Macroscopic and microscopic approaches toward bacterial adhesion

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Bacteria are, with respect to their dimensions, colloidal particles, and hence the process of bacterial adhesion is commonly described by the Derjaguin-Landau-Verweij-Overbeek (DLVO) theory of colloidal stability. The DLVO theory estimates the interaction forces between the interacting surfaces based on Lifshitz-Van der Waals (LW) and electrostatic (EL) interactions and their decay length with separation distance. The most important parameter determining the LW interaction is the Hamaker constant, which is a material property. The value of the Hamaker constant is in most cases rather uncertain, yet for microbial cell surfaces it is currently being estimated from contact angles measurements on bacterial lawns. On the other hand, calculation of electrostatic interactions requires knowledge of the electrostatic surface potential of the interacting surfaces. The electrostatic surface potential of microbial cell surfaces cannot be measured directly but is commonly estimated from measured electrophoretic or electro-osmotic velocities observed during dynamic measurements in electric fields. Yet, application of DLVO theory to explain bacterial adhesion to solid substrata has only been successful for a
limited number of strains and species. In general, the degree of success of the DLVO theory to explain bacterial adhesion frequently decreases as the complexity of the cell surface of the organisms under consideration increases. It is important to realize that bacterial cell surfaces are both chemically and structurally more complex and heterogeneous than most inert colloidal particles. Therefore, a need exists for microscopic characterization of the physico-chemical properties of bacterial cell surfaces. Subsequently, a generalized physico-chemical theory to account for bacterial adhesion to substratum surfaces might become in reach.

The introduction of the atomic force microscope (AFM) and its application to biological surfaces has offered new possibilities to obtain microscopic physico-chemical properties of bacterial cell surfaces. Using AFM, the interaction force between a single bacterium and a solid substratum (such as an AFM probe) can be directly measured down to the nanoNewton range. Yet, interpretation of the force involved as well as distinction between its different components remains difficult. The main challenge in extracting tip-surface forces is the exact determination of the distance origin relative to the tip location. Bacterial deformation under the applied load or tip penetration through extracellular material could likely overestimate the distance range at which interaction forces are measured. The most current approach to interpret AFM force-distance curves is still to assume that the bacterium is linearly elastic and that all non-linearity displayed on the curves is a consequence of electrostatic and (electro)steric surface forces (Razatos et al., 1998; Ong et al., 1999). It is worthy to note that poor agreement has been found up to date between AFM force-distance curve interpretations and bacterial adhesion to solid substrata as macroscopically observed (Burks et al., 2003). In addition, attempts to fit DLVO theory with AFM data have mostly failed since AFM often predicts higher forces operating over larger distances than theoretically expected (Camesano & Logan, 2000).

This thesis represents a further attempt to correlate microscopic surface properties of bacterial species as derived from force-distance curve analyses to their macroscopic adhesion to solid substrata as directly observed under well-defined flow conditions. In order to formulate generic relations, bacterial species differing with respect to their outermost surface were considered along the subsequent studies, i.e. an entire collection of fibrillated Streptococcus mitis strains, an extracellular polymeric substances (EPS)-producing Streptococcus thermophilus B strain and two lactobacillus strains with and without a surface layer protein (SLP) anchored to their cell envelope. In general lines, it was observed that

a) approach lines of force-distance curves correspond with the rate of initial macroscopic adhesion of the organisms to a substratum

b) the maximum distance over which attractive forces are probed by AFM upon retraction of the tip are influential on the area blocked by an adhering bacterium on a substratum

c) bacterial desorption from solid substrata does not show a relation with adhesive forces as probed by AFM unless the contact time between bacterium and substratum is taken into account.
These three aspects will now subsequently be discussed.

**Approach lines of force-distance curves correspond with the rate of initial, macroscopic adhesion of the organisms to a substratum**

Although the energy barrier detected upon approach of the AFM tip to the bacterial cell surface could be interpreted as an activation energy for the organisms to successfully deposit, the magnitude of the activation energies observed ranged between 2400 kT and 200000 kT (for *S. mitis* 244 in water and the lactobacillus strain with SLP in 100 mM KCl solution) among all strains considered. These activation energies are prohibitively high for their spontaneous adhesion observed on macroscopic substrata under laminar flow conditions. However, considering that most of the bacterial strains studied here bear extracellular material at their cell surface envelope, the following experimental situation could have been encountered during AFM:

(i) At a long separation distance between the tip and the bacterial surface, the force experienced by the AFM tip is zero. (ii) As the tip approaches the bacterial surface, the cantilever may bend upwards due to a repulsive force. (iii) At a certain distance, the tip gets into direct contact with the extracellular material known to surround the cell envelope. After this first contact, the repulsion observed is due to a nonlinear deformation dominated by the initial penetration of the AFM tip through the...
extracellular material. (iv) Subsequently, a few layers of water may need to be removed before intimate contact with the peptidoglycan layer is eventually reached. (v) Finally, deformation of the peptidoglycan layer could take place. This indicates that, the major part of the activation energy estimated belongs to a nonlinear deformation of the bacterial extracellular material. In the particular case of S. mitis strains, for instance, the non-linearity likely reveals an increased gradient on fibrillated material density towards the peptidoglycan layer. Additionally, the highest energy barriers detected for the lactobacillus strains correspond with a mechanically harder coat surrounding the envelope of these specific bacterial strains.

At high ionic strength, the DLVO theory for colloidal stability predicts for all S. mitis as well as for both lactobacillus strains studied a net attractive force at all separation distances between the bacterium and a substratum. Consequently and based on the previous interpretation of force-distance curves, the total force needed for the AFM tip to overcome in order to establish close contact with the bacterial cell surface would represent an estimation of the rigidity of the extracellular material surrounding the cell envelope whereas the distance range associated to such a force would make reference to the thickness of the extracellular material. Thickness values ranging from 50 to 314 nm were found for the polyelectrolyte layer surrounding S. mitis strains. Even though these strains are characterized as possessing extremely long fibrils at their cell surfaces, hydrodynamic measurements have revealed that long appendages of fibrillated strains collapse onto the cell surface upon increasing the ionic strength (Van der Mei et al., 2000a). Therefore, Figure 5.5 in Chapter 5 (showing that the rate of initial, macroscopic adhesion of S. mitis to a substratum increases as the force needed to achieve contact between the AFM tip and bacterial cell surface decreases) could be re-interpreted as follows:

a compressed and more rigid polymer layer results in higher bacterial adhesion. This phenomenon has been previously pointed out by Abu-Lail & Camesano (2003b) studying the role of biopolymer conformation on the adhesion of Pseudomonas putida KT2442. Additionally, the fibrillated Streptococcus salivarius HB has been reported to adhere in higher numbers (regardless of the substratum wettability) as the ionic strength of the medium increases (Sjollema et al., 1990a). This observation likely corresponds once more with a compressed and more rigid surrounding polyelectrolyte layer. Results quantifying the macroscopic adhesion behavior of the lactobacillus strains at high ionic strength overlapped and hence, conclusions cannot be drawn to predict adhesion as function of the rigidity of their outermost surface. Nevertheless, AFM reveals a harder coat for the strain having SLP, in agreement with the proteinaceous crystalline structure known to be present at the outermost surface of these strains. Remarkably, the distances associated to the AFM approach lines of these bacteria are higher (56 -214 nm) than the ones associated to the fibrillated material collapsed onto the cell surfaces for several S. mitis strains. Both lactobacillus strains have been reported to be sensitive to hyperosmotic conditions regarding secretion of extracellular substances
(Machado et al., 2004; Fernandez Murga et al., 1999). A thick extracellular layer present as a result of the bacterial immobilization method used for immobilization in AFM, i.e. physical adsorption on positively charged glass, could account for the larger distances over which forces are recorded for the AFM tip upon approach to both lactobacillus strains. This phenomenon was pointed out in Chapter 3 comparing the interaction forces obtained between an EPS-producing Klebsiella terrigena strain and the silicon nitride tip of the AFM for three immobilization methods. Based on the results obtained, it was concluded that physical immobilization on a positively charged surface likely stimulates the secretion of EPS by K. terrigena.

At low ionic strength, it was shown that bacterial deposition probably takes place in the secondary minimum of the total Gibbs energy. Interestingly, in 10 mM KCl the AFM tip recorded a repulsion force regardless the strain studied. The presence of extracellular material implies that electrosteric repulsive interactions dominate the DLVO forces. Van der Mei et al. (2000a) have reported AFM characterization of a mechanically trapped ‘bald’ Streptococcus salivarius HBC12 in water. Approach lines were always repulsive (acting range ~21 nm) and no attraction was recorded at the position of the secondary minimum or at short separation distances. AFM likely does not allow to measure the distance dependence of attractive forces at short separation distances. When the gradient of the attractive forces exceeds the spring constant of the cantilever plus the gradient of possible repulsive forces, an instability may occur and at a certain distance the tip jumps onto contact with the surface. In addition, the attractive force predicted by the DLVO theory prior to reaching the secondary interaction minimum can be calculated from the slope of the distance dependence of the total Gibbs energy and amounts to $10^{-13}$ N for S. salivarius HBC12 interacting with hydrophilic, negatively charged glass across a low ionic strength solution. This force may be at least an order of magnitude lower than the force resolution of the AFM. Therefore, AFM likely fails to detect DLVO forces when bacterial cells are investigated.

$\xi_{\text{bacterium}} = -8 \text{ mV}; \quad \xi_{\text{substrate}} = -50 \text{ mV}; \quad H = 6 \times 10^{-21} \text{ J}; \quad a = 500 \text{ nm}$
The maximum distance over which attractive forces are probed by AFM upon retraction of the tip are influential on the area blocked by an adhering bacterium on a substratum

This conclusion is based on the hypothesis that multiple adhesion forces upon retraction of the AFM tip from bacterial cell surfaces are due to multiple cell surface polymers adhering to and detaching from the AFM tip. This hypothesis is also supported in the literature (Abu-Lail & Camesano, 2003b) and here by the observation that the number of local maxima in adhesion forces was not very sensitive to ionic strength when the fibrillated S. mitis strains were investigated. In consequence, at the distance over which the bacterium exerts adhesive forces through the extension of fibrils of different lengths, it is likely that bacteria are brought closer together and the distance between adhering bacteria is reduced, yielding smaller blocked areas (shown on Figure 5.6, Chapter 5).

This mechanism has been observed by AFM at a more molecular level for flexible rod-like proteins associated to microtubules found in the cytoplasm of eukaryotic cells. The projection domain of these proteins was identified as cross-bridges between microtubules based on the distance range of the forces probed by AFM (Mukhopadhyay & Hoh, 2001).

Bacterial desorption from solid substrata does not show a relation with adhesive forces as probed by AFM unless the contact time between bacterium and substratum is taken into account

Although the adhesion force measured by the AFM tip upon retraction was expected to be indicative for the desorption of bacteria in the parallel plate flow chamber, no clear relation could be found when S. mitis or lactobacillus strains were studied. It was hypothesized that this has to do with the nature of the desorption process. Desorption in the parallel plate flow chamber takes place as a spontaneous process under prevailing shear conditions, while in AFM the contact between the bacterium and surface substratum is forced to break by application of
an external force. Actually, it was shown in Chapter 6 that spontaneous desorption is related with the adhesion forces probed by the AFM if the contact time between bacterium and substratum is taking into account. AFM indicated that bond strengthening between the tip and *Streptococcus thermophilus* occurred within the same time as bond aging inferred from residence time-dependent desorption on a macroscopic level. In addition, comparison of the interaction energies derived from AFM and macroscopic desorption seems to indicate that bond strengthening arises as a result of multiple attachments of extracellular polymeric substances to a substratum surface. The fact that bridging controls desorption thus became evident.

The main forces controlling spontaneous bacterial desorption are thought to be Van der Waals attraction and bridging events taking place between cell surface macromolecules and the substratum. In order to investigate a possible role of Van der Waals forces to macroscopic spontaneous desorption $\beta$, $\beta$ is plot vs. $L_0$ i.e. the equilibrium length of the bacterial surface polymers as calculated applying the electrosteric model for the collection of *S. mitis* strains,

![Graph](image)

At first sight, it can be observed that the relation between $\beta$ and $L_0$ appears strongly influenced by the bacterial cell surface hydrophobicity (expressed between brackets in the graph) whereas 60 degrees appears to be a critical contact angle for strong desorption to occur. It has been reported that at high ionic strength steric interactions determined by cell surface macromolecules dominate adhesion (Rijnaarts et al., 1999). The steric interactions either promote adhesion by bridging or inhibit it by steric repulsion. The surfaces of the *S. mitis* strains may contain amphiphilic macromolecules with hydrophobic parts would induce steric repulsion on hydrophilic glass (but likely bridging on an hydrophobic substratum) while hydrophilic parts would likely promote bridging. Yet, as a general trend, both for more hydrophobic bacteria and more hydrophilic ones, desorption decreases with increasing thickness of the polyelectrolyte layer.
Accounting for Van der Waals forces, mainly arising from the body of the bacterial cell, the decrease of desorption $\beta$ with the increase of $L_0$ could be readily understood if the dependency of Van der Waals forces with the separation distance (in this case coinciding with $L_0$ i.e. the thickness of the polyelectrolyte layer) is taking into account. In addition, note that $L_0$ ranges between 40 and 71 nm. At such distances, Van der Waals forces are known to still play an important role.

Surface hydrophobicity has proven its value in bacterial adhesion studies (Rosenberg & Kjelleberg, 1986). Yet, it has also been observed that adhesion of certain strains to solid substrata often does not proceed according to expectations based on cell surface hydrophobicity and hydrophobic strains do not always adhere best to hydrophobic substrata, as predicted by surface thermodynamics (Van der Mei et al., 2003). Indeed, a definite role for bacterial and/or substratum hydrophobicity in adhesion has not been yet established and the term hydrophobicity remains still poorly defined.

In practice, the effect of cell surface hydrophobicity in adhesion is generally studied by quantification of the number of attached cells on substrata of different wettabilities. Chemical modification of AFM tips with organic thiols terminated with selected terminal groups has likely provided a new avenue to prove the enigmatic ‘hydrophobic interactions’. Accurate quantification of the role of hydrophobic interactions in adhesion processes would require the investigation of simpler systems than bacterial cell surfaces. Nevertheless, when bacterial cells are investigated, short-range ‘hydrophobic interactions’ could be expected to be recorded by hydrophobic and hydrophilic AFM tips either upon approach to or retract from the bacterial cell surface. Upon approach, a short-range hydration repulsion force is expected if both the interacting surfaces are hydrophilic. Hydrophilic surfaces bind a few layers of water molecules and their removal will lead to a repulsion force when the surfaces are forced into intimate contact (corresponds to (iv) in tip deflection vs. separation distance). If both surfaces are hydrophobic, attractive forces are expected. Again, AFM likely does not allow to measure the distance dependence of attractive forces at short separation distances. Upon retract, the average adhesion force detected by the AFM tips has been observed to be stronger for the combinations of a hydrophobic bacterium/hydrophobic substratum and hydrophilic bacterium/hydrophilic substratum than for the hydrophobic/hydrophilic combinations (Chapter 7 & 8).
The strength of hydrophilic interactions may reflect the complementarily and weak interactions between molecular groups on the surfaces interacting, *i.e.* hydrogen bonds, ionic interactions and ‘hydrophobic interactions’. The formation of each of these weak bonds contributes to a net decrease in the free energy of the system. ‘At short range’ the cumulative effect of many small binding forces could be enormous. Integration of the area under the maximum adhesion peak after contact in the force-distance curve has been reported to yield interaction energies of $10^4 - 10^5$ kT between the tip and the EPS-producing *K. terrigena* (Chapter 3). In addition, the strength of hydrophobic interactions also result from the system achieving greater thermodynamic stability by minimizing the number of ordered water molecules, which would surround the hydrophobic surfaces in solution.

Based on related literature and on the results presented here it is concluded that AFM fails to detect long-range DLVO forces when bacterial cell surfaces are investigated whereas the major force contributions recorded are electrosteric, elastic, polymer extension and binding (examples of equations for these interactions using a sphere-plane geometry are presented in the Table below (Heinz & Hoh, 1999b)).

<table>
<thead>
<tr>
<th>Approach</th>
<th>Retraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrosteric</td>
<td>Polymer extension</td>
</tr>
<tr>
<td>$F_a = 50k_BTaL_o\Gamma^{-3/2}\exp(-2\pi h/L_o)$</td>
<td>$F(x) = (kT/l_0)L^*_{\Gamma}(x/Nl_0)$</td>
</tr>
<tr>
<td>$0.2 \leq D/2L_o \leq 0.9$</td>
<td></td>
</tr>
<tr>
<td>Elastic</td>
<td>Binding (bond aging)</td>
</tr>
<tr>
<td>$F(\delta) = 4E\sqrt{a} \delta^{3/2}/3(1-\nu)^2$</td>
<td>$F = (U-kTln(\tau/\tau_0))/A$</td>
</tr>
</tbody>
</table>

- $a$: radius of probe sphere
- $E$: elastic modulus
- $h$: separation distance
- $k_B$: Boltzmann’s constant
- $L_o$: brush thickness in a good solvent
- $L^*$: inverse Langevin function
- $l_0$: monomer length
- $N$: number of unit in polymers
- $T$: absolute temperature
- $x$: elongation of polymer
- $\delta$: indentation depth
- $\Gamma$: brush density per unit area
- $A$: characteristic length of the bond
- $\tau_i$: reciprocal of the natural bond frequency
- $\tau$: period over which the bond will rupture
- $\nu$: Poisson ratio
Additionally, short range interactions such as hydrogen bonds, ionic interactions and ‘hydrophobic interactions’ might dominate at very short separation distances. Yet, distinction between the components of the total force involved requires accurate knowledge of the exact position of the tip relative to the bacterial cell surface. On any of the force-distance curves recorded for this thesis a clear transition between the different force regimes was observed. Perhaps, performing force-distance curves measurement at lower scan rate and plotting the mathematical gradient (‘rate of change’) of the tip deflection vs. the separation distance (instead of the deflection vs. separation distance) could give a better picture of the transitions taking place.

An accurate AFM microscopic characterization of physico-chemical properties of bacterial cell surfaces might benefit from the use of well-defined geometry probes (i.e. micro-spheres) with known physico-chemical properties. This would likely provide new insight for the development of theoretical models for colloid-like interactions. Additionally, systematic experiments need to be performed with strains differing in their outermost layer (e.g. fibrils, fimbriae, pili, capsules, S-layers). If different bacteria are compared there may be the need to introduce an empirical, strain-dependent factor to account for the surface structures of the cell.

In the near future, functionalization of AFM probes with biomolecules might allow mapping of the distribution of individual specific surface components and specific recognition forces which are known to play an important role in certain microbial adhesion processes. An important challenge will also be to combine force measurements with high resolution imaging on living cells. This will make it possible to correlate force interactions with morphological observations.