Analysis of bacterial adhesion forces
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Summary
Bacterial adhesion initiates the formation of biofilms, which often cause persistent contamination of surfaces in many diverse applications or infection associated with biomaterial implants or devices. Chapter 1 gives an overview of the mechanisms involved in bacterial sensing and responding to substratum surfaces. It is suggested that the bacterial cell wall deforms to different extents corresponding to the strength of the adhesion forces. Atomic force microscopy (AFM) has been extensively applied to assess bacterial adhesion forces, but it is still unclear how the bacterial cell wall deforms during adhesion, due to the complexity of monitoring and quantifying the degree of the deformation. In this thesis we have proposed different ways to determine bacterial adhesion forces to substratum surfaces and associated cell wall deformation and evaluated their role in bacterial susceptibility to antimicrobials, either in solution or when immobilized to a substratum surface.

Bacterial adhesion to substratum surfaces on two different types of coatings, one composed of an anti-adhesive polymer brush-coating and one formed by a layer of immobilized quaternary ammonium compounds (QACs), was evaluated by adhesion force measurements using AFM, and adhesion forces were related to the susceptibility of adhering bacteria to antimicrobials and their survival upon adhesion, respectively. In the first part of Chapter 2, we demonstrate that nine strains of Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa adhered more weakly ((-0.05 ± 0.03) – (-0.51 ± 0.62) nN) to polymer brush-coated than to uncoated silicone rubber ((-1.05 ± 0.46) – (-5.1 ± 1.3) nN). Although bacteria could still grow into a biofilm on the polymer brush coating, they remained in a planktonic state due to the weak adhesion forces, and therewith remained susceptible to gentamicin, unlike the case for antimicrobial resistant biofilms formed on uncoated silicone rubber. S. epidermidis showed lethally strong adhesion forces on hyperbranched QAC
coatings, as presented in the second part of this chapter. The extremely high adhesion forces found indicate that quaternary ammonium molecules on hyperbranched polyurea may partially envelope adhering bacteria upon contact. The results from both studies propose that the response of bacteria to their adhering state and accompanying susceptibility to antimicrobials is determined by the magnitude of the force through which the organisms adhere to a substratum surface.

Although bacterial adhesion forces to a substratum surface can be directly measured using AFM, insight into the nature of the adhesion forces, i.e. the interplay between Lifshitz-Van der Waals (LW) and acid-base (AB) forces, requires further analysis. Chapter 3 reviews the statistics based Poisson analysis of the AFM measured adhesion forces and its underlying assumptions. By summarizing literature data for different combinations of bacterial strains and substrata, it was concluded that bacterial adhesion to surfaces is generally dominated by short-range, attractive AB interactions, in combination with long-range, weaker LW forces, in line with surface thermodynamic analyses of bacterial adhesion. The derived strength of a single short-range bond $f_{SR}$ varied between -0.1 and -1.1 nN, and was orders of magnitude higher than the typical rupture forces of a hydrogen bond (~ 0.01 nN). This indicates that short-range bacterial adhesion forces are composed of multiple molecular forces at discrete adhesive sites on the bacterial cell surface, rather than single molecular forces. The adhesion force arising from these cell surface sites and the number of sites available may vary from strain to strain. In order to avoid the tedious task of identifying the minor peaks in the AFM retract-force distance curves involved in the Poisson analysis of bacterial adhesion forces, we suggested to carry out Poisson analysis on the work of adhesion, as derived from retract-force distance curves as well. Poisson analysis on the work of adhesion confirmed that the short-range bond involves a discrete adhesive
bacterial cell surface site, composed of multiple AB bonds, rather than a single molecular bond.

Bacterial adhesion and detachment are closely related to the visco-elastic deformation of the contact volume between adhering bacteria and substratum surfaces. Deformation of the bacterial cell surface and associated changes in contact area in response to an applied external force are difficult to model and require knowledge of the visco-elasticity of the bacterial cell surface. Current elastic deformation models based on colloid science are unable to distinguish between the relatively soft outer bacterial cell surface and the hard-core of a bacterium, constituted by a cross-linked peptidoglycan layer. Therefore in Chapter 4, we present a new, simple model to derive the reduced Young’s modulus of the contact volume between adhering bacteria and substratum surfaces based on the relation between deformation and applied external loading force, measured using AFM. According to pairwise comparisons among six strains of *Streptococcus salivarius*, *S. epidermidis* and *S. aureus*, we were able to interpret the reduced Young’s moduli $E^*$ ($8 - 47$ kPa) and the initial dimensions $h_0$ ($11 - 91$ nm) of the contact cylinder on the basis of the cell surface features and cell wall characteristics, *i.e.* surfaces that were more rigid (either because of a lower degree of fibrillation, less extracellular polymeric substance (EPS) production or higher degree of cross-linking of the peptidoglycan layer) had shorter contact cylinders and higher reduced Young’s moduli. Application of an established Hertz model to our experimental data also yielded pairwise differences in reduced Young’s moduli, demonstrating differences in cell surface features and cell wall characteristics within each pair of strains, similar as our new, elastic deformation model. However, $E^*$ values derived from the Hertz model were up to 100 times higher for all strains investigated, likely pertaining for a major part to the bacterial hard-core; while our proposed model self-defines the dimensions of the contact
cylinder, and therefore accounts for the heterogeneous composition and viscoelastic properties of bacteria.

Based on the elastic deformation model introduced in the previous chapter, we propose an alternative method to decouple the short- and long-range contributions to the bacterial adhesion force in Chapter 5. In order to identify the effects of various bacterial cell surface structures on the adhesion force, four strains of \textit{S. epidermidis} and \textit{S. salivarius} were examined that allow pair-wise comparisons with respect to their surface features (\textit{i.e.} the capability to produce slime and the existence of a fibrillated layer). The proposed method involves a linear relationship between the adhesion force and the applied loading force in AFM measurements, which was confirmed by experimental data for three out of four strains investigated, the only exception being the bald \textit{S. salivarius} HB-C12 strain. In line with Poisson analysis of adhesion forces, the long-range forces were attractive in most cases and their magnitudes were below 1 nN regardless of the loading force. Both methods reported significantly higher short-range forces for the slime-producing \textit{S. epidermidis} ATCC 35984 and the fibrillated \textit{S. salivarius} HB-7 than for \textit{S. epidermidis} ATCC 35983 and the bald \textit{S. salivarius} HB-C12 strain, respectively. Our findings suggest that the existence of extracellular features affects bacterial adhesion mainly by impacting short-range forces.

By applying this method to two \textit{S. aureus} strains (NCTC 8325-4 and ATCC 12600) and their isogenic \textit{Δpbp4} mutants, we demonstrate in Chapter 6 that the long-range adhesion forces of wild type, parent strains (0.5 and 0.8 nN) amounted to only one third of these forces measured for their, more deformable mutants (2.7 and 1.6 nN) that were deficient in peptidoglycan cross-linking. Whereas the outermost cell wall structures might vary most across different strains, yet the overall composition of different bacterial strains is rather similar, which suggests that the variations observed in long-range adhesion forces might have other
Summary

sourcing than differences in chemical composition. Measured long-range LW adhesion forces matched those calculated from published Hamaker constants, provided a 40% ellipsoidal deformation of the bacterial cell wall was assumed for the Δpbp4 mutants. We also measured the deformation directly using the AFM in the PeakForce-QNM mode, which confirmed height reduction due to deformation in the Δpbp4 mutants by 164 and 98 nm. The difference in origin of external loads in both methods likely explains why the deformation calculated from matching measured and theoretically calculated LW forces was larger than directly measured using the AFM in the PeakForce-QNM mode. Our results support that Δpbp4 mutants were mechanically "softer" than their parent strains and deformed significantly under loading, which is consistent with the lack of cross-linked peptidoglycan strands in their cell wall. More importantly, extrapolating from the results of this chapter, we reveal that a small, hitherto neglected deformation of the bacterial cell wall may occur upon bacterial adhesion to surfaces, and therewith pave the way for a better understanding of poorly understood phenomena like bacterial “stress-deactivation” upon strong adhesion of micron-sized bacteria to a substratum surface.

As a sequel to Chapter 4 where we established a method to derive the elasticity of the contact volume of bacteria adhering to a substratum surface, Chapter 7 was devoted to the determination of the viscous nature of this bond for six strains under two different loading forces. Strains were chosen to allow pairwise comparisons to identify the effects of the density and length of fibrillar surface appendages in isogenic S. salivarius mutants, EPS in S. epidermidis strains and the degree of peptidoglycan cross-linking in two isogenic S. aureus mutants. To this end, a tailor-made script was written for an AFM, that enabled a constant loading force of 1 nN or 5 nN to act for 30 s upon a bacterium wrenched between a cantilever and a glass surface, while measuring its deformation. Time-dependent
deformation was fitted to a one element Kelvin-Voigt analogue of the bond to yield a characteristic relaxation time and viscosity of the bond. Within the pair of fibrillated streptococci, increasing fibrillar density and length yielded slightly longer relaxation times, but viscosities were similar. Within staphylococcal pairs, the presence of increasing amounts of EPS, or the deficiency in peptidoglycan cross-linking significantly increased the relaxation times, and yielded slightly larger viscosities. Viscosities of streptococcal bonds were smaller (< 20 kPa s) than of staphylococcal bonds (> 31 kPa s). Since staphylococci are relatively rich in EPS, while streptococci have mainly fibrillar appendages, it can be concluded that presence of EPS contributes more to a viscous response than fibrils. The viscous nature of the bond between an adhering bacterium and a substratum surface slows down the impact of external stresses and may provide the bacterium with more time to respond and protect itself against such stresses.

A discussion on the assumptions underlying our analyses and models as presented in this thesis, together with challenges and future applications of the AFM study of bacterial adhesion forces is presented in Chapter 8. An attempt to combine AFM data with results using a quartz crystal microbalance with dissipation for investigating the bond between an adhering bacterium and a substratum surface is also briefly reviewed. Finally, the major conclusions of this thesis are summarized.