Macrosopic and microscopic approaches toward bacterial adhesion
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Softness of the bacterial cell wall of *Streptococcus mitis* as probed by micro-electrophoresis

*Virginia Vadillo Rodríguez, Henk J. Busscher, Willem Norde and Henny C. van der Mei*

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2.1 INTRODUCTION

Accurate quantification of the physico-chemical properties of bacterial cell surfaces is essential in order to reach a better understanding of bacterial adhesion to a substratum. Current methods to study bacterial physico-chemical characteristics of cell surfaces usually yield overall properties and they do not account for structural and chemical heterogeneities, such as the occurrence of fibrils, fimbriae or other surface appendages (Hermansson, 1999). These structural and chemical heterogeneities have a definite function in adhesion and it has been found, for instance, that fibrillated *Streptococcus salivarius* strains carrying the Lancefield

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Group K polysaccharide antigen are more adhesive to buccal epithelial cells than strains lacking the antigen (Weerkamp & McBride, 1980; Handley et al., 1987). *Streptococcus parasanguis* carries fimbriae that are held responsible for adhesion to saliva-coated hydroxyapatite (Fives-Taylor & Thompson, 1985). Yet, the influence of structural and chemical heterogeneities on overall physico-chemical cell surface properties, such as zeta potential and hydrophobicity, remains to be identified for most strains.

Zeta potential and hydrophobicity are macroscopic corollaries of the overall chemical composition of the bacterial cell surface and their role in adhesion is generally assumed to be confined to the interaction at larger separation distances (more than a few nanometers). Bacterial surface appendages and chemical heterogeneities often exert localized attraction and consequently function to bridge a gap between otherwise repelling substratum surfaces (Matthyse et al., 1981; Smit et al., 1986). 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. Electro-osmotic flow of fluid may occur within these polyelectrolyte layers and this, in turn, has a strong impact on electrostatic interaction of bacteria with surfaces. The ease at which fluid can flow through the polyelectrolyte layer is referred to as the electrophoretic softness. Ohshima (1995) has forwarded a model that enables to derive the electrophoretic softness \(1/\lambda\) and the fixed charge density \(\rho_{\text{fix}}\) of polyelectrolyte layers from the dependence of the electrophoretic mobility on ionic strength, according to

\[
\mu = \frac{\rho_{\text{fix}}}{\eta \lambda^2} \left[ 1 + \left( \frac{\lambda}{\kappa} \right)^2 \frac{1 + \lambda / 2\kappa}{1 + \lambda / \kappa} \right]
\]  

(2.1)

is in which \(\mu\) is the electrophoretic mobility, \(\eta\) the viscosity of the solution and \(\kappa\) the reciprocal Debye-length of the polymer layer, which determined by the ionic strength. \(\rho_{\text{fix}}\) is related to the number density of charged groups \(N\), the electrical unit charge \(e\) and the valency \(z\) through

\[
\rho_{\text{fix}} = zeN
\]  

(2.2)

The theory assumes that \(\rho_{\text{fix}}\) and \(1/\lambda\) are invariant with the ionic strength. However, the thickness of a polyelectrolyte layer may shrink and the charge density may increase with increasing ionic strength, both as a result of reduced intramolecular electrostatic interaction. On the other hand, dense packing of polymer segments in the polyelectrolyte layer suppresses the charge density along the polymer chain. Then, adding low molecular weight electrolytes may cause an increase of the polyelectrolyte fixed charge density that causes the layer to swell. At first approximation, these effects may be disregarded and \(\rho_{\text{fix}}\) and \(1/\lambda\), are
estimated from a plot of the electrophoretic mobility against the ionic strength or, for that matter, the reciprocal Debye-length $\kappa$.

Accounting for the electrophoretic softness of bacterial cell surfaces in adhesion models leads to a reduction of electrostatic repulsions between bacteria and a substratum surface of the same charge sign (Morisaki et al., 1999; Poortinga et al., 2001). These decreased repulsions are more realistic. For instance, when a soft glass surface interacts with an ion-penetrable bacterium, their electric double layers overlap, whereby the diffuse double layer charges are driven into the ion-penetrable layers causing an effective decrease in surface potential and electrostatic repulsion (Poortinga et al., 2001).

*Streptococcus mitis* is one of the primary colonizers of hard surfaces in the oral cavity and colonizes both dental hard tissues as well as mucous membranes, most notable the cheek and tongue (Marsh & Martin, 1992). By comparison with other oral streptococci, *S. mitis* strains usually carry sparsely distributed but extremely long fibrils, as demonstrated by electron microscopy (Cowan et al., 1992), and their cell surfaces should be regarded as being soft.

The aim of this study is to determine the electrophoretic softness and fixed charge density in the polyelectrolyte layer of nine *S. mitis* strains according to a soft-particle analysis using measured electrophoretic mobilities (Ohshima, 1995). The results are compared with those reported for other bacterial strains having different surface morphologies.

### 2.2 MATERIALS AND METHODS

#### 2.2.1 Bacterial strains, growth conditions and harvesting

The nine *S. mitis* strains used in this study were all cultured in Todd Hewitt Broth (Oxoid, Basingstoke, UK). For each experiment, the strains were inoculated from blood agar in a batch culture. This culture was used to inoculate a second culture that was grown for 16 h prior to harvesting. Bacteria were harvested by centrifugation (5 min at 10,000 $g$), washed twice with demineralized water and resuspended in water. To break up bacterial chains, cells were sonicated for 30 s at 30 W (Vibra Cell model 375, Sonics and Materials Inc., Danbury, CT, USA). Sonication was done intermittently while cooling in an ice/water bath. These conditions were found not to cause cell lysis in any strain.

#### 2.2.2 Particulate microelectrophoresis

Electrophoretic mobilities were measured at 25 °C with a Lazer Zee Meter 501 (PenKem, Bedford Hills, NY, USA) equipped with an image analysis option for tracking and zeta sizing (Wit et al., 1997). Measurements were carried out in KCl solutions of various ionic strengths (pH 5) ranging from 1 mM to 150 mM. Prior to each measurement, an aliquot of the bacterial suspension was added to the appropriate KCl solution to obtain a density of approximately $1 \times 10^8$ cells ml$^{-1}$. The pH of the solutions did not change upon addition of the bacterial cells. The Lazer Zee Meter was set at 150 V for determination of the electrophoretic mobilities of the bacteria.
2.3 RESULTS
The electrophoretic mobilities of the nine \textit{S. mitis} strains are summarized in Figure 2.1 as a function of ionic strength. Although the electrophoretic mobilities clearly become less negative with increasing ionic strength, they do not approach zero at elevated ionic strength, which is a characteristic feature of soft particles. The electrophoretic softness (\(1/\lambda\)) and fixed charge density (\(\rho_{\text{fix}}\)), characterizing the properties of a soft particle, as obtained from fitting the data in Figure 2.1 to Eq. 2.1, are summarized in Table 2.1. At low ionic strength, \textit{i.e.} 1mM, the observed mobilities deviate considerably from the ones calculated by the soft-particle model, clearly because the model assumptions of constant fixed charge density and softness are not applicable in the low ionic strength region. Similarly, except for \textit{S. mitis} 272 the mobilities at 150 mM appear to be slightly above the best-fit theoretical curves; nevertheless those values have been considered in our analysis. Note that for \textit{S. mitis} T9 the electrophoretic mobility at 150 mM is zero, despite the fact that a stationary value appears to have been reached at lower ionic strengths.

Although the quality of the fit, as expressed by the coefficient of determination (\(r^2\)), is generally high (see Table 2.1), it varies with the ionic strength range considered. For most strains the fit is the best for the ionic strength range 10 – 100 mM (\(r^2 = 0.95\)), and reduces to 0.93 for the ionic strength ranges 15 – 150 mM and 10 – 150 mM. For some strains, \textit{i.e.} for \textit{S. mitis} 357, BMS and ATCC33399, the quality of the fit is the highest for the ionic strength range 15 – 150 mM. Fitting errors in electrophoretic softness and fixed charge density are around 0.3 nm and \(-0.3\times10^6\) C m\(^{-3}\), and slightly dependent on the ionic strength range applied.

Based on the analysis above, it is concluded that for each isolate an optimal ionic strength range should be chosen, based on the quality of the fit obtained, that yields the best estimate for the bacterial cell surface softness and fixed charge density. These values have been marked in bold in Table 2.1 and are the ones that are considered further in this study.

Figure 2.2 expresses the softness against the fixed charge density obtained. It is interesting to note that for the present collection of \textit{S. mitis} strains the softest appear to possess the least fixed charge.

2.4 DISCUSSION
The frequency of occurrence of extracellular surface structures on \textit{S. mitis} strains is very high and various appendages with different lengths up to several microns have been detected. Generally, the density of appendages on cell surfaces may vary considerably between strains, species and even isolates, but for \textit{S. mitis} isolates it has been described that the density of fibrils is sparse (Van der Mei \textit{et al.}, 1998b).

The present study clearly reveals that the cell surface of all nine \textit{S. mitis} strains are electrophoretically soft with fixed negative charge densities. The degree of softness and charge density of the fluid-penetrable layer varies between the different strains. The data presented in Figure 2.2 clearly show that the softest strain has the lowest charge density. This correlation possibly reflects the density distribution of the fibrils on the different strains. For sake of comparison, results of
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Figure 2.1. Electrophoretical mobilities in KCl solution (pH 5) of nine *S. mitis* strains as a function of ionic strength. The solid lines represent the best fit to Eq. 2.1.

Figure 2.2. The electrophoretic softness as a function of the fixed charge density for nine *S. mitis* strains (optimal values for these data were derived from Table 2.1).
Table 2.1. The cell surface softness $1/\lambda$ and charge density $\rho_{\text{fix}}$ of nine *S. mitis* strains, as derived from a soft-particle analysis of electrophoretic mobilities measured in KCl over different ionic strength ranges, together with the quality of the fit, expressed as $r^2$. Values associated with the best fit are marked in bold. All experiments were carried out in five-fold on separate bacterial cultures.

<table>
<thead>
<tr>
<th><em>S. mitis</em> strains</th>
<th>Ionic strength range (mM)</th>
<th>$1/\lambda$ (nm)</th>
<th>$\rho_{\text{fix}}$ $(10^6$ C m$^{-3}$)$^a$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>10 – 100</td>
<td>2.5</td>
<td>-1.2</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>15 – 150</td>
<td>1.9</td>
<td>-1.8</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>10 – 150</td>
<td>2.2</td>
<td>-1.4</td>
<td>0.92</td>
</tr>
<tr>
<td>272</td>
<td>10 – 100</td>
<td>2.1</td>
<td>-1.6</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>15 – 150</td>
<td>1.9</td>
<td>-2.0</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>10 – 150</td>
<td>2.0</td>
<td>-1.8</td>
<td>0.98</td>
</tr>
<tr>
<td>398</td>
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<td>-2.7</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>15 – 150</td>
<td>1.2</td>
<td>-4.4</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>10 – 150</td>
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<td>-3.3</td>
<td>0.88</td>
</tr>
<tr>
<td>244</td>
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<td>-2.3</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
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<td>1.3</td>
<td>-3.2</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>1.7</td>
<td>-2.2</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>15 – 150</td>
<td>-$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>10 – 150</td>
<td>-</td>
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</tr>
<tr>
<td>ATCC9811</td>
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<td>1.2</td>
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<td>1.3</td>
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<td>0.90</td>
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<td>15 – 150</td>
<td>1.2</td>
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</tr>
<tr>
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<td>10 – 150</td>
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<td>0.96</td>
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<tr>
<td>ATCC33399</td>
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<td>-2.6</td>
<td>0.97</td>
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<tr>
<td></td>
<td>15 – 150</td>
<td>1.1</td>
<td>-3.5</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>10 – 150</td>
<td>1.3</td>
<td>-2.8</td>
<td>0.97</td>
</tr>
</tbody>
</table>

$^a$ In the original Ohshima analysis, fixed charge densities are expressed as C m$^{-3}$. The conversion between C m$^{-3}$ and mM can be done dividing by the electric unit charge, Avogadro’s constant and a factor of $10^3$

$^b$ No convergence of the fit
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similar studies on other bacterial strains are given. The cell surface morphologies of
these strains together with that of S. mitis are schematically depicted in Figure 2.3. The S. salivarius HB, has a dense pack of fibrils on the surface with lengths up to
178 nm (Van der Mei et al., 1987) and yields an electrophoretic softness of 1.4 nm, and a fixed charge density of -1.3×10⁶ C m⁻³. On the other hand, a completely bald variant, devoid of any fibrillar surface appendages, had a softness of 0.7 nm and a
fixed charge density of -1.5×10⁶ C m⁻³ (Bos et al., 1998). Encapsulated staphylococci with a thick, contiguous polysaccharide-rich coat also presented
themselves as electrophoretically soft when grown in liquid cultures (1.7 nm) and on solid agars (2.8 nm), while their fixed charge densities were relatively high, i.e. between -2.3×10⁶ and -2.7×10⁶ C m⁻³ (Kiers et al., 2001).

The bald variant of S. salivarius appears to be by far the least soft, as expected. The other strains show comparable softness in spite of different surface
morphologies. It indicates that the Ohshima theory may be applied to indicate cell
surface softness, but that it does not provide information on the surface morphology
underlying the softness. Remarkably enough, the resistance to electro-osmotic flow,
determining the electrophoretic softness, appears to be hardly affected by the
density of the fibrillar layer and, moreover, is comparable to that in slime coatings.
In this respect it should be realized that, in view of the values for the softness
parameter, electro-osmotic flow occurs only through the very outer part (a few nm)
of the extracellular layer that extends over a thickness of hundred(s) of nm. Hence,
it seems that a tenuous distribution of extracellular structures, as is the case for the
sparsely fibrillated S. mitis, suffices to immobilize the solvent flow.

The differences in fixed charge densities obtained for the different morphologies
are even more difficult to understand. In particular, the finding that the fixed charge
densities in the outer region of the sparsely fibrillated S. mitis is about twice as high
as in the densely packed fibrillar coating of S. salivarius is highly unexpected. It
suggests that the charge density in the fibrils of S. mitis is much higher. Assuming a

Figure 2.3. Schematic presentation of four different bacterial cell surface structures.
(a) S. mitis, with long and sparsely distributed fibrils.
(b) S. salivarius HB showing a dense pack of fibrils with different lengths.
(c) S. salivarius HBC12, a completely bald, non-fibrillated organism.
(d) Staphylococci surrounded by a thick slime layer.
value of 0.20 nm for the radius of ionic groups in the polyelectrolyte, a fixed charge density of -2.5×10^6 C m^{-3} (being the average derived for the *S. mitis* strains) corresponds to a volume fraction of only 5×10^{-2} % in the soft part of the layer taken by ionic groups. Considering that the electrophoretic softness analysis reveals the charge density at the outer periphery of the extracellular layer, it would be instructive to compare that charge density with the charge density in the cell wall. The latter may be obtained by proton titration of whole cell walls. The charge densities reported for whole cell walls is typically three to four times higher (Van der Wal *et al.*, 1997; Carstensen & Marquis, 1968) than those derived from the softness analysis. The difference may be explained by the much higher density of the bacterial cell wall as compared to the extracellular layer.

Summarizing, we have demonstrated that *S. mitis* cell surfaces are electrophoretically soft. A comparison with surfaces of other bacterial strains that are reported to be soft as well reveals that the soft particle model as forwarded by Ohshima is not conclusive with respect to the surface structures causing the softness. The most probable reason is that for the various morphologies, in our case sparsely or densely packed fibrils or porous slimy coating, the electro-osmotic flow occurs only in the very outer region of a many-fold thicker extracellular surface layer. Nevertheless, irrespective of the surface morphology, establishing the surface softness is essential for the evaluation of the bacterial ζ-potential. Knowledge of that quantity is a necessity for quantitative assessment of electrostatic interaction in bacterial adhesion and aggregation.