Viscoelasticity of bacterial biofilms

Biophysics lab course for biochemist students

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1. Bacterial biofilms

Biofilms can grow on various surfaces and in many different environments, a phenomenon that constitutes major problems in industry and medicine (Fig. 1). The term “biofilm” describes a community of microorganisms that adhere to each other and/or to a surface. Biofilm architecture is provided by extracellular polymeric substances (EPS), a mix of polysaccharides, proteins, lipids, and nucleic acids. The EPS make up to 50–90% of the total organic material in biofilms, embed cells, and provide increased resistance to antibiotics and environmental stresses.

Biofouling can reduce mass and increase corrosion, and biofilms on surfaces in food production enhance the risk for product contamination with pathogenic microflora. Thus, biofilms cause a significant amount of economic damage every year. Of great concern are biofilm-associated bacteria on medical implants or catheters as they can cause serious infections and will decrease the functionality of the medical device. As a consequence, the deactivation and removal of biofilms from surfaces has become a main goal in biofilm research, and the mechanical properties of those biofilms have gained lots of attention during the past decade.

![Biofilm examples](image)

*Fig. 1. Biofilm examples. a) biofilm-infected medical catheter, b) biofilm contaminated pipe*

Biofilms can be formed by a variety of gram-positive as well as gram-negative bacteria. The formation of a biofilm comprises several phases starting with the attachment of single cells on the target surface, ending with the formation of the mature biofilm where the bacteria are embedded and protected by a self-produced matrix of exopolymeric substances [Fig. 2]. The detailed composition of those exopolymeric substances varies depending on the bacterial species, but many bacterial strains secret polyanionic polymers such as alginate or polyglutamate.
Bacteria inside biofilms are often highly resistant against a range of antibiotics and other toxic substances. This biofilm property is surprising considering that planktonic (= single floating) bacteria do not possess this high resistance. As a consequence, when living in the complex environment of a biofilm, the bacteria either acquire this resistance by adjusting their metabolism, or selected biopolymers in the biofilm matrix absorb the hazardous chemicals and prevent them from reaching the bacteria in the first place.

It has been shown that the presence of shear forces and different ionic conditions during the growth phase of the biofilm can have an impact on the biofilm mechanics. Moreover, also the viscoelastic properties of mature biofilms can be altered by shear forces and selected chemicals. When those shear forces are generated by flowing liquids, detachment of individual bacteria or erosion of biofilm fragments have been described. The erosion stability of biofilms depends on the biofilm species and on the chemical environment the biofilm is exposed to. However, it is not clear whether chemical conditions that are beneficial for the mechanical properties of a biofilm might be, at the same time, harmful for the embedded bacteria.

2. Goal of the experiment

The goal of your experiment is to compare the influence of different metal ions on the viscoelastic properties of mature biofilms and on the viability of the embedded biofilm bacteria. You will focus on metal ions that are typically found in pipes: Fe$^{3+}$, Cu$^{2+}$ and Zn$^{2+}$.

In your experiment, you will study biofilms formed by the model organism Bacillus subtilis B1, a non-pathogenic strain that resides in the soil and is e.g. found in oil fields.
3. Theory of viscoelasticity

Viscoelasticity describes a material property which combines both viscous and elastic characteristics when undergoing a deformation. Purely elastic materials respond immediately to an externally applied force \( (F) \) and the resulting deformation \( (\Delta x) \) completely recovers when the external force is released (see. Fig. 3). In contrast, purely viscous materials start to flow in response to an external force, and they remain deformed once the force is removed. Viscoelastic materials combine those two behavior types. Prominent examples are polymer melts or creams, cells and tissues, but also almost every material (with the exception of Newtonian fluids) displays a certain amount of viscoelastic behavior.

![Fig. 3. Linear material response to deformation. Elastic, viscous and viscoelastic material response to an applied step force, e.g. of a cubical volume in shear. Whereas an elastic material returns to its original state once the stress is removed, the deformation of viscous materials is irreversible. For viscoelastic materials the deformation process is partially reversible and the accumulation and recovery of the strain is delayed.](image)

For viscoelastic materials, the relation between stress (= force/area) and deformation (= strain) typically depends on the time scale of the mechanical stimulus, i.e. they might behave elastically when deformed quickly but could at the same time behave as a fluid when deformed slowly. To quantify the elastic and viscous behavior of a given material, two parameters are commonly used as described in [Table 1]. The **storage modulus** \( G' \) is a measure for the amount of energy that is stored in the material during the deformation process and represents the elastic behavior of the material. The **loss modulus** \( G'' \) reflects the viscous behavior of the material and represents the energy that is lost during the deformation process.

<table>
<thead>
<tr>
<th>G' Storage Modulus</th>
<th>G'' Loss Modulus</th>
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</thead>
<tbody>
<tr>
<td>Energy that is...</td>
<td>stored</td>
</tr>
<tr>
<td>Measure for...</td>
<td>elasticity</td>
</tr>
</tbody>
</table>

**Table 1. Physical meaning of storage and loss modulus**
However, these material parameters are only valid as long as the ratio of stress and strain is independent from the actual magnitude of the applied force or deformation. This is the so-called linear response regime, where Hooke’s law holds and the stress is linearly related to the strain: $\sigma \sim \gamma$. Typically, this linear relation is not valid anymore at large strains or stresses. For the measurements in this lab course, linear viscoelastic behavior is ensured since the deformation amplitude applied to the material is very small.

4. The macro-rheometer

The viscoelastic properties of the biofilms will be determined by oscillatory shear rheometry using the stress controlled rheometer Physica MCR102 (Anton Paar, Graz, Austria) (see Fig. 4). This will allow you to characterize both the viscous and elastic properties of the biofilm samples and compare those properties for untreated and chemically challenged biofilms.

![Rheometer](image)

**Fig. 4. Rheometer** a) Schematic drawing of the measuring head and the temperature controlled plate of the rheometer used for oscillatory measurements. b) Picture of the Rheometer Physica MCR102. By oscillatory rotation, the measuring head induces a shear stress on the sample. The deformation of the sample in response to the applied stress is recorded by the rheometer which then calculates the viscoelastic moduli $G'$ and $G''$.

By inducing a sinusoidal shear deformation (= strain),

$$\gamma(t) = \gamma_0 sin(2\pi ft)$$

on the sample with an oscillation frequency $f$, and measuring the stress response $\sigma$,

$$\sigma(t) = \sigma_0 sin(2\pi ft + \delta).$$

the elastic (= storage) modulus can be determined by

$$G'(f) = \frac{\sigma_0}{\gamma_0} cos(\delta)$$

and the viscous (= loss) modulus by

$$G''(f) = \frac{\sigma_0}{\gamma_0} sin(\delta).$$
\[ G''(f) = \frac{\sigma_0}{\gamma_0} \sin(\delta) \]

where \( \delta \) is the phase angle between the applied oscillatory deformation \( \gamma(t) \), and the stress response \( \sigma(t) \) (see Fig. 5).

![Diagram of phase angle](image)

**Fig. 5. Phase angle \( \delta \) between an applied oscillatory stress \( \sigma(t) \) and the resulting deformation \( \gamma(t) \)**

**Question 1:** How large would the phase shift between stress and strain be

a) for a purely elastic material (that does not possess viscous properties)

b) for a purely viscous material (that does not possess elastic properties)?

As the phase shift \( \delta \) depends on the frequency of the applied deformation, the two parameters \( G'(f) \) and \( G''(f) \) acquire a frequency dependence. When this frequency dependence is plotted, it is referred to as a frequency spectrum (see Fig. 6).

![Frequency spectrum](image)

**Fig. 6. Frequency spectrum of a *Pseudomonas aeruginosa* biofilm depicting both viscoelastic moduli. \( G'(f) \) [full circles] and \( G''(f) \) [empty circles] were plotted over a frequency range of 0.01 Hz to 100 Hz.**

**Measuring a frequency spectrum**

All rheological measurements will be performed using a 25 mm plate-plate geometry and a plate separation of 300 \( \mu \)m. To ensure that you record the viscoelastic properties of your
samples in the linear response regime, before each strain-controlled measurement a pre-experiment needs to be performed to determine the optimal strain for each frequency spectrum. Therefore, you should apply a torque of \( M_{\min} = 0.5 \, \mu \text{Nm} \) at a frequency of \( f = 1 \, \text{Hz} \) on the sample and record the resulting deformation. From our experience, 0.5 \( \mu \text{Nm} \) can be considered as the lower limit of torque that can be resolved reliably by the rheometer. You can therefore consider about 150% of \( \gamma_{\min} \) as the optimal strain for subsequent deformation controlled measurements with this sample. In your measurements, obtain frequency spectra in a range of 0.1 Hz to 10 Hz for each sample and collect 5 data points per decade.

The temperature of the sample can be controlled during the measurement with a peltier element that is integrated into the lower plate of the measuring unit. Choose a constant temperature of 21 °C for all your measurements! Also, a solvent trap can be used to prevent the samples from dehydration during the measurements.

**Question 2:** How could you verify experimentally whether a solvent trap is necessary for your measurements?

**Question 3:** Which are the input and output variables of the pre-experiment and the frequency sweep (= measurement of a frequency spectrum)?
5. Experiments

Biofilm formation (already conducted by supervisors)

- preparation of an overnight culture of *B. subtilis* B1 in liquid LB-media
- spreading out of overnight culture onto LB-Agar plates for biofilm formation

A. Influence of the metal ions on bacterial growth (4 conditions, n=2)

*Harvesting of biofilms*

- use 2 glass slides and gently scrape off biofilm. Be careful not to accidentally take agar
- pool the biofilm material
- transfer 8 samples of about 500mg biofilm material each into eppendorf tubes
- weigh each sample and note the mass

*Chemical challenge with Fe$^{3+}$, Cu$^{2+}$ and Zn$^{2+}$.*

- calculate the correct mixture ratios (5 %(v/w) ionic solution)
- n = 2 per ion solution and control group with pure ddH$_2$O conditions with n = 2, total of 8 samples
- pipette ionic solution into eppendorf tubes and mix with biofilm
- wait 30 min

*Release chemical pressure*

- add 10ml of LB to Falcon tube
- add biofilm + chemical to fresh LB-medium

*Monitoring growth of chemically challenged bacteria*

- measure OD values every 60 - 90 min, start with an initial time point

B. Influence of the metal ions on the mechanical properties of biofilm (5 conditions, n=2)

*Harvesting of biofilms*

- use 2 glass slides and gently scrape off biofilm. Be careful not to accidentally take agar
- pool the biofilm material
- transfer 10 samples of about 500mg biofilm material into eppendorf tubes
– weigh each sample and note the mass

*Chemical challenge with ddH$_2$O, Fe$^{3+}$, Cu$^{2+}$ and Zn$^{2+}$.*

– calculate the correct mixture ratios (5 %(v/w) ionic solution)

– $n = 2$ per ionic solution and control group with pure biofilm and biofilm with water 5 conditions with $n=2$, total of 10 samples

– pipette ionic solution into eppendorf tubes and mix with biofilm

– wait 30 min

*Rheological Measurements*

– write the test protocol for pre-test and the frequency sweep

– test your samples with both protocols, adjusting the main measurement according to the results of the pre-test
### 6. Timetable

<table>
<thead>
<tr>
<th>Time</th>
<th>What to do</th>
</tr>
</thead>
<tbody>
<tr>
<td>09:00</td>
<td>Meet at IMETUM, Room Nr.: 1.211</td>
</tr>
<tr>
<td>09:00 – 09:45</td>
<td>Harvesting and weighing of biofilm</td>
</tr>
<tr>
<td>09:45</td>
<td>Start chemical challenge</td>
</tr>
</tbody>
</table>
| 10:15       | End chemical challenge
1. OD measurement |
| 10:15 - 11:15| Seminar: Basics of Rheology and Discussion of Questions                   |
| 11:15       | 2. OD measurement                                                         |
| 11:15 - 12:15| Start of rheological measurements                                         |
| 12:15       | 3. OD measurement                                                         |
| 12:30 - 13:15| Lunch                                                                      |
| 13:15       | 4. OD measurement                                                         |
| 13:30       | Continue rheological measurements                                         |
| 14:15       | 5. OD measurement                                                         |
| 15:15       | 6. OD measurement                                                         |
7. Your protocol

General information for the structure of your protocol:

Submission date for the protocol: 14 days after your lab course date.

Please structure the protocol based on the different experiments and not along the time course of the day.

Introduction (15%)
- general introduction into the topic
- motivation, aims and goals of experiments

Materials & Methods (30-40%)
- how did you perform your experiments?
- denote materials and equipment entirely: (trade name, company, city, country)

Results (30-40%)
- what did you measure?
- present the data of your experiments, explain what’s shown in your figures
- don’t mix results with discussion

Discussion (15-20%)
- what did you find out and how do you explain it?
- answer the additional questions stated below.

References

In addition to the analysis of your experiments, answer the following questions:

- What is the main biopolymer component of B. subtilis B1 biofilms? What properties does this molecule possess?

- Also Pseudomonas aeruginosa can form biofilms. e.g. when the bacterium colonizes the lung of patients with cystic fibrosis. A main component of those biofilms is alginate, and similar to B. subtilis B1 biofilms, also P. aeruginosa biofilms exhibit a greatly increased storage modulus when brought in contact with Fe3+ ions. What could be a possible explanation for this similar behavior?

- Outside the laboratory, bacterial biofilms are often exposed to a variety of metal ions. With the observations made in your experiments and your answer to the previous question in mind, what chemical treatment would you suggest for weakening the mechanical properties of bacterial biofilms so that their removal from surfaces is facilitated?
- The ratio $G''/G' = \tan(\delta)$ is called “loss factor”, a dimensionless parameter that is used as a simple quantity to rate a given material as “mostly elastic” or as “mostly viscous”. Of course, also this quantity depends on the frequency of the applied deformation. Calculate the loss factor curve $G''/G'(f)$ for an untreated biofilm sample and for a biofilm that has been challenged with Fe$^{3+}$ ions. What do you observe? What alteration in this loss factor curve do you expect for a chemical challenge that induces a “fluidization” of the biofilm, i.e. for a chemical that liquefies the biofilm material? Plot this expected loss factor curve for a fluidized biofilm in your previous graph as well.

- Compare your results for bacteria growth in the biofilm under different conditions with the given date for bacteria growth in a liquid culture. Why are they different? Explain and discuss the reasons.

8. References


M. Morikawa et al., *Biofilm formation by a Bacillus subtilis strain that produces \(\gamma\)-poly-glutamate*, Microbiology (2006), 152, 2801–2807