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Macroscopic and microscopic adhesive properties of microbial cell surfaces

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1.1 INTRODUCTION
The study of microbial adhesion encompasses a broad range of scientific disciplines, ranging from medicine, dentistry and microbiology to colloid and surface science. Initially, the involvement of colloid and surface scientists originated from the simple realization that microorganisms are, with respect to their dimensions, colloidal particles and that their adhesion should be predictable by surface thermodynamics (Absolom et al., 1983) or Derjaguin-Landau-Verwey-Overbeek (DLVO)-theory like approaches (Bos et al., 1999). Considering the ubiquitous nature of microbial adhesion, such generalized predictive models would be extremely valuable.

Microorganisms have, from a physico-chemical point of view, generally been considered to be similar to inert polystyrene particles. However, microorganisms are not smooth particles and, in contrast to polystyrene particles, carry long, usually very thin surface structures protruding from the cell surface and radiating outwards into the surrounding liquid. These structures are responsible for adhesion to a variety of surfaces. Bacteria in particular carry a wide range of surface structures that have been described on the basis of their ultrastructure and distribution on the cell surface. There are many morphologically distinct types of surfaces structure and almost every bacterial strain or species carries its own type of surface appendage. Yet, the function of these surface structures in specific adhesion processes to different substratum surfaces as well as their influence on overall physico-chemical cell surface characteristics, remains to be identified for most bacterial strains.

Application of physico-chemical models to explain microbial adhesion to solid substratum have been successful for a limited number of strains and species (Van Loosdrecht et al., 1989), despite the macroscopic nature of the input data (i.e. Hamaker constants, acid-base properties, zeta potentials and contact angles). Therefore, relevant physico-chemical measurements on microbial cell surfaces require a microscopic resolution that can not be accomplished with most currently employed methods.

The aim of this chapter is on the one hand to point out the merits of a macroscopic physico-chemical approach toward microbial adhesion, while on the other hand emphasizing the need of a microscopic physico-chemical surface characterization.

1.2 MACROSCOPIC PHYSICO-CHEMICAL CHARACTERISTICS OF MICROBIAL CELL SURFACES

Bacterial cell surface hydrophobicity and charge are commonly accepted as influential on bacterial interactions with their environment. Therefore contact angles and zeta potentials of microbial cell surfaces are frequently measured for use as input data for predictive, physico-chemical models of their adhesion to surfaces. Furthermore, at a similar overall level as cell surface hydrophobicities by water contact angles and zeta potentials, chemical composition data of cell surfaces are being obtained by unexpected techniques, like X-ray photoelectron spectroscopy (XPS) (Amory et al., 1988) or secondary-ion mass spectroscopy (SIMS) (Tyler, 1997).

1.2.1 Cell surface hydrophobicity

Hydrophobicity is a general term utilized to describe the relative affinity of a surface for water. Microbial cell surface hydrophobicity can only be measured by placing water droplets on carefully prepared and dried microbial lawns (Busscher et al., 1984). If water molecules have a greater preference to surround each other than to contact a microbial cell surface, the surface appears as hydrophobic and water droplets do not spread. In contrast, if water molecules favor a microbial cell surface rather than each other, a water droplet spreads and the surface appears hydrophilic.
Spreading is determined in part by the intermolecular forces, such as Lifshitz-Van der Waals and acid-base forces (Van Oss, 1995), as can be calculated amongst others, from the Hamaker constants and measured contact angles with liquids. The only non-trivial step in contact angle measurements on microbial lawns involves the degree of drying of the lawns. It must be realized that drying of the lawns determines the degree of collapse of microbial surface appendages and therewith the contact angle measured (Busscher et al., 1984). Usually contact angles measured on microbial lawns increase as a function of drying time until a so-called “plateau” is reached. As long as the lawns are dried to the plateau for water contact angles, the cell surface is assumed to be in a physiologically relevant state. However, theoretically, cell surface hydrophobicity cannot be measured solely by water contact angles as this does not allow to account for acid-base interactions as occurring between water molecules and between water molecules and the microbial cell surface. Indeed, whereas water contact angles have been designated as the intrinsic surface hydrophobicity of a microbial cell surface, the thermodynamic cell surface hydrophobicity strictly speaking reflects its surface free energy, including Lifshitz-Van der Waals and acid-base components. Calculation of the thermodynamic cell surface hydrophobicity requires contact angle measurements with at least three different liquids with varying acid-base properties, as water, formamide, methyleneiodine and/or α-bromonaphthalene (Van Oss et al., 1987).

The cell surface hydrophobicity describes a macroscopic surface property. The hydrophobicity of surface appendages, like fibrils or fimbriae may be substantially different from the overall cell surface hydrophobicity. However, despite being an overall cell surface property, microbial contact angles vary greatly between different strains and even the presence of tufts of fibrils as on Streptococcus sanguis strains is reflected in the measured values of contact angles (Busscher et al., 1991). Both Streptococcus salivarius HBC12 and S. sanguis CR311 VAR3 have bald cell surfaces

Figure 1.1. Electron micrograph of S. sanguis PSH 1b labeled with colloidal gold at pH 10.2. Gold is attached only to the ends of the long fibrils. Cells were stained with 1% methylamine tungstate. Bar marker is 200 nm (adapted from Van der Mei et al. (1998b) with permission).
and are hydrophilic (water contact angles 21 and 31 degrees, respectively) compared with their peritrichously fibrillated (S. salivarius HB) and tufted (S. sanguis CR311) parent strains. Hence, hydrophobicity is conveyed to the cell surface by fibrils and fimbriae and unlikely by the bald cell surface. Handley et al. (1991), for instance, demonstrated that hydrophobic, colloidal gold particles only adsorbed to the tip of fibrils on S. sanguis PSH 1b and concluded that the cell surface hydrophobicity was confined to the ends of the long fibrils (see Figure 1.1).

Therefore, contact angles measured with liquid droplets on a microbial lawn are essentially representative of a fuzzy coat of cellular surface material, collapsed into a lawn. Therewith results are useful to interpret the long-range interactions between an organism and a substratum surface, but not necessarily for the interpretation of short-range interactions, which may be dominated by structural and chemical cell surface heterogeneities (Van der Mei et al., 1998b).

1.2.2 Zeta potentials
Particulate electrophoresis is the most common method to determine bacterial zeta potentials and surface charge densities (James, 1991). The measurement of microbial electrophoretic mobilities and the derivation of zeta potentials thereof by particulate microelectrophoresis proceeds according to the standard methodologies in physico-chemistry (James, 1991), although the calculation of zeta potentials from measured electrophoretic mobilities is not always straightforward (Van der Wal et al., 1997). Furthermore, it is important to realize that the plane of shear may be removed far away from the bacterial cell wall if long fibrils or fimbriae are present (e.g. as on Streptococcus mitis strains) and consequently, in these cases, the zeta potentials measured are not representative for the one of the true cell surface (Figure 1.2).

Moreover, long appendages may collapse onto the cell surface upon increasing the ionic strength, as demonstrated e.g. by dynamic light scattering (Van der Mei et al., 1994). Depending on ionic strength of the liquid, also electro-osmotic flow of fluid may occur within these polyelectrolyte layers.

Fibrils, fimbriae and even extracellular surface polymers around bacterial cells may be considered as a polyelectrolyte layer, possessing a number of fixed charges, i.e. ionic groups that are covalently linked to the polymer and thus have a strong impact on the electrostatic interactions of bacteria with surfaces. Hayashi et al. (2001) and Poortinga et al. (2001) have both described that this electrostatic repulsion is often overestimated due to the neglect of bacterial cell surface softness, i.e. the ease with which electro-osmotic fluid flow develops in the surface layer. Soft, ion-penetrable cell surfaces experience less electrostatic repulsion than similarly charged, hard, ion-impenetrable surfaces, since their diffuse layer charges are driven into the ion-penetrable cell walls causing an effective decrease in surface potential (see also Figure 1.2) and, hence, electrostatic repulsion. Recently, Morisaki et al. (1999) explained adhesion of a negatively charged marine bacterium Vibrio alginolyticus, onto a negatively charged substratum by considering the softness of the strains.

When analyzing microbial adhesion data in terms of electrostatic interactions, it should not be a priori assumed that the electrostatic interaction is repulsive, simply
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Figure 1.2. Schematic representation of the surfaces of a negatively charged ion-impenetrable (a) and ion-penetrable bacterium (b). The electrokinetic potential decreases exponentially from the ion-impenetrable bacterial core surface and the slip plane, determining its zeta potential close to the surface. The ion-penetrable bacterium is covered by a polyelectrolyte layer with fixed negative charges, through which electrophoretic fluid flow is possible. The slip plane is assumed to remain at approximately the same position as for the ion-impenetrable bacterium inside the soft layer. Therefore, the zeta potential is more negative than the potential $\Psi_0$ at the outside of the soft layer (taken from Kiers et al. (2001) with permission).
because the zeta potentials of the interacting surfaces are both negative. Indeed, nearly all biological surfaces carry a net negative charge. However, at a more microscopic level than the macroscopic level of particulate microelectrophoresis, microbial cell surfaces may have positively charged domains mediating adhesion through local electrostatic attraction despite overall repulsion, like described e.g. the interaction between *Treponema denticola* and human erythrocytes (Cowan et al., 1994). Obviously, a minor number of positively charged sites, while instrumental for adhesion, does hardly affect the macroscopic cell surface charge density.

1.2.3 Chemical composition of microbial cell surfaces

X-ray photoelectron spectroscopy (XPS) provides a mean to obtain the chemical composition of the outermost microbial cell surfaces (Amory et al., 1988). XPS spectra of microbial cell surfaces are fairly similar, with carbon, nitrogen, oxygen and phosphorous being the main elements detected, albeit in different amounts on different isolates. Decomposition of $C_{1s}$ and $O_{1s}$ electron binding energies has furthermore indicated the presence of lipids, proteins and polysaccharides. As XPS is a high vacuum technique, an extensive sample preparation, including washing, centrifuging and freeze-drying, is involved before microbial cell surfaces can be studied by XPS (Rouxhet et al., 1994). These steps obviously bring the cell surface in a state that is far remote from its physiological one. Some authors believe that the integrity of the vulnerable cell surface of especially Gram-negative bacteria, as compared with Gram-positive bacteria, is disrupted by this extensive preparation (Marshall et al., 1994) with a potential impact on the results.

However, the relevance of XPS for the analysis of microbial cell surfaces is supported by the relationships with other physico-chemical cell surfaces properties, preferably measured on cells in a more physiologically relevant state than in their dehydrated, freeze-dried state as for XPS. For instance, combinations of contact angle data on a collection of widely different streptococcal strains and XPS have demonstrated that hydrophobicity is conveyed to the cell surface by nitrogen-rich groups, concurrent with the possession of a high isoelectric point (the pH at which the zeta potential is zero) (Van der Mei et al., 1988b). Furthermore, the presence of tufts of fibrils on *S. sanguis* CR311 increased the N/C from 0.066 of a bald variant to 0.085 for the parent strain, indicating a nitrogen (protein) rich composition of the tufts (Busscher et al., 1991). In the same way, the progressive removal of fibrils from the cell surface of *S. salivarius* HB was accompanied by a significant decrease in the N/C elemental surface composition ratio (Van der Mei et al., 1988a). However, like almost all physico-chemical methods for the study of microbial cell-surfaces properties, the spatial resolution of XPS is inadequate to deal with chemical and structural heterogeneities, like sparsely or unevenly distributed fibrils or fimbriae on cell surfaces.

Secondary-ion mass spectroscopy (SIMS) is a surface sensitive technique, that also probes the chemical composition of a surface, but through a different principle as XPS. XPS involves the bombardment of a surface with X-rays and the subsequent measurement of the photo-emitted electrons. Since these photo-emitted
electrons have discrete kinetic energies that are characteristic of the emitting atoms and their bonding states, they can be applied for chemical analysis. In SIMS, the surface is bombarded with a focused beam of primary ions, the impact of which produces secondary ions that are collected and focused in a mass spectrometer where they are separated according to their mass. Tyler (1997) has shown that SIMS can also be applied to probe microbial cell surface chemistry. SIMS spectra of four freeze-dried strains, S. salivarius HB and three mutants, indicated the presence of proteins, hydrocarbons and carbohydrates on the bacterial cell surfaces, as well as of proteins and teichoic acid on the cell wall. The correlation between SIMS spectra and previous XPS analysis on those strains was excellent. Taking into account that SMIS is not only capable of providing accurate analysis of surface chemistry, but is also sensitive to the composition and orientation of bio-molecules, the potential of this technique to characterize bacterial cell surfaces seems promising but needs to be further explored.

1.3 MICROSCOPIC PHYSICO-CHEMICAL PROPERTIES OF MICROBIAL CELL SURFACES BY ATOMIC FORCE MICROSCOPY

Even though structural and chemical heterogeneities on microbial cell surfaces have an impact on the overall cell surfaces, methods to obtain detailed knowledge on cell surface heterogeneities are still lacking. Indeed, considering the importance of structural and chemical heterogeneities in microbial adhesion, the development of a generalized model for microbial adhesion to surfaces seems still far beyond reach. However, the introduction of the atomic force microscope (AFM) and its application to biological surfaces (Dufrêne, 2001) has offered new possibilities to obtain microscopic physico-chemical properties of bacterial cell surfaces.

AFM provides exciting possibilities for probing the structural and physical properties of living microbial cells. Using topographic imaging, cell surface nano-structures (e.g. appendages and flagella) can be directly visualized (see for instance Figure 1.3 and the changes of cell surface morphology occurring during physiological processes can be determined.

Force-distance curves, shown in Figure 1.4, can provide complementary information on surface forces and adhesion mechanisms at a square nano-meter scale, yielding new insight into the mechanisms of biological events such as microbial adhesion and aggregation.

Initial AFM studies on bacterial cell surfaces have focused on probing surface morphology and surface forces. For instance, Razatos et al. (1998) showed that the adhesion force between a silicon nitride AFM tip and Escherichiae coli was affected by the length of lipopolysaccharide molecules on the cell surface and by the production of a capsular polysaccharide. Furthermore, it was discovered using AFM that E. coli JM109 and K12J62 have different surface morphologies dependent on environmental conditions, while lysozyme treatment led to the loss of surface rigidity and eventually to dramatic changes of bacterial shape (Bolshakova et al., 2001). Camesano and Logan (2000) concluded that the interaction between
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Figure 1.3. AFM contact mode topographic image of *S. mitis* T9 immersed in water. The image reveals characteristic topographic features on the right hand side of the cell surface, *i.e.* lines oriented in the scanning direction, attributable to fibrils.

Figure 1.4. Force-distance curve of *S. mitis* T9 in water. The solid line represents the approach curve, while the dashed line indicated the retraction curve. Upon approach, a long-range repulsion, starting at a separation of ~ 100 nm, was detected, while no jump-to-contact was observed. Upon retraction, multiple adhesion forces were found.
negatively charged bacteria and the silicon nitride tip of an atomic force microscope was dominated by electrosteric repulsion. Only a limited number of studies have focused on the characterization of local properties of bacterial cell surfaces. Recently, for instance, the turgor pressure of a spherical bacterium, *Enterococcus hirae*, in deionized water was derived from the indentation depth caused by an AFM tip and found to be between 4 and $6 \times 10^5$ Pa (Yao *et al.*, 2002). However, in order to develop a ubiquitously valid physico-chemical model for microbial adhesive interactions, microscopic characterization of properties as hydrophobicity, surface charge density and chemical composition on microbial cell surfaces is required. Due to the structural and chemical heterogeneities that the cell surfaces present, it would be of interest to collect an array of force curves over the entire cell surface. Such an array would produce information about the distribution of different surface properties. For example, using charged or chemically functionalized AFM tips to probe the surface, would allow localizing more specific interactions at a microscopic level. At present, the only charge maps for biological samples have been made for bacteriorhodopsin membrane patches (Butt, 1992) and phospholipid bilayer patches (Heinz and Hoh, 1999a) on hard substrates. From the known surface charge density of the substratum, it was possible to calculate a reasonable value for the surface charge density of the membrane (Butt, 1992). Hydrophobicity at a microscopic level has been probed on spore surfaces of the fungus *Phanerochaete chrysosporium* (Dufrène, 2000) by using chemically modified AFM probes, terminated with OH (hydrophilic) and CH$_3$ (hydrophobic) groups.

1.4 TOWARD RELATIONS BETWEEN MICROSCOPIC AND MACROSCOPIC PROPERTIES

Overall properties are a macroscopic expression of interactions taking place at a microscopic level. Extension of a microscopic property of microbial cell surface to the entire surface should theoretically lead to the corresponding macroscopic property. However, it will be a delicate task to amalgamate microscopic properties derived from AFM measurements into the macroscopic cell surface properties. The different conditions under which the macroscopic and microscopic properties are measured, should be taken into account. Properties as hydrophobicity and surface charge, from a macroscopic point of view, are determined in a two component system, *i.e.* bacterium and liquid medium. At the microscopic level, the AFM tip interacts with the bacterium in a medium, and consequently, bacterium and medium would respond to this third component, *i.e.* the tip. Therefore, hydrophobicity and charge mappings derived from AFM measurements depend on the properties of the AFM tip as well. Macroscopic and microscopic properties estimated in both systems are related, but to find out how, constitutes an enormous challenge. It involves an accurate knowledge of geometry and physico-chemical characteristic of the AFM tip as well as of a theory describing long and short-range interactions in such a system.
1.5 CONCLUSIONS AND AIM OF THIS THESIS

Physico-chemical properties of microorganisms can vary widely and generalizations at the species or even strain level are virtually impossible. The degree of success of physico-chemical models to explain microbial adhesion frequently decreases as the complexity of cell surface appendages on the organisms under consideration increases. Understanding how microscopic properties can be amalgamated into the macroscopic properties previously determined by many different research groups all over the world for a large variety of different strains and species is an imperative next step in the characterization of microbial cell surfaces. Subsequently, a generalized physico-chemical theory to account for bacterial adhesion to substratum surfaces will become in reach. Recently, the introduction of the atomic force microscope and its application to biological surfaces has opened a new avenue to obtain microscopic physico-chemical properties of bacterial cell surfaces. It is a challenge for the future to develop models, based on these improved methodologies, that will allow predicting bacterial adhesion from the initial adhesion events.

This thesis is aimed to reach a microscopic characterization of physico-chemical properties of bacterial cell surfaces as well as to find out if and how microscopic properties could be amalgamated into macroscopic cell surface properties and related to macroscopic bacterial adhesion on solid substrata.