primary structure [107].

The study is carried out with the following polymers: 0.5 wt. % poly(ethylene oxide) from Roth in distilled water, and 0.05-0.25 wt. % Xanthan gum from Sigma Aldrich in distilled water. The poly(ethylene oxide) has an average molecular weight of $6 \times 10^5$ g/mol and contains 200-500 ppm butylated hydroxytoluene inhibitor. Xanthan gum has an average molecular weight of $2 \times 10^6$ g/mol. The polymer solutions are prepared according to standard protocols [117]: Polymer particles are dissolved in distilled water at a room temperature of (298.16 ± 0.1) K and gently stirring at 400 rpm for about 24 hours to achieve complete homogenization. Only fresh solutions not older than four days were used for the rheological studies.
3.3 Experimental results and discussion

3.3.1 Viscosity measurements

In this study, an aqueous 0.5 wt. % solution of poly(ethylene oxide) used to test the setup under study. Figure 3.4 shows its viscosity function, measured at gap widths of 100 µm and 20 µm. The open symbols indicate data values at a torque below 10 µNm (at low shear rates) and at a Reynolds number beyond 20 (at high shear rates). In this case, the Reynolds number was defined with the measured viscosity refer to Eq. (20). At a gap width of 100 µm this Reynolds number limit is reached at a shear rate below $10^4$ s$^{-1}$. Reducing the gap width to 20 µm moves the limit to a shear rate of about $10^5$ s$^{-1}$. The minimum shear rate due to the low-torque limit does not depend on the gap width. As seen in Figure 3.4, at a gap width of 20 µm the data scatter is quite large at considerably higher shear stresses than in 100 µm gap. This fact which is attributed to the dust particles in the ambient laboratory environment [37]. Measuring the disk-surface temperature on the gap side after maintaining the shear rate at $10^5$ s$^{-1}$ for 100 s did not show any marked deviation from ambient temperature. Increasing the shear rate to $10^5$ s$^{-1}$ like in Figure 3.4 resulted in a temperature increase of 0.2 K for the poly (ethylene oxide) samples. Finally, it has been remarked that the zero-shear viscosity could not be determined at a gap width of 100 µm for the poly (ethylene oxide) solution due to low-torque limits of the device.

![Figure 3.4: Viscosity function of an aqueous 0.5 wt. % poly (ethylene oxide) solution. Triangles and circles indicate measurements at gap widths of 100 µm, and 20 µm, respectively. Open symbols identify data points obtained below a torque of 10 µNm or beyond a Reynolds number of 20. Solid symbols show data within these limits.](image)
At both gap widths, 20 µm and 100 µm, the shear-thinning regimes are well-distinguished, which follow the power-law with exponents of 0.830±0.005 up to shear rate of about $2\cdot10^5$ s$^{-1}$ and 0.870±0.003 at a shear rate up to about $5\cdot10^4$ s$^{-1}$. As flexible polymers, shear degradation is particularly severe in PEO solutions [118]. Therefore, PEO tends to degrade at high shear rates making it impossible to reach a second Newtonian plateau at the initial molecular weight [88, 119]. We hypothesize that it is due to polymer shear degradation, why it is not possible to reach the second Newtonian branch with this measurements even at high shear rate range.

Pipe et al. [8] studied the rheological properties of 0.1 wt.% PEO solution at high shear rates up to $10^3$ s$^{-1}$ using a cone and plate geometry. They showed the polymer solution has a slightly shear-thinning response and the second Newtonian branch was not possible to reach. This is in line with our findings. In the current study even at $10^5$ s$^{-1}$ the second Newtonian branch is not reached. Subsequently, we studied strongly shear-thinning semi-dilute aqueous xanthan solutions to investigate the second Newtonian branch at high shear rate limits that do not show degradation. According to Wyatt et al. [73], in Figure 3.2, aqueous xanthan solutions are semi-dilute in the concentrations range between 400 ppm to 2000 ppm.

Figure 3.5 (a) shows cases for different gap widths of an aqueous solution of xanthan with a concentration of 0.25 wt.%. The diagram indicates that the second Newtonian branch is well defined within the measurement range of the apparatus. Defining the Reynolds number with the measured viscosity, the solid symbols indicate data obtained at Reynolds numbers below 20, a limit well below the established one of about 100 [22]. At a gap width of 1000 µm and shear rate below $10^3$ s$^{-1}$, this Reynolds number limit is attained. Reduction of the gap width to 100 µm shifts the limit to a shear rate below $10^4$ s$^{-1}$. Further, reducing the gap width to 20 µm shifts the shear rate beyond $10^5$ s$^{-1}$, wherein we reach the maximum possible angular velocity of the rheometer. Higher shear rates could be reached by further narrowing the gap. Within the limit defined by the Reynolds number, the data obtained at different gap widths coincides well within the remaining uncertainty of the gap width even at Reynolds numbers beyond 20. At low shear rates, slight deviations occur. They are apparently due to wall slip at the present high viscosities similar to those observed, for instance, in oil paints [120]. Viscous heating could result in lowering the viscosity. Measuring the disk-surface temperature on the gap side after maintaining the shear rate at $10^4$ s$^{-1}$ for 100 s did not show any deviation from ambient temperature. Increasing the shear rate to $10^5$ s$^{-1}$ resulted in a temperature increase of 0.2 K. Besides, the power-law exponent is 0.33, which corresponds to the theoretical value obtained for dilute and semi-dilute solutions of dumbbells and semi-flexible polymers at high shear rates [121,
122]. Furthermore, the extrapolation of the power law to high shear rates would result in the solvent viscosity at a shear rate of about $2 \cdot 10^4 \text{ s}^{-1}$.

In Figure 3.5 (b), 0.23 wt. % xanthan gum solution was tested in cone and plate geometry with cone angle of 1° and 25 mm radius using an MCR 301 rheometer. The result is compared with parallel-disk geometry at 20 µm gap width. It has been seen that within the shear rate limits from $1 \text{ s}^{-1}$ to $10^4 \text{ s}^{-1}$ defined by the Reynolds numbers beyond 20, the data at different geometries overlapped well within the remaining uncertainty of power-law fitting exponent of $0.42 \pm 0.01$ at cone and plate geometry for shear rate up to about $10^3 \text{ s}^{-1}$. The exponent of the power-law fitting is $0.45 \pm 0.01$ of parallel-disk for shear rate up to about $10^4 \text{ s}^{-1}$.

![Figure 3.5: Viscosity functions of aqueous xanthan solutions at different gap widths (a) and in comparison with cone and plate data (b). Open symbols in (a) identify data points obtained beyond a Reynolds number of 20. Solid symbols show data within this limit, (b) cone angle of 1° and plate diameter 25 mm, parallel-disk at 20 µm gap thickness. Temperature (297.86±0.1) K, xanthan concentration: 0.25 wt. % (a), 0.23 wt. % (b).]
The viscosity function of an aqueous 0.25 wt. % xanthan solution is fitted with Cross model [68] as shown in Figure 3.5 (a). The Cross model is described by the following equation:

\[
\eta(\dot{\gamma}) = \eta_0 + (\eta_\infty - \eta_0) \left[1 + \lambda (\dot{\gamma})^m \right]^{-1}
\]  

where \( \lambda \) is a constant with units of time and \( m \) is a dimensionless constant [68]. To fit the raw data to these models, the Excel® Solver option was used as proposed in [123].

The aqueous semi-dilute xanthan gum solutions show a second Newtonian plateau at concentrations as low as 0.05 wt. % after a shear-thinning region as shown in Figure 3.6. The viscosity functions are well-described by the Cross-model (see Eq. 28). The zero-shear viscosity is concentration dependent as shown before by Wyatt et al. [73]. Thereafter, the polymer solutions exhibits a shear-thinning regime with a power-law behaviour. The degree of shear-thinning of its viscosity function is concentration dependent as Figure 3.6 shows. The second Newtonian branch occurs at shear rates beyond \( 10^4 \text{ s}^{-1} \). The infinity-shear viscosity increases with the concentration.

![Figure 3.6: Viscosity functions of aqueous xanthan solutions at gap width of 20 µm with different concentrations. The data at low shear rate obtained below a torque of 10 µNm or beyond a Reynolds number of 20. Parallel-disk at 20 µm gap thickness. Temperature (297.86±0.1) K.](image)
Figure 3.7: (a) Power-law exponent of the aqueous xanthan solutions for concentrations ranging from 0.05 wt. % to 0.25 wt. % in the shear-thinning regime in Figure 3.5 (a). Diagram (b) depicted the infinity-shear viscosity minus the solvent (water) viscosity.

Figure 3.7 (a) depicts the power-law exponent of the shear-thinning region defined from fitting the data with the Cross model for shear rate range from $2 \cdot 10^{-1}$ s$^{-1}$ (at a torque beyond 10 µNm) to $10^5$ s$^{-1}$ (at a Reynolds number below 20) and the error bars in the diagram are due to the fitting uncertainty. The power-law exponent and the concentration are related inversely. The power-law exponent for 0.25 wt. % xanthan solution is 0.33, which corresponds to the theoretical value obtained for dilute and semi-dilute solutions of dumbbells and semi-flexible polymers at high shear rates [121, 122].

The difference between the infinity-shear viscosity at a shear rate up to $10^5$ s$^{-1}$ and the solvent viscosity ($\eta_\infty - \eta_s$) in Figure 3.7 (b) shows that it increases with xanthan polymer concentrations. The curve shows two power-law regimes. Up to concentration of 0.2 wt. %, the exponent of a power-law is about 0.5±0.01, which is agree well with the Fuoss law [71] and typical for semi-dilute disentangled polyelectrolyte polymer solutions [74]. At higher concentrations the viscosity changes with concentration according to a power-law with exponent 1.6±0.01, which is typical for entangled semi-dilute solutions. The dependence of viscosity on the concentration is much weaker than for natural polymers, which ($\eta_\infty - \eta_s \sim c^{15/4}$) [59]. It was found that the semi-dilute entanglement $c_e$ concentration in this study is moved from 400 ppm for $\eta_o$, see Figure 3.2 to 2000 ppm as shown in Figure 3.7 (b).
3.3.2 Normal force measurements

Apart from measuring the viscosity function, the setup permits the determination of the normal force on the plates caused by normal-stress differences up to shear rates of $10^5$ s$^{-1}$. For two different gap widths, Figure 3.8 (a) shows the normal force for the xanthan solution after baseline correction. The normal force after subtracting the contribution due to centrifugal forces is shown in Figure 3.8 (b). Centrifugal forces result in a negative normal force $F_{N,\text{Inertia}}$ on the plates [22]

$$F_{N,\text{Inertia}} = -\frac{3\pi}{40}\rho\Omega^2R^4 = -\frac{3\pi}{40}\rho(\dot{\gamma}HR)^2$$  \hspace{1cm} (29)

where $\rho$ is the liquid density. Since the inertial contribution is opposite to that by normal-stress differences, the measurement range of the rheometer is extended. As shows Figure 3.8 (a), the normal force becomes measurable within the limits of the rheometer at shear rates of about $10^2$ s$^{-1}$. At thicker gaps, it is hardly possible to measure normal forces in this sample. Yet, thinner gaps allow studying the normal forces at high shear rates. With the parallel-disk configuration, the difference between the first and second normal stress differences, $N_1 - N_2$, can be determined from the normal force on the plates [22]:

$$N_1 - N_2 = \frac{F_N}{\pi R^2} \left( 2 + \frac{d \ln F_N}{d \ln \Omega} \right)$$  \hspace{1cm} (30)

where $F_N$ is the normal force without the contribution due to centrifugal forces. Figure 3.8 (c) shows the result. The slope correction $\left( \frac{d \ln F_N}{d \ln \Omega} \right)$ was obtained by using a third-order polynomial fit to the logarithm of the data. At higher shear rate, where the viscosity deviates strongly from the power-law and in the second Newtonian branch shown in Figure 3.5 (a), a power-law fit to the normal-stress differences yields an exponent of $0.65\pm0.1$, while the exponent is slightly larger in the shear-thinning regime. This value is close to the exponent $2/3$ that is predicted for the first normal-stress difference in semi-flexible polymers at high shear rates [121, 122].

In the cone and plate measurements, it was found that at a cone angle of $1^\circ$ and plate diameter of 50 mm, it is not possible to measure the first normal stresses at the maximum cone height of 440 $\mu$m, which is consistent with the normal force measurement with the parallel-disk geometry in Figure 3.8 at larger gap widths.
At shear rates beyond up to $10^4 \text{ s}^{-1}$, the power-law exponent for the normal stress differences is about $0.65 \pm 0.1$ and seems to be independent from concentration in the range studied as seen in Figure 3.9. These results are in line with theoretical results on semi-flexible and rigid polymer solutions [73, 121, 122].

Figure 3.8: (a) Normal force of an aqueous 0.25 wt. % xanthan solution after baseline correction. (b) Normal force due to normal-stress differences up to Reynolds numbers of 20 for the data from Figure 3.5 (a). (c) Shows the normal-stress differences obtained from the data in (b). Here, the slope correction was obtained by using a third-order polynomial fit to the logarithm of the data. The straight line shows a power-law fit to the data at the height shear rates. Temperature: (297.86±0.1) K.
Figure 3.9: Power-low exponent of the normal-stress differences in xanthan solutions at shear rates beyond $10^4 \text{s}^{-1}$. Temperature: (297.86±0.1) K.
3.3.3 Birefringence measurements

The birefringence of samples under shear is related to the deformation and orientation of its microstructure. The setup with transparent rheometer plates allows detecting the optical anisotropy of the sample in the shear flow with respect to the turning axis. In the present study, it is shown that the change in the microstructure can be observed by measuring the samples’ birefringence. The setup is similar to a polarization microscope [124]. A white-light is passed perpendicularly through the rheometer plates and to the sample and finally into a digital camera. On either side of the parallel-disk configuration, there is a polarizer, as sketched in Figure 3.10. During rheological runs, both polarizers are oriented perpendicular to each other at an angle of 45° with respect to the local flow direction. Optically isotropic samples do not change the light polarization and hence, no light passes through the analyzer, i.e. the second polarizer. As the polymers are deformed in flow direction, they become anisotropic. Since they are oriented at an angle of 45° with respect to the incident light, they change the polarization of the light like a wave plate. The phase shift $\Delta \varphi$ between the waves polarized in flow direction and perpendicular to it depends on the wavelength of the light $\lambda$, the gap width $H$ and on the difference in the refractive index $\Delta n$ between the two directions:

$$\Delta \varphi = \frac{2\pi}{\lambda} H \cdot \Delta n$$  \hspace{1cm} (31)

The difference in the refractive index itself depends on the strength of the polymer deformation. At stronger polymer deformation, the light component polarized in the direction of the analyzer, and hence the light intensity detected with the camera, increases.
With the polarization of the analyzer perpendicular to the first polarizer, the phase shift $\Delta \phi$ results in a light intensity $I = I_o \cdot \sin \Delta \phi$ detected by the camera, where $I_o$ is the maximum intensity. This yields for the difference in the refractive index $\Delta n$:

$$\Delta n = \frac{\lambda}{2\pi H} \cdot \arcsin \frac{I}{I_o}$$  \(32\)

$I_o$ can be determined from turning the analyzer by 90°, i.e. to the same polarization as the first polarizer, measuring the light intensity with the rheometer at rest. As a proof-of-principle, Figure 3.11 shows the viscosity and the light intensity as a function of the shear rate at a gap width of 1000 μm and 20 μm. At low shear rates, there is a plateau where apparently polymers are not deformed. Beyond a shear rate of about 0.1 s$^{-1}$, the intensity increases. Although the scatter is quite significant using a white-light source, Figure 3.11 shows that birefringence measurements with polymer solutions are possible in 20 μm thin layers. At low shear rates up to about 3 s$^{-1}$, the data obtained at different gap widths coincides well within the remaining uncertainty of the gap width, even at Reynolds numbers beyond 20 only slight deviations occur. They are apparently due to wall slip at the present high viscosities similar to those observed.

At the first Newtonian branch, the hydrodynamic forces are too weak to deform the polymer microstructure, therefore the flow birefringence does not show any response. On the other hand, in the shear-thinning regime up to shear rates of about $10^3$ s$^{-1}$ the polymers are increasingly deformed and lead to a stronger birefringence. At the second Newtonian branch, the birefringence does not
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seem to change within its range of uncertainty. However, it seems desirable to minimize the measurement uncertainty, which may be obtained by using a laser instead of a white light source.

![Graph](image)

**Figure 3.11**: Viscosity function (closed symbols) and intensity of white-light source (opened symbols) of an aqueous 0.25 wt. % xanthan solution. The birefringence signal at 1000 µm (squares) and 20 µm (circles) gap width. The light was detected in a small area at a distance between 20-22 mm from the rheometer’s turning axis. The error bars indicate the uncertainty of image light intensities.

To conclude, several findings for the xanthan polymer solutions in this study are not covered by the former studies. First, the infinity-shear viscosity was measured at high shear rates up to $10^6$ s$^{-1}$. This regime cannot be reached with commercial rotational rheometers due to their zero-gap errors. In addition, the infinity-shear viscosity, which is measured in the second Newtonian branch for both semi-dilute disentangled and semi-dilute entangled polymer solutions is concentration depending and represented by power-law with exponents in very well agreement with scaling laws known from zero-shear rate viscosity studies. Furthermore, in the entangled concentration range the infinity-shear viscosity shows a dependence on concentration and this agree with polyelectrolyte scaling theory of rigid and flexible polyelectrolyte polymer solutions. The semi-dilute entanglement $c_e$ concentration in this study is moved to 2000 ppm compared with its onset in former studies that is 400 ppm. Next, in the second Newtonian branch the normal-stress differences were measured at high shear as well and the data were fitted to the power-law rather than the previous studies. Finally, the birefringence of the samples under shear is related to the deformation and orientation of its microstructure and the deformation of the xanthan polymers show slightly variation in the second Newtonian branch at lower gap thickness.
4 Viscoelasticity and adhesion limits of biological cells

This chapter focuses on the mechanical behaviour of cells. Different harmonic oscillation tests are implemented to identify the linear viscoelastic regime of fibroblast cells in a monolayer as an example. Finally, cell adhesion properties are studied and are compared with previous work.

4.1 Introduction and background

Rheological properties of biological cells have vital functional implications such as mechanical stability, adjustment to environmental load, migration, proliferation, phagocytosis, contraction and are important, for instance, for health care products and vaccines [125]. The mechanical properties of the cells are closely related to their physiological activities. Cancer, for example, changes the mechanical cell properties and Red Blood Corpuscles cannot transport oxygen through narrow capillaries unless they change their shape [126]. In eukaryotic cells, the cytoskeleton, which is a complex, contractile network of protein filaments covering the entire cell body, is responsible for controlling the mechanical properties, cell shape, locomotion, and division [127]. The cytoskeleton itself is a complex gel and it comprises of three components: microfilaments, intermediate filaments, and microtubules [128, 129]. It has the ability to support large elastic stresses. At the same time it is able to generate internal stresses.

Given the importance of cells, the need to develop an understanding of cells and the countless applications in medicine and biotechnology, rheological studies on cells have taken a firm standing. In the past decades, different techniques like optical and magnetic tweezers, atomic force microscopy, magnetic twisting cytometry, micropipettes, microplates, cell poking, and particle tracking micro-rheology have been employed to measure the viscoelastic behaviour of single cells (Figure 4.1).[127, 130, 131].
Comparing the numerous sequential experimental runs, it is desirable to obtain this data from a single measurement. In particular, it seems appealing to apply well-established techniques such as the commercial rheometers. In a former study, Fernández et al. [26] showed the ability to measure rheological properties with a rotational rheometer. They carried out amplitude and frequency sweeps with a monolayer of a large number of fibroblast cells that were adhered between parallel disks. However, since they did not determine the number of cells, they did not obtain quantitative data from their measurements for typical or average cell properties.

A non-reversible deformation of biological cells where the rheological properties are deviated noticeably from the linear viscoelastic regime (LVE) is known as non-linear viscoelastic behaviour [26, 40, 132]. The non-linear viscoelastic regime has been extensively studied by several groups. In single cell study, Fernández et al [132-134] established that the cell response in frequency experiments shows power law creep function in nonlinear regime via using microplate rheometer. Kollmannsberger et al. [125, 135] recorded creep response of single cell by apply stepwise increasing forces to magnetic beads via magnetic tweezers. They found that the cells can adapt to a wide range
of mechanical conditions by controlling the amount of stress stiffening and fluidization in response to large external forces. For a monolayer, Fernández et al. [26] studied the response to stepwise loading by creep experiments in the nonlinear regimes. They found that initially nonlinear responses become linear over time. In the current study, a higher strain amplitude range is applied in oscillatory mode to reach the non-linear viscoelastic regime. Furthermore, changing the gap width allows studying the rheological behaviour of the cells under pre-set stress conditions.

The properties of cell-substrate adhesion can strongly affect the cell mechanical properties. The cell deformation and stiffness depends on cell adhesion to the substrate and can be affected by the surrounding cytoskeleton [136]. Therefore, measuring cell adhesion has become critical and important for biological applications [137]. For example, in chromatographic separation of blood components some blood cells are retained temporarily or permanently when they passed through a packed column of beads or fibers [138, 139]. Several techniques have been used to measure the cell adhesion. They can be classified as cell-to-cell and cell-to-substrate adhesion. The most common method is averaging adhesion strength of a cell network to a substrate by define shear stress level [140]. This measures the shear force at which cells detach from the surface [141]. A simply rheometry technique is developed to measure the cell adhesion strength of cells attached to synthetic polymer surfaces [137]. In this method, the cell-seeded polymers were exposed to a constant shear stress and the cells detected optically in the radial position where the stress varies. The effects of externally unidirectional shear stress have been used to determine the cell adhesion strengths for cells in fibrillary extracellular matrix (ECM). The cell adhesion has been probed via unidirectional shear stress increased successively up to the load limit, where cell detachment took place [142, 143]. Quantifying critical limits is particular important for many industrial processes, which process windows are restricted by cell damage or detachment from the substrate.
The single-cell studies have been carried out at frequencies up to about $10^4$ Hz. In a wide frequency range, they show a power-law rheology in the linear viscoelastic regime like soft glassy materials. The exponents are typically between 0.1 and 0.5. They are smaller for stiffer cells [127]. At frequencies below 0.1 Hz a second branch with higher exponent may occur [131, 144]. Depending for instance on the life cycle, shape, structure and level of proteins [145], cells show large cell-to-cell variations. The moduli may vary by orders of magnitude [134, 146]. Therefore, it is of great importance to determine typical or average values for the cell behaviour. Cai et al. [146] used for their study a microarray with single cells and measured the cell response in an atomic force microscope to determine mean properties from samples of up to about 200 cells. To average over a million cells, Fernández et al. [26] studied cell monolayers in a rotational rheometer in harmonic oscillation experiments and step shear or step stress experiments. They, however, did not reach the linear viscoelastic limit but studied the nonlinear viscoelastic regime. Furthermore, they did not quantify the number of cells in their monolayers and, consequently, were not able to obtain quantitative data for the moduli.

Determining average rheological properties of biological cells is still a complicated issue. The principle problem in measuring the mechanical properties of the cells is the requirement of high precision and sensitive devices to detect the cell responses to the different strains. To detect their properties, for instance, in a parallel-disk rheometer, the biological cells must cover a total area of order of $20 \text{ cm}^2$ to let the rheometer resolve forces [134]. Another issue is the need to coat both plates with thin-film adhesion protein that make the cell monolayer adhered on. The modified setup, which was introduced in chapter 2 has overcome these difficulties. A well-controlled parallelism of both plates at a given position and both plates aligned perpendicularly to the rotating axis met the requirements of detecting the cell coverage in the monolayer, and as a result, in this study, the average cell moduli have been determined and explored in the linear viscoelastic regime. Moreover, the impact of biochemical treatments on the cells has been quantified that would otherwise be difficult to determine on the background of strong cell-to-cell variations. With this modification, this method may also be used as a diagnostic tool to identify variations in the rheological cell behaviour, for instance, due to diseases. Furthermore, changing the gap width allows studying the rheological behaviour of the cells under pre-set stress conditions.
In the present work, the limitations of the former monolayer study have been overcome. Additionally, the linear viscoelastic regime has been reached and the average moduli has been determined by detecting the cell concentration in the gap optically. This results in determining average cell properties with less than a factor of 2.

As an example for the applicability of the narrow-gap rheometer to cell rheology, 3T6 Fibroblast cells are studied. Fibroblast cells are part of the connective fibrous tissue and maintain its structural integrity by continuously secreting precursors of the extracellular matrix [145]. Fibroblasts may have various shapes and sizes. Adhering to surfaces, fibroblasts are flat and elongated. In medicine, fibroblasts are of utmost importance. They are needed in the healing process as an injection for patient’s own fibroblasts to the site of the injury [147]. Biochemical cell modifications due to aging, diseases or drugs can result in changes of the mechanical cell properties and thus can be detected by rheometry [148-150].
4.2 Materials and methods

This section describes the cells and chemicals used in experimental in detail. The modified rotational rheometer with parallel-disk configuration with its associated specification is given in elsewhere (see Chapter 2).

4.2.1 Cell culture

Swiss 3T6 fibroblasts cells from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany are used in cell monolayer study (Figure 4.2) and Human Embryonic Kidney 293 (Hek293) from ATCC are used in the cell adhesion studies. Their biological properties are described by Todaro and Green [151]. The same culture protocol was used for both cell experiments. After thawing the cells from cryo-storage, the fibroblasts were grown in an incubator at a temperature of 310.16 K for at least one week and no longer than one month according the following standard protocol [152]: As culture medium, Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) is used. The medium contains 25 mM HEPES and 0.5 mM NaHCO3. The medium has a pH value of 7.4 and was prepared at a temperature of 298.16 K.

![Figure 4.2: (a) Standard transillumination microscopy and fluorescence imaging in the rheometer (b). Cell coverage: 50% (a) and 42% (b).](image)
4.2.2 Cell preparation

The cells are prepared for the rheological experiments in same procedure as in former studies [26] using the method described by Freshney [152]. The procedure shown below:

1. The culture medium is removed from the culture flask with a pipette as a first step for cell detachment from inner surface of the culture flask.
2. The flask is rinsed with 5 ml of phosphate buffered solution from Sigma-Aldrich (PBS) to break the ionic bonds between the cells and inner surface of the culture flask.
3. After gently shaking the flask, the PBS is removed and discarded.
4. A Trypsinization process is necessary with 5 ml trypsin enzyme (Accutase solution from Sigma-Aldrich), which is added to the culture flask. The flask is incubated at room temperature for 30 minutes to let the cells detached from its inner surface.
5. The cells are checked under an inverted microscope to determine if at least 90% of the cells were detached and floated, which is identified by their round shape. If cells are not detached after 30 minutes, they are incubated for another 1-2 minutes.
6. As soon as cells are floating, 10 ml fresh culture medium is added to the culturing flask to inactivate the trypsin.
7. The sidewalls of the flask are rinsed down several times with new culture medium to wash all cells off the plastic wall and into the solution.
8. The liquid that contained the cells is removed and transferred into a 50 ml conical tube. It was centrifuged in the tube for 8 minutes at 1000 rpm, which corresponds to 180 g to separate the cells pellets from the culture suspension.
9. The supernatant is discarded and the cells are counted under the microscope using a hemocytometer to estimate the percent of the cells that is needed in each individual experiment.

4.2.3 Coating the glass plates

The adhesive protein solution preparation follows the protocols mentioned in previous work [26]. The rheometer glass plates are coated with a fibronectin solution to adhere the cells to the plates. The solution consisted of 30 µg·ml⁻¹ fibronectin from Sigma-Aldrich, an adhesion promoting protein, in phosphate buffer solution (PBS). It was left between the glass plates for one hour at a gap width of 50 µm. Then, the plates were rinsed three times with PBS. Therefore, the upper plate is lifted up and down and the solution is sucked into a pipette.
4.2.4 Introducing cells between the glass plates
The cells are introduced between the parallel-disks based on the protocol from previous works [26].
The procedure is listed below:

1. The suspension of fluorescence labeled cells (see section 4.2.6) is introduced between the
glass plates via sucking it with capillary forces.
2. The suspension is left on the lower glass plate of the rheometer for 20 minutes to let the cells
settle down and adhere to the plate.
3. Afterward, the upper glass plate of the rheometer is lowered at minimum speed of the
rheometer to confine the cells at a gap width of 5 µm.
4. The cells are left in this position for one hour to promote perfect adhesion between both plates.
5. The images are taken to determine the cell coverage in the gap. The cells are now ready for
the rheological measurements.

4.2.5 Microscopy cell detections
To quantify the cell coverage in the gap, the cells are detected from underneath with a camera
equipped with a 5x objective lens to cover a wide area of cells. Since deformation is strongest at
maximum distance from the rheometer’s turning axis, the rheometer signal is mainly due to the cells
at the outer rim, as also shown by Fernández et al. [26]. 40 fluorescent field images and occasionally
gray-scale pictures with area of 0.775 mm² are taken around the edge of the upper plate. As light
source, light-emitting diodes, VLP IntraLED 2020/W (Volpi) is used connected to a light guide
(Lumatec 3716) for transillumination images and LUXEON Rebel (Lumitronix) for fluorescence
images. The latter was mounted to a filter block (C-FL Epi-FL FITC, EX 465-495, DM 505, BA 515-
555, Olympus), which was incorporated in the lens tube. The pictures were grabbed with a CCD
camera with a primary resolution of 1280×960 pixel (1.3 MP Chameleon, 1/3” CCD, Point Grey
Research). Images of fluorescent cells were segmented and quantitatively analyzed using a modified
version of DetecTIFF® software.[153] For further details about the method see Appendix B.
4.2.6 Chemicals

To determine the cell coverage in the rheometer gap optically, 0.2 vol. % of the fluorescent agent calcein Am (Sigma Aldrich) is added to the cells. Calcein Am is an indicator to quantify living cells that passes through the cytoplasmic membrane. It has an excitation maximum at 485 nm. Its fluorescent signal was detected at wavelengths of about 530 nm. Thereafter, the cells are incubated at a temperature of 310.16 K for about one hour to let the material go through the cell membrane.

Apart from experiments with untreated cells, the impact of drugs that affect the cytoskeleton structure of the cells is studied. As biochemical drugs, the pure cells are treated with 1 vol. % ethanol with a purity of 99.8% from Roth, 0.1 vol. % glutaraldehyde and 150 micromolar of blebbistatin from Sigma-Aldrich. Ethanol depolymerizes the actin filaments that are one of the building blocks of the cytoskeleton and hence responsible for the mechanical cell properties [148]. Glutaraldehyde is a fixation agent that arrests the dynamic response of the cells and makes them purely passive [149]. Blebbistatin inhibits myosin motors that can generate contraction in the actin network [154]. To allow good interaction of these drugs with the cells, they are mixed into the culture medium just before leaving the cells between the glass plates.

4.2.7 Error uncertainty

The error in measurement uncertainty of the instruments and apparatus that have been used during the study period can be shown below:

- Digital thermometer measurement ± 0.1 K
- Precision glass pipettes max. ± 0.02-0.08%
- Micropipettes ± 0.01-0.02%
4.3 Experimental results and discussion

4.3.1 Cell monolayer rheology

4.3.1.1 Linear viscoelastic regime

The amplitude sweeps are carried out at controlled strain in the range between 0.1% and 10%. The gap width was fixed at 5 µm and the frequency at 1 Hz. Typical amplitude sweeps are shown in Figure 3.4. The cells in this test covered 37% of the outer area between the plates at 5 µm gap width. The data is typical for viscoelastic solids. From the diagram, the linear viscoelastic regime ranges up to strain amplitudes of about 1%.

![Figure 4.3: Amplitude sweep at a gap width of 5 µm (a) and 10 µm (b) at a frequency of 1 Hz. Storage modulus and loss modulus are indicated by closed and open symbols, respectively.](image)

While Fernández et al. [26] focused on the nonlinear viscoelastic regime at amplitudes of 2% and more, Figure 4.3 shows that it is possible to study the linear viscoelastic regime with the monolayer configuration. Figure 4.3 reveals that the linear viscoelastic regime of the fibroblast cells extends to amplitudes of less than 1%, a result that favourably compares with data on the stiffness of single fibroblasts in oscillatory microplate experiments [133].

Figure 4.3 (a), however, also displays strong scatter for the loss modulus at low strain amplitudes making it difficult to obtain precise data for this quantity in the linear viscoelastic regime. Therefore, in amplitude sweeps the loss modulus in the linear viscoelastic regime is determined from its valley in the strain-amplitude range between 0.3% and 1%. The scatter is apparently mainly due to the torque limit of the used rheometer, which is commonly taken to be considerably larger than the value
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provided by manufacturers [42, 43]. This explains why the relative scatter in the loss modulus is much larger than that in the storage modulus. At strain amplitudes beyond 1%, the loss modulus increases considerably at a gap width of 5 µm while this happens at a gap width of 10 µm only beyond 10%, as shows Figure 4.3. This may point to the fact that the cells are compressed in the narrow gap and the nucleus may interfere resulting in larger friction.

4.3.1.2 Cell aging test

It is pointed out that the cytoskeleton’s mechanical properties may be affected by aging [149, 150]. Measuring the long-time changes in the cell mechanical properties namely, due to aging, the dynamic moduli are detected for about 10 hours, as shown in Figure 4.4. During the test, the cells are exposed to constant deformation amplitude of 0.2% at a constant frequency of 1 Hz. As Figure 4.4 shows, the storage modulus increases by about a factor of 1.5 during the test, while the loss modulus slightly increases by about a factor of 1.15.

The entire measurement time for the amplitude and frequency sweeps shown in section 4.3.1.3 is about 30 min. Only the study on the impact of the gap width took about 4.5 hours. Yet, as shown in Figure 4.4 the rheological properties of the cells hardly change during the time interval of the sweeps. The storage modulus increases slightly after the first 200 min and remains rather constant for the rest of the 10 hours lasting experimental run while the loss modulus remains rather constant during the entire experiment. We expect that rather large recording times can be realized due to the fact that the cells are still within their medium, of which the contribution to the rheological measurements is negligible.

Figure 4.4: Aging test for the fibroblast cells with constant strain amplitude of 0.2% and constant frequency of 1 Hz at a gap width of 5 µm. Storage modulus and loss modulus are indicated by closed and open symbols, respectively.
4.3.1.3 Average rheological cell properties

The Figure 4.6 (a) shows the storage and loss moduli of the cell monolayers in the linear viscoelastic regime for different cell coverage up to about 50% of the area covered by the cells. At higher cell concentrations, cell agglomeration becomes significant making it difficult to obtain a monolayer. The error bars in the moduli indicate the standard deviation for the linear viscoelastic regime. For the loss modulus, this was determined only for the scatter around the minimum value, see Figure 4.3, since the data at smaller strain is below that corresponding to 20 times the minimum torque provided by the manufacturer, which has been found to be a realistic lower limit as discussed by Oliveira et al. and Soulages et al. [42, 43]. The error bars in the cell coverage show the standard deviation between the 40 locations around the outer part of the gap. The moduli of the cell monolayer depend strongly on the cell coverage. Figure 4.6 (a) shows that the storage modulus is roughly proportional to the cell concentration within the rheometer gap. The moduli of the medium are much smaller than those of the cell monolayers. The storage modulus of the medium is about 21 Pa and the loss modulus is about 3 Pa (see Figure 4.5). Hence, the contribution of the medium to the moduli of the monolayer is negligible.

Figure 4.5: Amplitude sweep of cell culture medium at a gap width of 5 µm at a frequency of 1 Hz. Storage modulus and loss modulus are indicated by closed and open symbols, respectively.
Then, from the measured storage modulus, $G'$, and loss modulus, $G''$, and the measured mean cell concentration in the outer part of the gap, $c$, the average moduli of the cells, $\langle G'_\text{cell} \rangle$ and $\langle G''_\text{cell} \rangle$, can be determined:

$$\langle G'_\text{cell} \rangle = \frac{G'}{c}$$

Figure 4.6: Storage modulus and loss modulus of the fibroblast monolayers in the linear viscoelastic regime obtained from amplitude sweeps (a) and average moduli per cell (b) as a function of the cell coverage. Storage modulus and loss modulus are indicated by closed and open symbols, respectively. The dashed lines in (b) indicate the mean values of the data points for the respective moduli. Gap width: 5 µm; frequency: 1 Hz.
Figure 4.6 (b) shows the average storage modulus of the cells obtained in this way. The dashed lines indicate the mean values of the data points for the respective moduli. Within measurement uncertainty, the average moduli per cell fall within a narrow interval. Specifically, the data for the storage modulus per cell scatter around a mean value of about 17000 Pa with standard deviation of about 19%.

As shown in Figure 4.6, it is indeed possible to obtain representative or average storage and loss moduli for the cells for a large range of the cell coverage between a few percent up to about 50%. The main restrictions seem to be rheometer torque resolution at low strain amplitude and multilayers at high cell coverage. Within the studied range, the moduli per cell are independent of cell coverage. The storage modulus per cell varies by less than a factor of 2 between the single measurements; see Figure 4.6 (b). The standard deviation for the cell storage-modulus is about 19%. This is much smaller than typical cell-to-cell variations [134, 146]. As is also apparent from Figure 4.6, the main uncertainty originates from variations in the cell distribution, see also Figure 4.2. Hence, it seems near at hand that improving cell distribution may noticeably improve the precision.

4.3.1.4 Frequency sweeps test

Figure 4.7 shows a frequency sweep in the range between 0.1 Hz and 10 Hz at an amplitude of 0.2%. This is well within the linear viscoelastic regime see Figure 4.3. A power-law fit yields exponents of 0.13±0.01 for the storage modulus and 0.11±0.01 for the loss modulus. In the studied range, \( G' \) and \( G'' \) show a power-law dependence with an exponent typical for soft glassy materials [155, 156]. The value is in the range typically found for the linear viscoelastic range of the animal cells [127].
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Figure 4.7: Frequency sweep for the fibroblast cells in the linear viscoelastic range. Storage modulus and loss modulus are indicated by closed and open symbols, respectively. The lines are power-law fits to the data. Cell coverage: 44%; strain amplitude: 0.2%; gap width: 5 µm.

Many rheological studies focus on frequency-dependent cell properties [127]. In a wide frequency range, they usually find a power law for rheological quantities such as the storage or loss moduli that is typical for soft glassy materials. The frequency sweeps are measured in present study within the linear viscoelastic regime and cover a range of frequencies between 0.1 Hz and 10 Hz. The exponent of about 0.1 obtained at a gap width of 5 µm is at the lower end of the exponents obtained in previous single-cell studies [127].

4.3.1.5 Gap dependence, pre-stress

Depending on the gap width, the adhered cells may be compressed or elongated. The dependence of the cell moduli on pre-set compression or elongation is studied by changing the gap width after adhering the cells to both plates. The experiment started byzeroing the normal force before bringing the upper plate into contact with the cell suspension. This allows quantifying the pre-stress exerted on the cells in the monolayer at different gap widths. Thereafter, the cells are confined at a gap width of 5 µm. At this gap width, the amplitude sweep is carried out at a frequency of 1 Hz and at strain amplitudes between 0.1% and 5% to determine the linear viscoelastic range. Thereafter, frequency sweeps between 0.1 Hz and 10 Hz are carried out well within the linear viscoelastic range at a strain amplitude of 0.2% for the different gap widths that are increased stepwise up to 16 µm. At the end of the experiment, an amplitude sweep and a frequency sweep are carried out again at a width of 5 µm to quantify changes during the experimental run. The entire experimental run took about 4.5 hours.
Figure 4.8 (a) shows the normal force, \( F_N \), on the plates as a function of gap width. It is positive at the gap width of 5 µm and negative at larger gaps indicating cell compression at the narrowest gap and elongation at larger gaps. We note that incremental changes in the normal force are large at small deformations and decrease at larger deformations. With the detected cell coverage, the normal stress per cell, \( \langle \sigma_{\text{cell}} \rangle \), can be determined analogously to the dynamic moduli:

\[
\langle \sigma_{\text{cell}} \rangle = -\frac{F_N}{cA}
\]

where \( A \) is the surface area of the upper plate and the negative sign indicates that the normal stress acts in the opposite direction of the compression. Hence, the normal stress per cell ranges from about -1 kPa at 5 µm gap width to about 7 kPa at 16 µm gap width.

Figure 4.8: Gap-width dependency of the normal force (a) and of the dynamic moduli (b) in the linear viscoelastic range. The experiment was carried out by stepwise increasing the gap width from 5 µm to larger gap widths. In (b), the closed and open symbols show the exponent of storage and loss moduli, respectively. The triangles show the data measured at the end of the experiment at 5 µm gap width. Cell coverage: 36%; strain amplitude: 0.2%; frequency: 1 Hz.
As shows Figure 4.8 (b), the pre-set strain has a strong impact on the dynamic moduli. The dynamic moduli strongly decrease with increasing gap width. At a gap width of 16 µm, the storage modulus is about a factor of 50 smaller than at 5 µm. Returning from the maximum gap width to the minimum by the end of the experiment, the initial high storage modulus is almost recovered: The decrease by about 18% is supposedly to be due to detachment of a corresponding amount of cells during the elongation phase. In this case, the tensile stress at maximum gap width increases to about 8 kPa.

In line with the previous monolayer study in the nonlinear regime by Fernández et al. [26], the moduli weaken considerably with increasing gap width, see Figure 4.8 (b). It is apparent from Figure 4.8 (a) that the cells are compressed at a gap width of 5 µm and elongated at larger gap widths. Taking a gap width with zero normal force as reference, the maximum gap width studied corresponds to a stretch ratio of about 3. With the detected cell concentration, normal stresses ranging from -1 kPa to about 7-8 kPa are obtained. In the studied range, the normal force and hence, the normal stress show a nonlinear dependence on the gap width. The weaker increase of the absolute normal force at larger elongations cannot be explained by cell detachment as quantified from the data for the storage modulus and indicates softening of the cells at larger elongation. This behaviour was also reported for single cells during step-wise increase of elongation [157]. These authors also found for 3T3 fibroblasts at a temperature of 310 K normal stresses in the range of a few kPa.

Figure 4.9 (a) depicts some exemplary frequency sweeps for different gap widths. The larger the gap width the stronger the moduli increase with frequency. Figure 4.9 (b) shows the exponent of a power-law fit to the data. Both exponents increase with gap width where the exponent of the storage modulus is somewhat larger than that of the loss modulus. The storage and loss moduli in linear viscoelastic regime as well as their power-law exponent strongly depend on the gap width and thus on the pre-set stress created by elongating or compressing the cells with the rheometer plates as show Figure 4.8 and Figure 4.9. While the dynamic moduli become weaker the more the cells are elongated, the power-law exponent increases with the gap width as shown in Figure 4.9 (b). This means that the cells become more fluid-like the stronger the cells are elongated, a typical behaviour of cells under large stresses [127]. It is due to the slight compression of the cells at 5 µm gap width, see also Figure 4.8 (b). This might be due to considerable scatter for the loss modulus at the small strain amplitudes studied.
Figure 4.9: Frequency sweeps (a) and their power-law exponents (b) as a function of the gap width. The experiment was carried out by stepwise increasing the gap width from 5 µm to larger gap widths. Closed and open symbols show the data for the storage and the loss modulus, respectively. The triangles show the data measured at the end of the experiment at 5 µm gap width. Cell coverage: 36%; strain amplitude: 0.2%.
4.3.1.6 Impact of biochemical modifications

Biochemical treatment may affect the dynamic moduli and their frequency dependence. Figure 4.10 (a) shows the moduli per cell in the linear viscoelastic regime. Compared to untreated cells, the storage modulus is lowered by blebbistatin; glutaraldehyde and ethanol tend to enhance the storage modulus. While blebbistatin slightly alters the loss modulus within range of uncertainty, the other drugs yield an increase of the loss modulus. Note that for the strain amplitude the upper limit of the linear viscoelastic regime does not change by using the drugs except for blebbistatin, which results in a higher limit. Figure 4.10 (b) shows the power-law exponents obtained from the frequency sweeps. While treatments with glutaraldehyde and ethanol hardly alter the exponents within the range of uncertainty, the blebbistatin treatment yields much smaller exponents.

![Figure 4.10: Dynamic moduli per cell (a) and power-law exponent (b) for untreated fibroblast cells and cells treated with different drugs. The storage modulus, the loss modulus and their respective exponents are indicated by closed and open symbols, respectively. The data in (a) was obtained from amplitude sweeps. In (b) the error bars indicate the uncertainty of the fit. For the untreated cells, the data shows the mean moduli per cell averaged over all experiments of Figure 4.6. (b) For cells treated with blebbistatin, glutaraldehyde and ethanol, the cell coverage was 31%, 22% and 8%, respectively. Frequency in (a): 1 Hz; amplitude in (b): 0.2%; gap width: 5 μm.](image)

Our approach is suitable to quantify the impact of drug treatment on cell rheology (Figure 4.10). Since Fernández et al. [26] did not quantify the cell coverage in their monolayer study, they needed to apply the drugs to their cells once these were already adhered inside the gap to be able to determine quantitative differences between treated and untreated cells. To study pre-treated samples or to identify infected cells this is not useful. The present approach does not rely on this treatment but rather compares different cell samples allowing for different concentrations. Although it suffers from the additional uncertainty of detecting the cell coverage, it is able to distinguish the impact of the treatment well within measurement uncertainty: Ethanol is known to be toxic for animal
cells at higher concentrations [158] and dose-dependently inhibits ERK-induced JNK-signalling [159]. Glutaraldehyde works as a fixative crosslinking protein side chains thus terminating protein functionality, stopping all network activities but preserving the cytoskeleton [26, 149]. Cells previously treated with these drugs have larger moduli than untreated ones as shows Figure 4.10 (a). In particular, the loss modulus is strongly affected. The power-law exponents, on the other hand, hardly change, see Figure 4.10 (b). The monolayer study by Fernández et al. [26] also showed stiffening for cells treated with glutaraldehyde. This is also in line with single-cell studies [132, 134, 155]. On the contrary, inhibiting myosin-II motors with blebbistatin rather lowers the storage modulus compared with untreated cells. This is consistent with findings in single-cell studies [160-162]. Furthermore, it observes that the power-law exponents of the blebbistatin-treated cells are considerably lower than those of the untreated cells. In particular, the one for the loss modulus is considerably smaller than that for the storage modulus. This is similar to the behaviour of myoblasts treated with blebbistatin found by Balland et al. [160].

4.3.1.7 Oscillatory study at higher amplitudes

Cells and cytoskeletal biopolymers are shear-softening materials as also shown by former studies [26, 132, 133]. Their cytoskeletal networks consist of actin and the flexible cross-linker filamin A. Under sufficiently large external stresses they show nonlinear responses [135, 163]. Therefore the strain-controlled oscillatory study was extended into the nonlinear range up to amplitudes of the order of 100%. The gap width is fixed at 10 µm and the frequency at 1 Hz. A typical amplitude sweep is shown in Figure 4.11. As shows the diagram (a), the nonlinear viscoelastic regime starts at strain amplitudes of about 0.2%, where the storage modulus begins to deviate noticeably from the LVE plateau. The crossover point where the dynamic moduli are equal $G'=G''$ is called flow point [40]. Beyond this point, the material flows. In the cell monolayer, the flow point may be reached due to cell rupture or due to the lack of adhesion of the cells to the disks. According to Figure 4.11 (a), the flow point is between the strain amplitude of 30% and 70%. Figure 4.11 (b) shows the same data as strain amplitude vs. shear stress. In this representation one can identify two nonlinear viscoelastic regimes that follow a power law. The first one occurs at a strain rate up to about 30%, has an exponent of $1.3\pm0.01$, which shows a shear softening response. The exponent decreases with increasing cell stiffness and increasing contractile signal [125, 144, 164, 165]. The other one between strain rates of 5% and 50%. Here the exponent is $3.6\pm0.01$. We hypothesize that some cells already detach from one of the plate surfaces meanwhile other big cell clusters still adhere to both surfaces. As reported in the former studies for single cell [133] and cell monolayers [26], the decrease
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of the cell dynamic moduli in nonlinear viscoelastic regime are similar as shown in Figure 4.11 (a) due to the fact that the cells are soft-solid matter and their cytoskeleton has strongly nonlinear response [166]. As shown in Figure 4.11 (b), at high shear rate the stiffening force becomes a power-law with an exponent of 1.3 that is in line with what was reported by Gardel et al. [167] who indicated a power-law exponent of 3:2 in their study.

![Figure 4.11: Amplitude sweep at a gap width of 10 µm and at a frequency of 1 Hz. (a) Storage modulus and loss modulus are indicated by closed and open symbols, respectively. (b) Strain amplitude vs. shear stress for the same data in (a).](image)

4.3.2 Unidirectional strain controlled and cell detachment experiments

To figure out whether the flow point is reached due to lack of adhesion or due to a break-up of the cells, unidirectional strain-controlled experiments were carried out, yet in this case it is easier to distinguish between the two cases optically. Again, the strain-control is used, yet it results in a maximum shear stress at the yield stress. Figure 4.12 shows again two different nonlinear regimes like in Figure 4.11 (b) for oscillatory the test. At a strain up to about 5%, the cells show a power-law response with an exponent of 1.3. The other power-law regime in Figure 4.12 has an exponent of 4.6 at strains up to about 120%. At a strain of about 200% the maximum shear stress is reached. This value is close to the flow point in the oscillatory study, see Figure 4.11.

The images depicted in Figure 4.12 show pictures of cells close to the disk radius. The arrows indicate the corresponding data points in the diagram. Image (a) and (b) show the cells adhere to the surface and did not detach. Image (c) was taken at the second power-law regime. It shows slightly deformed cells that still adhere to the two plate surfaces. Yet, at slightly larger strain all cells were detached in
image (d) and the shear stress start to decrease. At the yield stress, it is clear that most of the cells detached from one of the plates and some move with the upper plate.

![Graph showing shear stress vs. strain percentage]

*Figure 4.12: Unidirectional strain-controlled measurement with 3T6 fibroblast cells at 10 µm gaps. The figure shows two nonlinear viscoelastic regime follows the power-law with exponent of 1.3 and 4.6, respectively. The images are taken corresponding to different strain regimes. Image (a) is corresponding to 0.2% strain amplitude, image (b) is corresponding to 10% strain amplitude and image (c) is corresponding to 185% (d) is corresponding to 200%, respectively.*

The experiment was repeated with another cell line to compare the critical strain limits. Hek293 monolayer cells have about the same shape and size as 3T6 fibroblasts. They were adhered between the parallel-disks at a gap width of 10 µm using the same procedure as for fibroblasts. Figure 4.13 shows the measurement with Hek293 cells in comparison with the fibroblasts in unidirectional strain-controlled experiment. Like the fibroblasts, the Hek293 cells show two nonlinear regimes. The exponents are quiet similar in Figure 4.13, 1.3 and 4.7, respectively. They detached already at strain amplitude of 70%. Despite the Hek293 cells show two clearly nonlinear regimes at strain rate up to about 4% and 50%, respectively and power-law exponents in these regimes close to fibroblast cells, they started to detach at strains below the detachment strain of fibroblasts.
Figure 4.13: Unidirectional strain control of 3T6 Fibroblast cells and Hek293 cells until cell detachment taken place at 10 µm gaps.

As pointed out by Pullarkat et al. [168], the different cytoskeleton elements play a role in untreated fibroblast cells, which can undergo deformations of about 100% without showing damage and significant stiffening. Therefore, to study the impact of the cell deformability of actin filament cytoskeleton and microtubules inhibitors, the fibroblast cells were treated with 1 vol. % ethanol, and 150 micromolar blebbistatin. Figure 4.14 shows the unidirectional experiments. To compare the strain limits of untreated cells and treated one, the actin depolymerization cells treated with ethanol, show detachment at a strain of 135% while microtubules inhibited cells with blebbistatin shows detachment at a strain of 340%. Due to the fact that the ethanol is a fixation agent and all dynamical processes of actin cytoskeleton activates are arrested by exposing the cells [149], the cells become stiffer than untreated cells. As shows Figure 4.10 (a), we hypothesize that the lower deformation of the stiffer cells causes the easily detachment at lower strain. In addition, they show only one nonlinear regime with power-law exponent of 1.4 and the cells are detached at 135% strain in contrast with untreated cells. On the other hand, blebbistatin treated cells show two nonlinear responses with power-law exponents of about 1.2 and 10, respectively. Inhibiting myosin-II motors with blebbistatin rather increase the critical strain to about 340%. Furthermore, one observes that the power-law exponent of the blebbistatin-treated cells is considerably higher in the second nonlinear regime than that of the untreated cells. In addition, single cell studies by Fernández [134] did not show the strong effect of blebbistatin.
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As shown in Figures 4.12 - 4.14, it is distinctly that in the first nonlinear viscoelastic regime the power-law exponent is of about 1.3±0.1 regardless untreated or treated cell like semi-flexible biopolymers [169], therefore, biological cells shows a strongly nonlinear responses of the cytoskeletal actin network with increasing deformation limits [127, 170].

Figure 4.14: Unidirectional shear strain controlled of 3T6 fibroblast cells shows normal cells, treated with blebbistatin, and ethanol at 10 µm gaps.
5 Conclusions

The main contribution of this study is to overcome the significant errors in the gap width of commercial rotational rheometers caused by squeeze flow of air during zero-gap error setting, misalignment of the parallel-disks and insufficient flatness of the plates while zeroing the device plates. To minimize zero-gap error, the rheometer has been modified and the gap width has been measured independently from the rheometer reading. A procedure has been developed to align the parallel-disk to each other within uncertainty of ±0.7 µm gap width. This means the zero-gap precision is improved by a factor of 30 and more that enables to extend the range of applicability of rotational rheometers to measure low viscosities, high shear rates and normal stresses with a parallel-disks configuration comparing with commercial rheometers. For oscillatory studies, the uncertainty can be further reduced to about ±0.2 µm. The contribution offers the advantage of using the full capacity and versatility of commercial rheometers and extends the measurement range to large shear rates and to lower viscosities.

Extending the measurement range to high shear rate up to about $10^5$ s$^{-1}$ and low viscosities less than 1 mPa·s, enables to access, for instance, the second Newtonian regime of polymer solutions. Experimentally, this has been done for xanthan aqueous solutions. The values for the infinity-shear viscosity of the second Newtonian branch are obtained and the data shows power-law behaviour according to Carreau-Yasuda and Cross models. Besides providing data on the infinity-shear viscosity, this modification provides normal-stress differences and rheo-optical data on the microstructure sample like birefringence at high shear rates for the polymer solution. Hence, with this setup it is possible to push forward considerably the knowledge about the rheological behaviour of polymer solutions.

Moreover, the high precision in parallelism between the two faces of the plates makes it possible to measure the mechanical properties of biological cells. As an example, 3T6 fibroblasts were studied. While cell-to-cell variation is typically very large, detecting the area covered by the cells in the rheometer gap enables to quantify the average moduli of the cells. The modification allows to determine average cell properties like their storage and loss moduli in the linear viscoelastic range and normal stress in monolayers. The cell concentration within the gap had to be detected optically.
The average cell properties could be detected in a large range of cell concentrations. The setup permits varying the pre-stress of the cells by changing the gap width. Depending on the chosen gap width, the cells may be compressed or elongated. The dynamic moduli strongly increase with compression, while the power-law exponent of the frequency dependence decreases. Measuring the average cell properties and their subsequent changes under different environmental conditions could render this apparatus as a diagnostic tool in modern medicine. As a first proof of concept, the impact of biochemical modifications on the rheological properties of the cells is quantified.

Cell adhesion can strongly affect the cell mechanical properties. For instance, cell rupture or detachment due to high deformation or high shear stress can be affected by the surrounding cytoskeleton. It has been established that fibroblast cells are detached from the fibronectin layer, which coated both plates’ surfaces. They flow at a critical shear stress in which their dynamic moduli are equal. The crossover point is called the flow point. Comparing fibroblasts with Hek293 cells show that Hek293 needs less strain in unidirectional strain-controlled test to detach.
6 Bibliography


7 Appendixes

7.1 Appendix A: Confocal spectral interferometry (CSI)

The confocal spectral interferometry sensor (CSI) according to the operation manual of the manufacturer company STIL-S.A. is designed for dimensional metrology (3D digitalization) and in particular for measuring the microtopography of objects, the roughness of surfaces, or the thickness of thin transparent samples [55]. The principal parameters of the optical pen are confocal imaging and spectroscopic analysis of white light interferograms (SAWLI). Important parameters are the working distance and the numerical aperture (NA). Images in CSI are based on interferometry imaging and the chromatic coding of the optical axis.

In this setup an optical system images a point source on the surface of the object. The background light is collected by the same optical system, which images the light spot on a pinhole. The pinhole is placed in front of photodetector (spectrometer). It filters the light rays that can reach the spectrometer and for this reason it is also called filter. The confocal setup is characterized by an exceptional Signal-to-Noise ratio. SAWLI consists in spectral analysis of the interferogram generated by mixing two wave fronts in order to compute the difference in their respective optical paths. When measuring thickness, the two wave fronts are generated by reflections on the two faces of a transparent sample. When measuring distance (i.e. the microtopography of a sample), one wave front is generated by reflection on the surface of the observed sample, and the other one by reflection on a reference window. In the first case, the optical path difference is proportional to the sample thickness; in the latter case, it is proportional to the thickness of the air gap between the sample and the reference window. Figure 7.1 shows a CSI sensor operating in the “Distance” measuring mode, in other words, with a reference window. In this figure the window is placed on the sample.
The interferometric signal is a channeled spectrum. The sample thickness (or the air gap thickness in present study) is derived from the spectral phase of this signal, which is computed with a subnanometric resolution using an advanced algorithm. The characteristic properties of CSI optical pen (Figure 7.2) are listed below [55]:

- Having interferometric objective.
- An interchargeable collimator (MG lens). The MG lens determines the spot size on the sample surface.
- The optical pen does not require a specific calibration.

Figure 7.1: Principal of confocal spectral interferometry imaging where backscattered beam is focalized on the pinhole [55].

Figure 7.2: A modular optical pen with its fiber optics cable [55].
7.2 Appendix B: Image analysis routine for cell detection

The DetecTiff® source code that were used image acquisition for cell detection was written in LabView® 7.0 (National Instruments, Austin, TX) with the optional Vision Development Module in the Microsoft Windows XP Professional platform. LabView® is a graphical programming language with applications called virtual instruments (VIs) that are composed of a front panel, the user interface (Figure 7.3), and a hidden block diagram that represents the program structure and may run within the LabView® development environment or as a stand-alone application. DetecTiff® is a software package consisting of 108 sub-VIs (components in the diagram of a higher level VI: 75 are for image processing, 12 for quantification data handling and report generation, 8 for error handling, and 13 for other applications). DetecTiff®-VIs are organized in 11 hierarchical levels. The methodology and structure-recognition principle presented here can be easily customized for other bioassays to address a variety of experimental applications [153].

![Figure 7.3: DetecTiff® user interface. [153.](image)]
The iterative procedure of DetecTiff® to detect objects has been described in [153]. The software allows different file formats as image source to deal with. There is an ability to select the region of interest within the image for analysis. For structure recognition, first the original image is filtered to remove speckle noise and then using a mask for subsequent quantitatively analysis from the original image. DetecTiff has an iterative procedure. The general steps followed in the procedure for structure recognition can be summarised as follows:

1. The acquired images are uploaded in DetecTiff in a 16-bit tiff format. Whole or every part (by manual selection) of the acquired images can be analysed.
2. These are filtered by a median filter to get rid of the background noise intensities. A copy of these filtered images is temporarily saved in an 8-bit tiff format.
3. Iterative cycles of intensity- (brightness threshold) and size- (area threshold) based structure recognition (IBSR and SBSR respectively) are performed to generate masks for subsequent quantitative analyses.
4. **IBSR:** Intensity threshold is predefined by the user. Therefore, by iterations, the areas in the image with intensity below the threshold value are neglected. This results in a black-and-white (BW) image (where B is background with intensity 0 and W represents object with intensity 255).
5. **SBSR:** Also has a predefined area range by the user. The BW image is proceeded via particle filtration. The objects falling out of this range are discarded.
6. Then the iteration cycles begin. Here the number of iteration cycles is also defined in the beginning by the user, in the user interface. This cycle has a lower intensity threshold than the previous cycle. The level of intensity can be set by the user in the form of *Intensity reduction factor*. Therefore, the darker structures, i.e., smaller or in the inner parts of the cell, are analysed now. These basically correspond to the cell organelles.
7. After each cycle, the resulting images are saved as BW-images. The whole procedure is automated, after the user defines the required parameters.
8. Lastly, segmentation of the images is performed. Here, the detected objects are dilated, i.e., the shape of the object is extrapolated based on the original image.
9. Hence, final template is obtained from which quantitative information can be extracted.