Biophysics practical course (R3a)
for master students

Viscoelasticity of semi-flexible polymer networks

Introduction

Any given substance has characteristic material properties. While their macroscopic properties are often known and easy to investigate, little is known on the corresponding microscopic (local) properties and how we can deduce a microscopic value from the macroscopic one. In this practical course, we want to investigate the macroscopic visco-elastic properties of a polymer network using rheology[1] and employ the obtained information to predict, if a particle of a given size is able to freely diffuse within the polymer network. Using time-lapse microscopy and particle tracking, we will perform a microscopic analysis of the particle motion within the polymer gel to verify if our predictions hold true. Furthermore, we want to answer the following questions: 1) Can I understand at which polymer concentration the particle is able to move? And 2) is the particle motion confined or do we observe free diffusion?

Complex shear modulus and complex viscosity

In order to investigate the macroscopic visco-elastic properties of a material one typically uses macro-rheology. Here, using a rotational rheometer with a plate-plate geometry one performs an oscillatory experiment e.g. the so called amplitude sweep. By applying an oscillatory shear strain γ with rising amplitude at a constant frequency and measuring the corresponding shear stress response σ, one can determine the complex shear modulus.

Let us consider an oscillatory strain:

$$\gamma(t) = \gamma_0 \, \sin(\omega t)$$

Then the resulting time dependent stress, will show the same frequency, but depending on the material, might exhibit a phase shift

$$\sigma(t) = \sigma_0 \, \sin(\omega t + \delta)$$

Decomposing the phase shifted oscillation into sin and cos terms, the stress is seen to contain a component which is in phase with the strain and another component which is in phase with the shear rate: $\dot{\gamma}(t) = \dot{\gamma}_0 \, \cos(\omega t)$.

By definition it follows

$$\sigma(t) = \sigma_0 \, (cos\,(\delta)\sin(\omega t) + \sin(\delta)\cos(\omega t)) = G \cdot \gamma(t) + \eta \cdot \dot{\gamma}(t)$$

This relation can be written as a complex stress-strain relation:

$$\sigma(t) = \gamma_0\big(G' \, \sin(\omega t) + G'' \, cos(\omega t)\big) = G^* \cdot \gamma(t)$$

where the shear modulus, G*, is now a complex quantity constituted by a real and imaginary part:

$$G^* = G' + iG'' = \frac{\sigma_0}{\gamma_0}\,(cos\,(\delta) + i\sin(\delta))$$

With G' the storage or elastic modulus and G'' the loss modulus. G' thereby corresponds to the elastic (solid) properties of a substance, while G'' corresponds to the viscous (water-like) properties of a substance.

Similar to the shear modulus, the viscosity has a complex form:

$$\eta^* = \eta' + i\eta''$$

With the viscous component η', which is the dynamic viscosity, and the elastic component η''.

The dynamic viscosity is thereby connected to G'' by: $\eta' = G''/\omega$

Strain-stiffening of semi-flexible polymers

Hydrogels derived from biopolymers often show strain-stiffening[2]. This means that the elastic modulus (G') increases under stress and the substance gets stiffer. This is an important feature in biology as e.g. cell migration depends on the visco-elastic properties of the biopolymer the eukaryotic cells are migrating on. Here, cell contraction itself can induce long-ranged stress stiffening in the underlying biopolymer[3]. Strain-stiffening of a semi-flexible polymer depends on the persistence length l_p, the contour length L_c and the topology of the connectivity (**Fig. 1**) of the polymer gel[4]. For semi-flexible polymers we find: $l_p \sim L_c$. In addition, the strain-stiffening of a network is correlated with a low connectivity $z < 6$, with z being the number or fibrils originating from one connection point. Besides the connectivity z, also the number of connection points is an important factor determining the mesh size ξ of a given polymer network. The mesh size, is thereby connected to the elastic modulus by: $G'(\omega) = \xi^{-3}\, k_B T$.

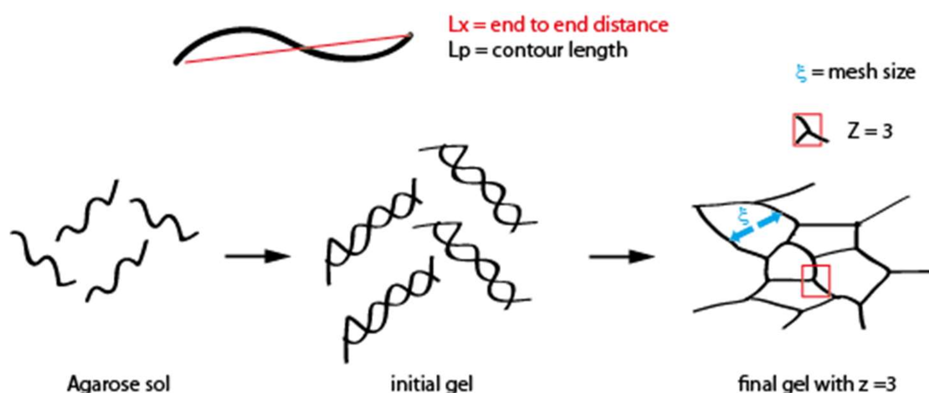

Fig. 1: Agarose as an example of a hydrogel with strain-stiffening properties. In solution, the agarose sugar chains are not connected with each other. Upon gelation first helices are built and then finally a polymer network is formed with z = 3.

To investigate and quantify if an observed strain-stiffening is strain or stress controlled one can calculate the differential modulus K = $\partial\sigma/\partial\gamma$ (**Fig. 2a**). Near the zero shear

stress, K is close to the zero stress storage modulus G_0 (**Fig. 2b**). By plotting K versus shear strain, one can also determine the critical stress, σ_c, which is the stress at which the stiffening sets in (**Fig. 2b**). Below the critical stress, we find the linear visco-elastic region (LVR).

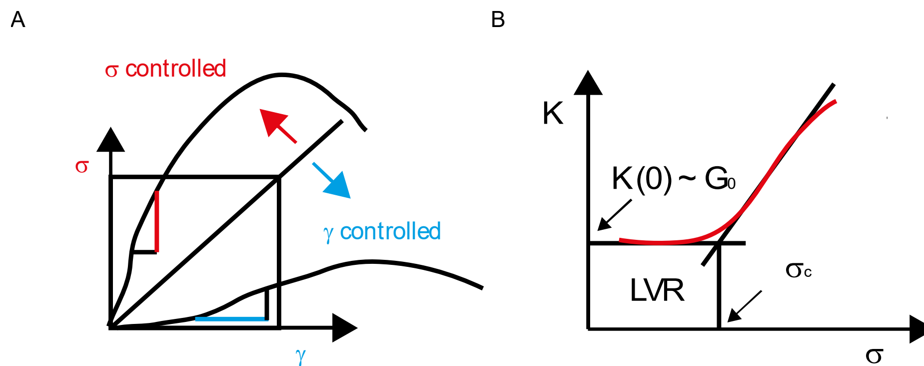

Fig. 2: Strain-stiffening can be stress or strain controlled (A) and can be analysed by the differential modulus K (B).

The polymer agarose

The polymer used in this practical course is agarose and serves as a model polymer that shows strain-stiffening as a hydrogel[4]. Agarose is a linear polysaccharide that consists of alternating D-galactose and 3.6-anhydro-L-galactopyranose units linked by α-(1-3) and ß-(1-4) glycosidic bonds. Agarose forms a gel once it has been heated and allowed to cool again. As a sol, the linear sugar chains are not connected with each other. During gelation first helices are formed and then in the final gel these helices are connected with each other with a low connectivity of z = 3 for various agarose concentrations. The mesh size of an agarose gel (**Fig. 1**) ranges from 0.01 -1µm [4] for a broad range of agarose concentrations. However, the number of connection points decreases with decreasing agarose concentrations, leading to a higher average mesh size. Consequently, while a 0.6 µm bead particle should be trapped within the polymer network at high agarose concentrations, one should observe free particle diffusion within an agarose network of low agarose concentration.

Mean square displacement (MSD)

Mean square displacement is a measure of the deviation of the position of a particle with respect to a reference position over time and can therefore be used to quantify the motion of a particle within a polymer network. The MSD = x^2 is given by:

$$x^2 = < \; < |x(t) - x_0\,(t)|^2 > = \frac{1}{N}\sum_{i=1}^{N}\left| x^i\,(t) - \; {x_0}^i(0)\right|^2$$

With X_0 being the position of the particle at time-point t_0. Clearly, freely diffusing particles have a higher MSD. Consequently, the MSD is connected to the diffusion coefficient D of a particle by: MSD(t) = 2Dt, with t being the time.

With $D = k_B T/f$ and $f = 6\pi\eta r$ one obtains the Stoke-Einstein relation:

$$D = \frac{k_B T}{6r\pi\eta}$$

With k_B the Boltzmann constant, T the absolute temperature, r the radius of the particle and η the dynamic viscosity of the medium.

Consequently, one derives an equation that describes the movement of a particle in a viscous medium in one dimension:

$$\text{MSD}(t) = \frac{k_B T}{3r\pi\eta} t$$

This constitutes also a direct relation between the MSD over time with the dynamic viscosity of the polymer network that one can determine by macro-rheology. In a polymer the viscosity increases with polymer concentration and hence particle displacement is reduced. This effect can be even more pronounced if the polymer is cross-linked.

Further recommended reading

- [1] You can find the basic theoretical principles of rheology described in the manual of the F-practical course for bachelors, where you also find information regarding the used instrument (https://www.softmatter.physik.uni-muenchen.de/teaching/fortgeschrittenenpraktikum/r3_rheologie/fpraktikumrheologiebenglish.pdf).
- [2] Storm *et al.,* Nonlinear elasticity in biological gels, Nature, 435,191-194, 2005
- [3] Han *et al.,* Cell contraction induces long-ranged stress stiffening in the extracellular matrix, PNAS, 115, 4075-4080, 2018.
- [4] Bertula *et al.,* Strain-Stiffening of Agarose Gels, ACS Macro Letters, 8, 670-675, 2019.

Your experiments:

In the morning from 9am to 12pm:

Rheometer:

You will use the rotational rheometer Kinexus Ultra+ for your experiments using a plate-plate geometry setting. Detailed instructions on how to use the instrument, you will find in the handout lying next to the instrument. Use upper geometry: CP1/60 SR4753SS and lower geometry: PLS61 S3629SS. You will perform a series of oscillating experiments.

Amplitude sweep

Prepare the following solutions: 0.1 / 0.05 / 0.03 / 0.025 /0.02 / 0.01 / 0 % agarose in water. Prepare each solution directly before you actually use it!

The agarose has to melt. Once dissolved, it has to be loaded to the rheometer at once, so that the agarose can form a gel on the rheometer. So the rheometer has to be ready for loading a new probe prior you melt each agarose concentration!

Use the microwave to melt the agarose until bubbles can be seen (power 90). Your adviser will instruct you on how to use the microwave. Be aware of boiling retardation -> hold glass in that way that the open side shows away from your eyes. Repeat this step at least twice to ensure that the agarose is completely dissolved. Than load the agarose solution to the rheometer.

Wait at least for 5 min before you start the program: Toolkit_O001 Amplitude Table (Strain control). T = 20°C, 10 steps per decade, strain: 0.1-100%, frequency 1Hz.

Save your data: final results, right mouse click, select all, right mouse click, export data. Save as csv files. This format can be read by any standard software including exel.

Data analysis

1) Plot G’ versus strain for all agarose concentrations. Indicate at which shear strain you observe strain stiffening.
2) Plot G’’ versus strain for all agarose concentrations.
3) Plot G’ for low strain versus agarose concentrations.
4) Plot shear stress versus shear strain. Is the shear-stiffening stress or strain controlled? Obtain the differential modulus K and plot K versus shear stress.
5) Normalize K and σ (K/K_{LVR} and σ/σ_{KVL}) and plot K/K_{LVR} versus $\sigma/\sigma_{KVL.}$ Fit the data with $K = A\,\sigma^x$ to quantify that the strain-stiffening is stress controlled.
6) Determine the critical stress for each agarose concentration and plot the critical stress versus agarose concentration.
7) Obtain G_0 and plot it versus agarose concentration.
8) Quantify if K and G_0 show the same dependencies if plotted versus agarose concentration. What does this tell you?

9) Calculate the average mesh size for each agarose concentration using the measured G' value ($\omega = 2\pi f$, f = 1Hz). At which agarose concentration would you expect the 0.6 um particle to be able to move within the agarose mesh? When do you expect free diffusion?

In the afternoon from 1 pm to 4 pm:

Inverse time-lapse microscopy

Time-lapse acquisition

Detailed instructions on how to use the instrument will be given by your instructor. Open the software micromanager 1.3. Choose 'Hellfeld' as a channel, 20 ms exposure time. 100% left -> all to camera. Lamp 3.2 volt.

Prepare again the following solutions: 0.1 / 0.05 / 0.03 / 0.025 /0.02 / 0.01 / 0 % agarose in water. Prepare each solution directly before you actually use it!

Take a microscope slide and 'paint' a rectangle with silicon paste on top.

Take 0.5 ml of each solution and ad 0.1µl of 0.6µm beads (diameter) and mix immediately (vortex).

Now pour 100µl of the agarose-bead solution into the silicon paste rectangle and seal it with a cover glass (smoothely).

Let the agarose form a gel within this setup for 10 min. Then put the slide on the microscope.

Use the 100x objective PH3, oil. -> Ad oil on top before you add the slide.

Chose 'live image' to find the particles in the gel. Focus on them so that they appear black in the image (this is important for image analysis later on).

Once you have done that, open 'burst'.

First, hit the stop button, than the start button. (50 images, 20 ms exposure time). This leads to in average 52 ms between each image. Save the obtained sequence in a new folder.

Take several sequences between 5 and 10 min. Choose start images on which the moving beads are not too close together, so that you are able to track them later easily.

Then go on to the next probe.

Image analysis:

Use image J to analyse your images.

- Image import image sequence
- Use the rectangle tool to create an ROI (region of interest) around the particle that you want to track
- Go to image scale to create a new sequence of only this ROI
- Go to image adjust threshold. Set the threshold in that way, that the particle is completely red.
- Go to analyze: set measurements. Choose area and center of mass.
- Then go to analyze: analyse particles. Size 5-30 as a start, vary if needed. Choose display outlines. Choose show results and exclude on edges.
- You will now obtain a results table showing the area as well as the x and y position of the particle. (Check if you only got the particle you wanted, if not correct threshold and or 'size' to get rid of 'wrong' particles.
- Save your final data.

Use a program of your choice to perform the following analysis:

1) Create a plot of the movement (y versus x position) for one 'nice' particle per agarose concentration. Do you find movement as expected?
2) Obtain the MSD over time for 3 particles per agarose concentration. Plot one of these MSDs (nicest one) over time for each agarose concentration in one plot (for binning 2 using the 100x objective: 1 pixel = 0,155μm).
3) Obtain the maximum MSD for each agarose concentration and plot this max MSD versus agarose concentration. What does this tell you?
4) Calculate the theoretical G'' value for pure water (look the viscosity value of water for 20°C in the literature, $\omega = 2\pi f$, frequency used was 1 Hz!).
5) Calculate the theoretical dynamic viscosity for the highest measured agarose concentration using the measured G'' value at zero strain.
6) Obtain the theoretical MSD over time for pure water and the highest measured agarose concentration by using your information obtained in 4) and 5) and fitting. Add these two theoretical data curves to your MSD figure created in 3).
7) Calculate the diffusion constant of the 0.6 μm bead within the agarose gel, using your MSD data.
8) Plot G' versus MSD. Indicate regions for which the particle is able to move using the data obtained with rheology.
9) Plot mesh size versus MSD using the data obtained with rheology.