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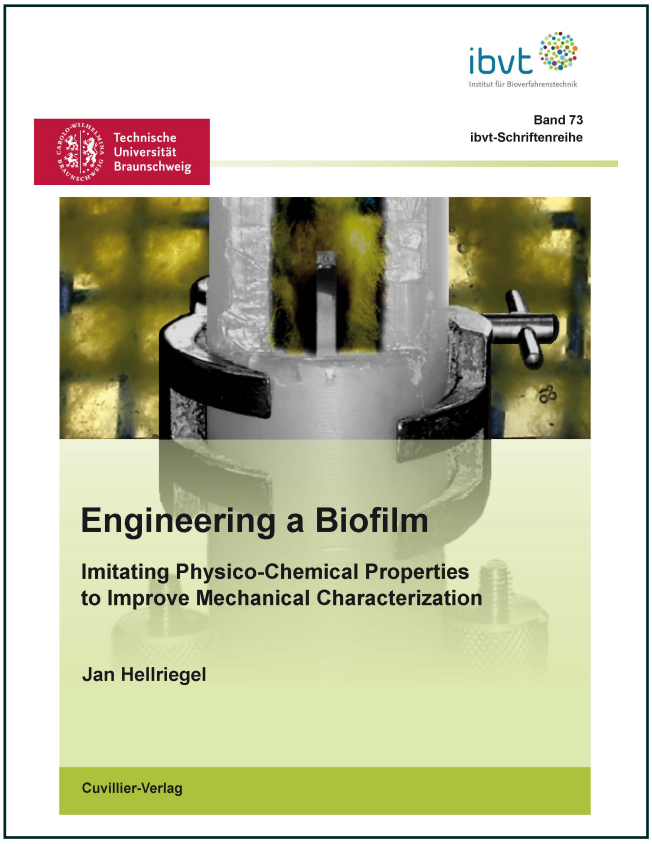
Engineering a Biofilm

Imitating Physico-Chemical Properties to Improve Mechanical Characterization



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1 Introduction

1.1 Biofilms Today

Biofilm-forming microorganisms are ubiquitous and protected by a matrix of biopolymers, proteins and other organic substances [87]. In industrial processes these characteristics are responsible for a reduced plant productivity or poor product quality. Removal of these unwanted biofilms is still difficult. However, biofilms are able to degrade recalcitrant compounds and play an important role in the biological industrial wastewater treatment or as catalytic biofilms which are able to synthesize a wide variety of metabolites, potentially used to produce pharmaceuticals [26, 47]. Some of the metabolites are only available in well-defined mixed biofilm biocoenoses, increasing the interest in a controlled biofilm formation for their production [115]. By today researchers exploit biofilm-based biotransformations to produce biological toxic bulk chemicals, e.g., styrene oxide [57, 58].
Nevertheless, it is still difficult to predict biofilm growth and structural properties or cultivate biofilms with constant material characteristics. Limited knowledge of subpopulations, fluid-structure interactions of biofilm formation and detachment as a result of mechanical stresses do hitherto not allow reliable prediction and control of biofilm development. Recent studies suffer from a deficiency in experimental data to validate developed models [12, 13, 41].

1.2 Objectives

The main objective of this thesis is the development of a hydrogel based physico-chemical biofilm model to investigate fluid mechanical interaction and its influence on growth and detachment effects of biofilms. Therefore, a representative and reproducible biofilm is required. Thus a microbial test system needs to be established which allows further analysis of planktonic and sessile growth in a controlled environment.
A further objective is to obtain a mono-septic culture biofilm to reduce the complexity of a multispecies biocoenosis. Targeted surfaces for biofilm development are either the walls of the tubing, surface modified object slides, plastic or iron net-

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tings and membrane filters. As far as possible automated on-line analytic should be used to measure dissolved oxygen, pH, surface and planktonic cell growth during cultivation. A suitable image acquisition technique to measure and visualize surface attached biofilm development in terms of growth and detachment processes is required.

Established measurement techniques are validated with real biofilms and with the help of the established hydrogel model. For future visualization of cell distribution within the biofilm and hydrogels with a confocal laser scanning microscopy (CLSM) the model strain *P. putida* KT2440 is genetically modified to produce the green fluorescent protein (GFP).

The development of a physico-chemical biofilm model based on highly hydrolyzed gellan hydrogels is supported by experimental design techniques. The resulting gellan-based hydrogel should be validated to be a mechanically similar biofilm imitate. Finally, the predicted model system should withstand a comparison with real biofilms and a physical biofilm model based on worm-like-chains. Its purpose is to mimic the viscoelastic properties of biofilms in terms of storage (G') and loss (G'') moduli. The physico-chemical model is supposed to imitate biofilms of different strength by changing its composition resulting in biofilm like viscoelastic behavior.

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2 Theory

2.1 General Knowledge of Biofilms

Biofilms are considered to be a community of microorganisms attached to surfaces or phase boundaries such as solid-liquid, solid-gaseous or gaseous-liquid [28, 34]. They have been around for two to three billion years and grow ubiquitously on all terrestrial surfaces. The first discovery of microbial life is linked to the breakthrough of the microscopy initiated by Robert Hooke (1635 - 1703), who first illustrated fruiting structure of molds and published it in 1665. Eleven years later Antoni van Leeuwenhoek used a simple microscopic construction to first visualize bacteria [95]. It was not until the nineteenth century that research in microbiology became of general interest [95] and not before the mid twentieth century that surface attached microorganisms were found to play a major role [16], even though it is the most common mode of growth for microorganisms on earth [28, 151, 155]. The production and secretion of exopolymers allow bacteria to modify the hydrophobicity of surfaces and assist adsorption and colonization of any substratum [111]. Some intensively studied bacteria are *Pseudomonas aeruginosa* or *Streptococcus mutants* which as pathogenic microorganisms are responsible for a wide range of infections. They produce bacterial alginates or biopolymers that form a hydrogel-like environment as protection matrix. Most biofilms are heterogeneous and are characterized by a nutrient gradient from the surface to the substrate as a result of limited diffusion through the exopolymeric matrix. The matrix characterized by extracellular polymeric substances (EPS) also provides mechanical stability and functions as reservoir for nutrients or water [111].

2.1.1 Structure and Composition of Biofilms

According to Mayer et al. [98] biofilms are highly hydrated viscoelastic systems with a water content between 92 to 94 % and a high storage (G') but a small loss (G'') modulus (compare Chapter 2.3.1). They found a bacterial concentration of 5 to $7 \cdot 10^{11}$ cells$\cdot g_{BDW}^{-1}$. Costerton et al. [34] reported a biomass content for dried biofilms between 10 to 25 % and 75 to 90 % EPS, respectively. Biofilm structures and properties are influenced by medium composition and environmental conditions.

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Fig. 2.1 shows a biofilm model adapted from Möhle [103]. The rough biofilm surface topology facilitates an efficient nutrient uptake and allows a constant fluid flow ($\vec{\omega}$) through the channels [134]. Fluid induced stresses (σ) into the biofilm cause detachment by erosion and sloughing (X_{det}) which eventually leads to stronger more compact biofilms and an increased strength of the EPS-matrix [3]. In laminar flows, biofilms tend to be fluffy and in general thicker than in tubular flow, which results in thinner and denser biofilms and promotes the formation of streamers [104, 148, 157]. Slow flowing fluids promote the biofilm to build mushroom-like structures. The controlling instance of biofilm formation is still poorly understood.

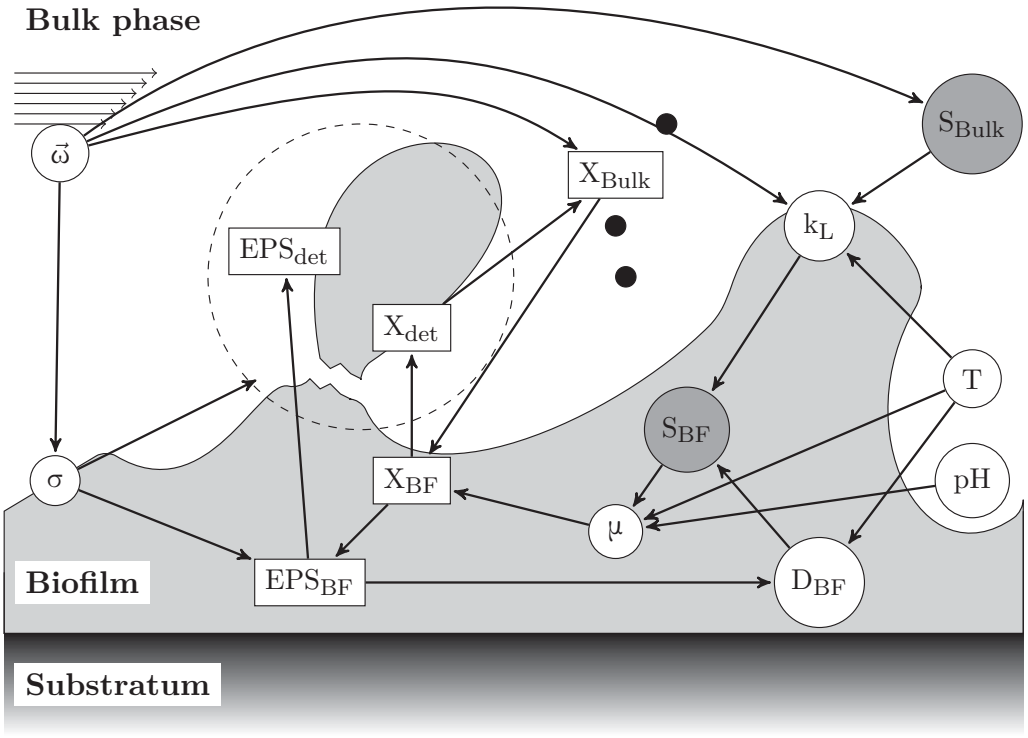

Figure 2.1: Biofilm model, adapted from Möhle [103] and modified. Fluid flows over the biofilm surface ($\vec{\omega}$), induces stress (σ) into the biofilm and transports biomass (X_{Bulk}) and substrate (S_{Bulk}). The stress influences the EPS structure (EPS_{BF}) and causes erosion or detachment (EPS, X_{det}). Planktonic biomass (X_{Bulk}) (re)attaches to the substratum, increasing the biomass of the biofilm (X_{BF}). Substrate (S_{Bulk}) is transported over the boundary layer (k_L) and diffuses (D_{BF}) through the biofilm. The specific growth rate (μ) is influenced by the substrate availability (S_{BF}), temperature (T) and pH (pH) in the biofilm and related the production of biomass (X_{BF}) as well as the EPS (EPS_{BF}).

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Wäsche [172] related the biofilm topology to the substrate availability and stress intensity. High substrate concentration at moderate flow velocities resulted in increased biofilm growth but in a weak and fluffy EPS-matrix. The author confirmed that at high stresses the fluid-biofilm interaction exceeds the influences of substrate and determines the biofilm surface structure and thickness.
Tolker-Nielsen et al. [165] investigated the development and dynamics of *Pseudomonas* sp. biofilms with confocal laser scanning microscopy (CLSM). Their published data proposes that the initial biofilm attachment is caused by simple growth on the substratum. They also identified fast movement of *P. putida* OUS82 bacteria inside microcolonies and discussed the possibility of bacteria being in different physiological states when sessile or planktonic. However, growing on surfaces *Pseudomonas* sp. strain B13 bacteria formed ball-shaped microcolonies and *P. putida* OUS82 showed loose protruding structures, displaying different kinds of biofilm formation. Nancharaiah et al. [109, 110] quantitatively described a *P. putida* KT2440 biofilm in situ and used GFP, a red fluorescent protein (DsRed) and a nucleic acid stain (SYTO 60) to study conjugal gene transfer. Their flow cell experiments showed a percentage surface coverage of 80 % in 48 hours. This proved the possibility to monitor biofilms with a CLSM and showed a significant correlation between the GFP and SYTO 60 stain distribution.
Nilsson et al. [116] described two gene clusters, pea and peb, in *P. putida* KT2440 to stabilize the biofilm. They postulated that biofilm development might occur without exopolysaccharides since these only function as stabilizers. In contrary, Klausen et al. [77] saw no reason of biofilm development of the model biofilms *P. aeruginosa* and *P. putida* in genetic program, but in a number of different mechanisms to colonize surfaces. The nutrient availability in a biofilm is defined by the growth limiting substrate concentration in the bulk phase (S_{Bulk}), the mass transport through the boundary layer (k_L) and the diffusion coefficient within the biofim (D_{BF})[172]. Biofilm growth (μ) is a function of substrate concentration (S_{BF}), pH and temperature (T) and closely linked to the production of EPS (EPS_{BF}).
Certain environmental conditions, e.g., ion and substrate concentrations, pH, humidity or toxicity, can promote or reduce biofilm growth and strengthen or weaken the EPS-matrix [88]. EPS production is often increased by temperatures lower than optimal and high carbon to nitrogen ratios as well as a lack of nutrients. Chang et al. [25] published data showing that for the bacteria *P. putida* mt-2 the production of alginate is linked to the availability of water functioning as reservoir.

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2.1.2 Composition of the Extracellular Polymeric Substances

According to Flemming et al. [48] most of the carbohydrate fraction of the EPS are polysaccharides and have been intensively studied since their commercial application as gelling agent, flocculates, foam stabilizer, hydrating agents and biosurfactants. Their gel-like behavior stabilizes and gives structure to the biofilm. Literature differentiates between non-bacterial and bacterial polysaccharides because they have dissimilar molecular weights and properties. It is, therefore, only possible to compare data qualitatively [87]. The average molecular length ranges from 500 to 2000 kDa. Bacterial polysaccharides can be capsular or released to the surrounding. Capsular polysaccharides are believed to be connected to the cell surface, possibly even covalently bonded while the released ones are free [1, 83, 154]. There is a wide variety of bacterial polysaccharides, some of them are homo-polymers like dextran, cellulose or sialic acid. However, most of them consist of multiple mono sugars, e.g., emulsan, xanthan, alginate or gellan [153].
Celik et al. [19] cultivated *P. aeruginosa* G1 and *P. putida* G12 on glucose, mannose, fructose and xylose and characterized the EPS of these strains. The monosaccharide composition of the biofilms can be analyzed by HPLC. Celik et al. used a PAP medium with glycerol, 2 % glucose (w/v), 2 to 6 % mannose, fructose or xylose (w/v) as carbon source for EPS production and found a high content of neutral sugars (92.0 to 99.2 %) and acetylated amino sugars (0.8 to 8.0 %) in the EPS. Kachlany et. al. [72] investigated the structure and carbohydrates involved in the exopolysaccharide capsule of *P. putida* G7. Special for *P. putida* G7 is the ability to produce capsular exopolymers which surround the young bacteria cells in planktonic and sessile state, respectively. Older cells lost the capsular EPS, thus resulting in cell associated and non-associated EPS. The EPS was purified and analyzed. It contained the monosaccharides, glucose, rhamnose, ribose, N-acetylgalactosamine and glucuronic acid. Jahn et al. [69] described the composition of *P. putida* biofilms. They had a maximum specific growth rate of $\mu_{max} = 0.34\ h^{-1}$ on 10 gl^{-1} citrate and of $\mu_{max} = 0.28\ h^{-1}$ on 10 gl^{-1} glucose in batch at 30 °C. The composition of the biofilm in a continuous cultivation varied depending on the limitation of either the carbon source or oxygen supply. The authors found high concentrations of proteins, polysaccharides, uronic acid and DNA. It is common that one bacterial strain produces multiple polysaccharides [154], thus the EPS always is a group of different chemical molecules.

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2.1.3 Biofilm Mechanics and Extracellular Polymeric Substances

Determining the mechanical properties of real biofilms or EPS-matrix is still difficult. The produced polysaccharide and the amount depend on the availability of certain substrates and nutrients. The nitrogen or phosphorus ratio to carbon can determine if a cell uses energy for growth or EPS production. A high carbon concentration usually promotes EPS production [153]. Mayer et al. [98] used *P. aerigonosa* SG81 biofilms grown on agar in petri dishes for 24 h at 36 °C to prepare a bacterial EPS-solution. To separate the biomass from its matrix they carefully removed the bacterial lawn from the agar surface, suspended and homogenized it by vigorous stirring in sterile water. Extraction was achieved by centrifugation and filtration, finally resulting in an EPS-solution. The authors found three binding forces, London dispersion forces, electrostatic interactions and hydrogen bonds, between the EPS molecules. Their strength depended on the arrangement of the monomers, linear or branched, and their composition and conformation. High uronic acid content usually stabilizes the hydrogel while a high arabinose content induces cell aggregation [98].

Körstgens et al. [81] described the mechanical stability of biofilms upon uniaxial compression. They stated that the mechanical properties of the EPS-matrix are mostly defined by the polysaccharidic skeleton. Körstgens [79] and Wloka [178] proceeded similar to Mayer et al. [98] to separate the EPS from the mucoid bacterial lawn and rheologically characterized an EPS-solution. However, the procedure required scraping off the biofilm and this destroyed the original matrix of the EPS. The characterized EPS-solution had little in common with the EPS-matrix of a biofilm. The mechanical data for the cohesive strength, storage, loss, elastic or shear moduli reported in literature varies over several magnitudes [12]. Reasons are a wide range of measurement methods, such as centrifugation or tension devices, fluid shear techniques, erosion or compression experiments and rheometry.

Furthermore, alternating species of biofilms or different culture strategies change the general structure of the biofilm. Möhle et al. [104] reported a cohesive strength of 6.0 to 7.7 Pa for a mixed culture biofilm while Poppele et al. [133] found up to $1.6 \cdot 10^4$ Pa for *P. aeruginosa* biofilms. The elastic modulus for *P. aeruginosa* biofilm was given with 1.0 to 5.0 Pa by Klapper et al. [76] or with up $2.3 \cdot 10^4$ Pa for *Staphylococcus epidermidis* by Hohne et al. [60]. Values for the storage (G') and loss (G'') moduli reported by Characklis [27] for a mixed biofilm are 60 Pa and 120 Pa, respectively, while Moresi et al. [105] found them to be $4.2 \cdot 10^4$ and 400 Pa for the EPS gel extracted from *Azotobacter vinelandii* biofilms.

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Fig. 2.2 shows some sample data for G′ and G″ for a gellan based hydrogel, a biofilm of *S. aureus* 6ME and the EPS-solution. The hydrogel contained $x_{Gellan} = 0.7$ %(w/v) and $c_{Ca^{2+}} = 0.03$ %(w/v), probe hight was given with 5.5 mm, $\gamma \approx 1.8$ % and data was extracted from Oliveira et al. [127]. The *S. aureous* biofilm was stressed with 3 Pa and data taken from Di Stefano et al. [37]. Wloka et al.'s [179] EPS-solution was measured after addition of 7 mM Ca^{2+} at a strain of $\gamma \approx 10$ %. The values for G′ and G″ are in the same magnitude for the hydrogel and the biofilm, with approximately 10 to 40 kPa for G′ and 2 to 30 kPa for G″, respectively. The reported values of the EPS-solution are several magnitudes below the values for the biofilm with $20 \cdot 10^{-3}$ for G′ and $20 \cdot 10^{-4}$ for G″. Thus, the analogy of the EPS-solution to the biofilm is questionable, whereas the hydrogel and the biofilm clearly show similarities. A comprehensive summary of mechanical methods and experimental determined data on biofilms can be found in the review by Böl et al. [12].

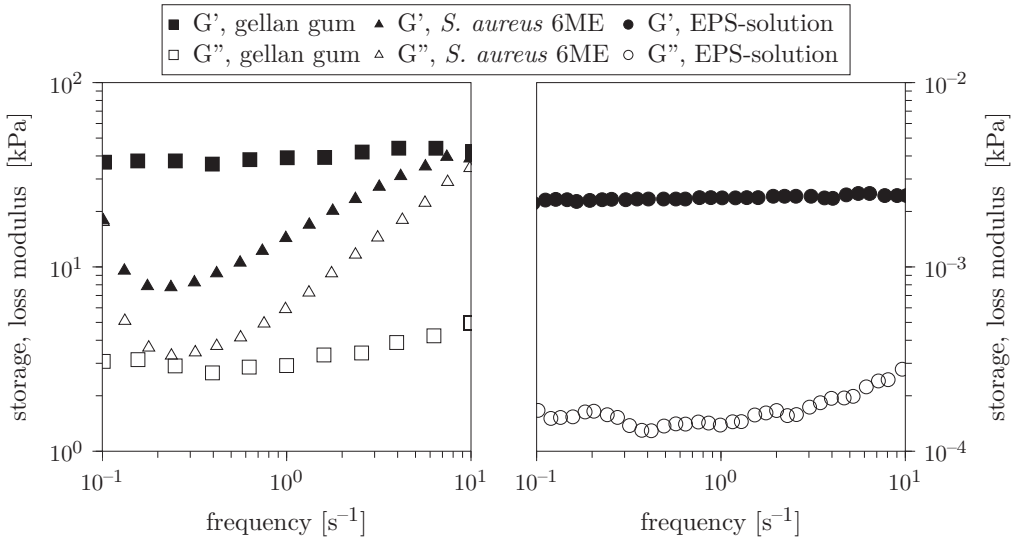

Figure 2.2: Storage (G′) and loss (G″) moduli for a hydrogel (squares), a biofilm of *S. aureus* 6ME (triangle) and an EPS-solution (circle). The hydrogel contained $x_{Gellan} = 0.7$ %(w/v) and $c_{Ca^{2+}} = 0.03$ %(w/v), probe hight was 5.5 mm, $\gamma \approx 1.8$ %. Data was extracted from Oliveira et al. [127]. The *S. aureus* 6ME biofilm was stressed with 3 Pa. Data was extracted from Di Stefano et al. [37]. The EPS-solution was measured after addition of 7 mM Ca^{2+} at a strain of $\gamma \approx 10$ %. Data extracted from Wloka et al. [179].

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2.1.4 Biofilm Detachment

The biofilm life cycle is a balance between microbial growth and detachment processes [163]. There are multiple reasons besides flow induced stresses [132] that promote detachment effects such as quorum sensing [137], nutrient completion [62] or the growth history [163]. Earlier events such as substrate limitations or changes in hydrodynamic forces have influenced the structure during the biofilm development [65]. Mushroom-like shaped biofilms are more easily destroyed by an increase in flow induced stresses than smooth surfaces. The cohesive strength of the biofilm is a result of the EPS-matrix and as such contingent on the availability of nutrient [75, 172].
Detachment is usually categorized into two types, sloughing and erosion [132]. The first is described as the tearing off of large biomass fragments, resulting in rougher surfaces or even complete removal of the biofilm [169]. The latter is the removal of smaller biofilm fragments which smoothen the biofilm surface [163]. Fast grown biofilms show a higher detachment rate than slowly grown biofilms if stressed similarly since high growth rates cause a weaker EPS-matrix and thus unstable biofilm accumulation [146]. Erosion and sloughing also provide biomass for re-attachment or population of other surfaces further down the stream [135].

2.1.5 Influence of Metal Ions

Körstgens [79] described a decreasing stability in the order of these metal ions: Cu^{2+} > Al^{3+}, Ca^{2+}, Fe^{3+} > Mn^{2+}, Fe^{2+} and Co^{2+}. The concentration of these ions play a major role for the biofilm strength and often have an optimal concentration after which no change or a decrease in stability was monitored. The author explained the strengthening effect of the ions by an increased electro static interaction between the multivalent cations and the extracellular polysaccharides, e.g., alginate. Wloka [178] analyzed the influence of Ca^{2+}, Mg^{2+} and Mn^{3+} on the storage and loss moduli of *P. aeruginosa* biofilms. He described entanglements and Coulomb forces as major reasons for the cross-linking of the EPS-solution and the biofilms with a decreasing influence of Ca^{2+}, Mn^{3+} and Mg^{2+}. An increase in the ion concentration changed the dominating attraction from entanglements to electro static forces.
Fig. 2.3 shows the results from Wloka et al. [180] for *P. aeruginosa* biofims with 1 or 10 mM Ca^{2+} in comparison to data published by Lieleg et al. [88] who investigated the influence of Ca^{2+}, Cu^{2+} and Fe^{3+} on the biofilm elasticity. There is an obvious increase in the biofilm storage and loss moduli due to the addition of Ca^{2+}

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concerning Wloka's data. However, the data from Lieleg et al. [88], who cultured *P. aeruginosa* on LB medium, is within the same range. But LB medium is known for its low Ca^{2+} and Mg^{2+} concentration [175]. This shows that the comparability of the data between different authors and experiments is difficult.

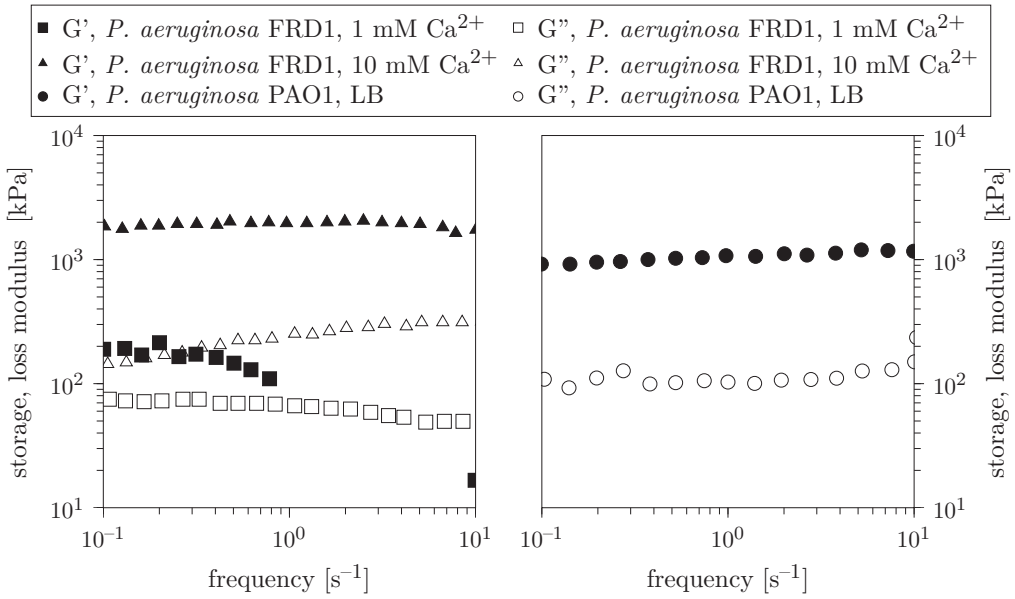

Figure 2.3: Storage (G′) and loss (G″) moduli of *P. aeruginosa* FRD1 and PAO1 biofilms. They were grown with 1 and 10 mM Ca^{2+} or in LB medium. Data extracted from Wloka et al. [180] (squares, 1 mM Ca^{2+}, triangles, 10 mM Ca^{2+}) and Lieleg et al. [88] (LB, circles).

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