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Influence of Ionic Strength on Elasticity of Bacterial Cell Surface Appendages as Characterized by Quantitative Nanomechanical Atomic Force Microscopy

by Ivan E. Ivanov

April 26, 2012
Influence of Ionic Strength on Elasticity of Bacterial Cell Surface Appendages as Characterized by Quantitative Nanomechanical Atomic Force Microscopy

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Submitted by:

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Submitted to:

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ABSTRACT

Bacterial adhesion is the initial step towards the establishment of a biofilm and the process can be influenced by a number of environmental factors, including salt concentration of the bulk solution. The adhesive bond stiffness of bacteria to a surface can be studied acoustically using a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). Experimental observations of the adhesive bond stiffness of streptococci with different cell surface appendages at different buffer ionic strengths have not allowed for the exact determination of the bond stiffness due to instrument limitations. Qualitative comparison based on the coupled resonance model indicated that decreasing ionic strength decreased the bacterial bond stiffness. These results were validated in this study by Quantitative Nanomechanical Atomic Force Microscopy (AFM) imaging. *Streptococcus salivarius* HB7 and HBV51 demonstrated decrease in the Young’s modulus and increase in the deformation of the cell surface appendages layer upon decrease in the buffer ionic strength. *S. salivarius* HB7, which expresses a dense layer of 91 nm long fibrils, exhibited higher stiffness and lower deformation of the surface appendages layer than *S. salivarius* HBV51 possessing a sparse layer of 63 nm long fibrils at both ionic strengths tested. The variation in the elasticity of the bacterial surface appendages is due to conformational differences of the layer in the two buffers tested, which affect the magnitude of the steric repulsion forces arising upon compression of the layer by the AFM tip. This study validates the use of the QCM-D as a convenient tool for the investigation of bacterial bond stiffness upon natural contact, which will facilitate the development of methods to prevent bacterial adhesion and dissemination.
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1. INTRODUCTION

1.1 Bacterial adhesion and the dissemination of biofilms

Microbial adhesion is the initial step towards the establishment of a biofilm in a new environment. According to the physico-chemical interpretation of the phenomenon, it involves the balance of attractive Lifshitz-van der Waals forces and attractive or repulsive electrostatic and acid-base interactions. Upon initial attachment of a microorganism to a surface, under favorable conditions, the cells can divide and begin to form a biofilm. Biofilms are microbial communities, encased in extracellular polymeric substances (EPS), of significant clinical and industrial importance. They are ubiquitous and difficult to eradicate. After maturation of the biofilm, individual cells or cell clusters may break off and colonize uninfected surfaces. The cell detachment if believed to proceed according to a viscoelastic mechanism. The adhesion and detachment processes in bacterial cells are influenced by their physical characteristics (surface charge and hydrophobicity), phenotype (production of EPS and surface appendages, expression of adhesion proteins, etc.), and environmental factors (presence of foreign conditioning agents, pH, ionic strength, etc.) among others.

1.2 Theoretical models of adhesion phenomena

Empirical studies of the adhesion process have led to the development of theoretical models, which can predict its behavior. According to the classical DLVO theory, adhesion of particles in liquids involves the balance of attractive van der Waals forces and repulsive electrostatic forces:

\[ W(d) = W(d)_A + W(d)_R \]  

(1)
where the interaction energy, $W(d)$, is presented as the sum of attractive van der Waals interaction energy, $W(d)_A$, arising from the fluctuations of atomic polarization, and repulsive electrostatic interaction energy, $W(d)_R$, caused by the formation of electrical double layers around charged particles in liquids. Generally, van der Waals forces act over larger distances as compared to electrostatic forces. Thus the adhesion outcome will be determined by the strength of the prevailing force.

Adhesion is non-conservative and additional work is required to separate two particles which have come in contact. Figure 1 provides an illustration of distinctive steps during the process of adhesion of a rigid spherical particle to a soft flat surface and the associated applied load as a function of penetration depth.

![Figure 1](image.png)

Figure 1. Contact mechanics in particle adhesion. As the particle is brought into contact with a flat plane (1), attractive forces arise (2). Indentation of the rigid particle into the soft plane results in net repulsive force (3). Detachment of the particle requires additional work and causes the formation of a neck around the area of contact (4).
At large particle-surface separation, the applied load, $F$, is equal to zero (Figure 1.1). As the particle comes in contact with the surface, attractive forces arise, which brings the load necessary to keep the particle and the surface in contact to negative values (Figure 1.2). With an increase in the applied load, the rigid spherical particle deforms the elastic flat surface to a certain penetration depth $h$ (Figure 1.3). The area of contact depends on the applied force, the radius of the particle, and the stiffness of the two objects. During the unloading cycle, the applied force is decreased, which reduces the penetration depth. Hysteresis between the loading and the unloading cycles typically arises and additional work, $W_a$, is needed to separate the particle and the surface. Before loss of contact, the surface forms a neck around the particle due to the presence of attractive forces (Figure 1.4). The total work required to separate the two objects is the sum of London dispersion forces, dipole-dipole interactions, induction interactions, hydrogen bonding, $\pi-\pi$ bonding, acid-base bonding, and electrostatic interactions.

1.2.1 The Hertz model

The first attempt to investigate in more detail the contact mechanics of two bodies was carried out by Heinrich Hertz in 1882.\(^5\) The problem examines the contact between two spheres of isotropic elastic media under small deformations. In order to simplify the problem, it is assumed that there is no hysteresis between loading and unloading, and that there is no adhesion forces between the bodies on contact. The two spheres are characterized by their Young’s moduli, $E$ and $E'$, and their Poisson ratios, $\mu$ and $\mu'$. Under applied load, the two bodies deform, which gives rise to a circular contact area, due to the axial symmetry of the problem. The radius of contact is denoted by $a$ (Figure 2).
Figure 2. Contact between two elastic spheres. The two spheres of radii $r$ and $r'$ are in contact at a circular area of radius $a$ under applied force $F$, which causes penetration depths of $h$.

As a simplifying notation, the reduced radius is defined as:

$$\frac{1}{R} = \frac{1}{r} + \frac{1}{r'}$$  \hspace{1cm} (2)

and the effective Young’s modulus, $K$, of the two materials is given by:

$$\frac{1}{K} = \frac{3}{4} \left( \frac{1-\mu'^2}{E'} + \frac{1-\mu^2}{E} \right)$$  \hspace{1cm} (3)

Thus, at small deformations, the model relates the penetration depths, $h$, and the radius of contact by:

$$h = \frac{a^2}{R}$$  \hspace{1cm} (4)

The solution of the Hertzian problem also gives the applied load as a function of penetration depth as:

$$F = \frac{KA^3}{R} = K\frac{h^{3/2}}{R^{1/2}}$$  \hspace{1cm} (5)

It can be seen that according to this theoretical prediction, the applied force will vary with the penetration depth raised to the power of $3/2$. An important special case of the model is also the
limit where \( r' \) goes to infinity. This represents the case of contact between a sphere and a flat surface. As such, the reduced radius will be equal to the radius of the sphere \( (R = r) \).

It is important to note that the underlying assumptions of the model limit its applicability. Cases of elastic contact between bodies of isotropic composition under no hysteresis and surfaces forces are rather rare in nature. However, this classical development of contact mechanics still finds applications in research due to its simplicity and is often used for an order-of-magnitude estimation before the application of more complex models.

### 1.2.2 The DMT model

It was not until almost one hundred years later that the Hertzian solution was expanded in order to account for adhesive forces between the two bodies in contact. The model was developed by Derjaguin, Muller and Toporov in 1975 and came to be known as the DMT model. It accounts for long-range attractive interaction around the area of contact between the two bodies, but restrains the contact area to Hertzian.

![Figure 3. Attractive forces during DMT contact. The DMT model considers long-range attractive interactions around the area of contact between the two bodies](image)

Thus, this model gives the relationship between applied force and penetration depth as:
where $\sigma$ is the adhesion work. As can be seen, the area takes the same expression as in the Hertz model, but the force is offset in order to account for the attractive interactions, which are considered by this model. This gives rise to a sharp discontinuity in slope at contact. The DMT model applies to rigid samples of small curvature, which give rise to low adhesion, but may underestimate the true area of contact. This model also ignores hysteresis between the loading and unloading cycles.

### 1.2.3 The JKR model

Shortly before the development of the DMT model, a different formulation of contact mechanics was developed by Johnson, Kendall and Roberts, which came to be known as the JKR model. According to this development, short-ranged attractive forces operate within the area of contact between two bodies and the geometry is not constrained to Hertzian.

![Diagram of attractive forces during JKR contact](image)

**Figure 4. Attractive forces during JKR contact.** The JKR model considers short-ranged attractive interactions within the area of contact between the two bodies.

The relationship between applied force and penetration depth is given by:

$$h = \frac{\alpha^2}{R}$$

(6)

$$F = \frac{k\alpha^3}{R} - 2\pi R \sigma$$

(7)
The JKR model accounts for hysteresis between the loading and unloading cycles. During unloading, a connective neck is formed between the two bodies, which ruptures at negative applied force. This model best applies to highly adhesive systems of large radii of curvature and low stiffness.

1.2.3 The Maugis model

Historically, the JKR solution preceded the DMT model. Derjaguin and his coworkers rejected the model proposed by Johnson, Kendall, and Roberts as neglecting the adhesive forces across the gap between the two objects and developed their own solution. This dispute proved to be instrumental for the development of later models of contact mechanics. In the beginning of the 1990s, Maugis came to the conclusion that the DMT and the JKR models are extreme cases of the same phenomenon and developed a model, which smoothly transitions between the two.

A plot of applied force versus penetration depth for the three models shows that the DMT model predicts stronger repulsive forces between the two objects in contact than the JKR model at all values of the penetration depth. Out of the three, however, the JKR equations are the only ones which take into account the non-conservative nature of the adhesion process (Figure 5).
Figure 5. Normalized load as a function of penetration depths for the Hertz, DMT and JKR models. The applied load increases with penetration depth. The Hertz model does not account for attractive interactions between the two bodies, whereas the JKR model predicts hysteresis between the loading and unloading cycles.

The Maugis model postulates that attractive forces act over an annular region around the area of contact between the two bodies. It is a more accurate representation of contact mechanics, which can be applied to systems of unconstrained radii of curvature or adhesive interactions. However, it is also more complex and requires a parametric solution.

Figure 6. Attractive forces during Maugis contact. The Maugis model considers attractive forces over an annular region around the area of contact between the two bodies.
The parameter $\lambda$ is used to characterize the range of material properties:

$$
\lambda = \frac{2.06}{\zeta_0} \left( \frac{R\sigma^2}{\pi K^2} \right)^{\frac{1}{3}}
$$

(10)

where $\zeta_0$ is the interatomic distance. Large values of $\lambda$ correspond to adhesive compliant materials and vice versa for small values of $\lambda$. The parameter $m$ is used to relate the area of contact to the reduced radius of the bodies:

$$
1 = \frac{\lambda a^2}{2} \left( \frac{K}{\pi R^2 \sigma} \right)^2 \left[ \sqrt{m^2 - 1} + (m^2 - 2) \arctan \sqrt{m^2 - 1} \right] + \frac{4\lambda^2 a}{3} \left( \frac{K}{\pi R^2 \sigma} \right)^{\frac{1}{3}} \left[ 1 - m + \sqrt{m^2 - 1} \arctan \sqrt{m^2 - 1} \right]
$$

(11)

Thus, the applied force and the penetration depth are given by:

$$
h = \frac{a^2}{R} - \frac{4\lambda a}{3} \left( \frac{\pi \sigma}{KK} \right)^{\frac{1}{3}} \sqrt{m^2 - 1}
$$

(12)

$$
F = \frac{K a^3}{R} - \lambda a^2 \left( \frac{\pi \sigma K^2}{R} \right)^{\frac{1}{3}} \left[ \sqrt{m^2 - 1} + m^2 \arctan \sqrt{m^2 - 1} \right]
$$

(13)

It can be seen that in the limit of $\lambda \to \infty$, the Maugis model approaches the JKR theory, while the limit of $\lambda \to 0$ corresponds to the DMT solution. As such, the Maugis model provides an encompassing analytical solution to contact mechanics, but at the expense of ease of use.

Theoretical models of contact mechanics are formulated on the basis of experimental observations of the interactions between particles at the nanoscale. They allow for characterizing the mechanical properties of a sample based on the sample deformation under applied load and the adhesion force which arises between the sample and the indenter. A sensitive tool often used to measure the nanoscale interaction forces between a sample and a sharp tip is the Atomic Force Microscope (AFM).
1.3 Atomic Force Microscopy

Atomic Force Microscopy is a versatile technique, which can be used to image surfaces at the nanoscale, and obtain quantitative information about the surface of interest, such as adhesion force, elasticity, mechanical and electrochemical properties, frequency- and time-dependent behavior, etc. The instrument is based on the piezoelectric effect and uses a sharp (1 – 10 nm in radius) tip to obtain topographical and other data about the sample. It finds broad application in material science, semiconductor and data storage industry and research. More recently, AFM has been applied to study properties and phenomena within cells and living organisms such as ligand-receptor interactions, mechanical and structural properties of proteins and nucleic acids, surface characterization of cells, etc. When evaluating the mechanical properties of cells, due to the stark difference in radius of curvature between the AFM tip and the cell, the cell membrane is often assumed to be flat elastic media and the AFM tip – rigid spherical body. Thus the models of contact mechanics discussed in Chapter 1.2 can be used to elucidate the elasticity of the cell surface.

1.3.1 Quantitative Nanomechanical Imaging

A novel scanning mode of atomic force microscopy is the PeakForce QNM® (Quantitative Nanomechanical Property Mapping) developed by Bruker AXS. The technique enables extraction of mechanical data without inflicting sample damage. During scanning, the tip is oscillated at approximately 1 kHz and each time the tip and the sample are brought into contact, a force curve is recorded. A typical force curve on soft sample is shown in Figure 7.
Figure 7. Characteristic force versus separation curve. As the separation is decreased, attractive forces cause the AFM tip to snap into contact with the sample. Indentation of the sample gives rise to repulsive forces, which decrease during the unloading cycle. Contact is lost when the adhesion forces are overcome.

As the AFM tip approaches the sample, attractive forces (primarily capillary forces, van der Waals interactions and electrostatic forces) arise between the two bodies, which cause the tip to snap into contact. With further decrease in the separation between the two bodies, the tip begins to indent the sample applying a load to balance the repulsive forces which arise at contact. After a user-defined value of the applied force is reached, the AFM initiates the retraction cycle. Figure 7 clearly indicates the non-conservative nature of the adhesion process. As the tip retracts, additional work is needed to separate the two bodies in contact. Eventually, the tip returns to its initial position and initiates another force curve cycle.

Force curves allow the direct measurements of parameters necessary to elucidate the mechanical properties of the sample. The adhesion force needed to separate the two objects is
defined as the minimum force value during the cycle, which occurs at the pull-off point. The sample deformation, i.e. the penetration depth of the ATM tip into the sample, corresponds to the horizontal distance between the point of contact and the tip position at the maximum applied force. The work required to separate the tip and the sample, which arises because of the energy dissipation during the adhesion process, can also be found by integrating the area between the approach and the retraction curves.

The PeakForce QNM mode uses the DMT model of contact mechanics in order to derive sample properties in real time. Quantitative determination of parameters, however, requires careful determination of the stiffness and radius of curvature of the tip used for scanning the sample. Furthermore, in order to decouple the effects of both indenter and sample deformation, it is assumed that the AFM tip is infinitely hard. Additionally, in order to obtain the Young’s modulus of the sample, instead of the reduced stiffness, it is required to supply the Poisson’s ratio for the sample. The Poisson’s ratio is a measure of the ratio between contraction (transverse strain) to extension (axial strain) of a body under applied load. The definition of Poisson’s ratio limits the values it can take to the range of -1.0 to 0.5 for stable, isotropic, linearly elastic materials. Most steels and rigid polymers exhibit values of about 0.3, while materials such as rubber and clay approach the upper limit of 0.5. Thus after proper calibration, the PeakForce QNM mode can be used to map the Young’s modulus, adhesion, sample deformation, and energy dissipation during imaging in addition to the height profile of the sample (Figure 8).
Figure 8. Quantitative Nanomechanical AFM imaging of bacteria. The PeakForce QNM mode allows for the quick and convenient mapping of the sample topography, elasticity, adhesion, deformation, and energy dissipation.

The AFM thus represents a powerful surface characterization technique. However, it requires sample immobilization and allows for the determination of mechanical properties only upon forced contact. Recent theoretical developments have allowed for the study of bond stiffness of particles adhering to the surface of an acoustic sensor upon natural contact.
1.4 Quartz Crystal Microbalance

The Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) has emerged as a convenient technique for nondestructive study of events at the surface. The technique is based on the reverse piezoelectric effect, where an AT-cut quartz crystal is oscillated in response to an applied current.\textsuperscript{15} The frequency of the oscillating crystal is affected by the attachment of mass to the surface or changes in the bulk fluid properties, while the frequency decay time as the oscillator is intermittently disconnected is proportional to the rigidity of the layer on the sensor surface. The technique has been successfully applied to a broad range of applications ranging from the study of biological macromolecules and cells to gas absorption in polymeric films and corrosion of fuel cell electrodes.\textsuperscript{16-18} The setup of the instrument also allows for electrochemical measurements and integration with other techniques such as microscopy or ellipsometry.

1.4.1 Conventional mass-loading theory

The quartz crystal in QCM-D is oscillated at its fundamental frequency (5 MHz) and its overtones (e.g. 15 MHz, 25 MHz, 35 MHz, etc.). Upon binding of molecules to the sensor surface, the oscillation frequency is decreased proportionally to the mass of the adsorbed layer. The relationship is given by the Sauerbrey equation:

\[ \Delta f_n = -\frac{n}{C} \Delta m \]  

(14)

where \( n \) is the overtone number (1, 3, 5, etc.) and the proportionality constant, \( C \), is equal to 17.7 ng Hz\textsuperscript{-1} cm\textsuperscript{2} for a 5 MHz quartz crystal.\textsuperscript{19} This relationship, however, only applies to rigid, laterally homogeneous films. Soft viscoelastic films do not couple completely with the sensor and thus give rise to energy dissipation. The dissipation factor, \( D \), is defined as the ratio of the lost and stored energy of the oscillator:
\[ D = \frac{E_{\text{lost}}}{2\pi E_{\text{stored}}} \]  

QCM-D is also sensitive to changes in the properties of the bulk fluid, according to:

\[ \Delta f_n = -\frac{1}{C} \sqrt{\frac{\rho_l \eta_l}{2\omega_F}} \]  

where \( \rho_l \) and \( \eta_l \) are, respectively, the fluid’s density and viscosity, and \( \omega_F = 2\pi f_F \) is the angular fundamental frequency.\(^{20}\) Thus a reference measurement in the same fluid as the sample of interest is always necessary to separate the bulk fluid contribution from the film properties. Additionally, the dependence of the film properties on changes in the bulk fluid can be studied by accounting for the contribution of changes in the bulk fluid to the frequency and dissipation shifts.

### 1.4.2 Coupled resonance model in QCM-D

In certain cases, such as the adhesion of spherical particles or cells, positive frequency shifts are observed with the addition of mass to the QCM-D sensor surface, which is in contradiction with the conventional mass-loading theory.\(^{21-23}\) This has led to the development of the coupled resonance model, which takes into account the discrete nature of such adhering bodies.\(^{24}\) This model simulates the particles as mass \( m \) attached via a spring of stiffness \( k \) to the sensor, which is also characterized by its effective mass \( M \) and internal stiffness \( K_n \), in the spirit of mechanical equivalent circuits (Figure 9).
Figure 9. Mechanical equivalent circuit of the coupled resonance model. A spherical particle of mass $m$ is connected through a spring of stiffness $k$ to the QCM-D sensor, which is characterized by its effective mass $M$ and internal stiffness $K$

When current is applied across the quartz crystal, both the sensor and the particle come into motion with a frequency equal to:

$$\omega_n = 2\pi f_s \sqrt{\frac{k_n}{M}}$$ (17)

$$\omega_p = 2\pi f_p \sqrt{\frac{k}{m}}$$ (18)

Thus the QCM-D response will be determined by the ratio of the resonance frequency of the sensor to the resonance frequency of the particle. At low sensor frequencies, the particle can move along with the motion of the surface, which causes the QCM-D sensor to experience an increase in its effective mass leading to a frequency decrease. At high sensor frequencies, the movement of the particle and the sensor surface decouple. The sensor experiences counteracting forces, proportional to the stiffness of the bond between the particle and the surface, which lead to a frequency increase.

This development of the coupled resonance model disregards dissipative energy losses, which, however, cannot be ignored when particle attachment in liquid is considered. Energy
dissipation can be incorporated in the model by considering a complex quantity for the stiffness of the bond between the particle and the surface, provided that the small load approximation holds. The validity of the small load approximation has been confirmed for particle attachment in both air and liquid. Furthermore, in addition to the discussed dissipation factor, energy losses in QCM-D can be quantified by the half-bandwidth of the resonance peak at half-maximum, $\Gamma$. The bandwidth and the dissipation factor are related through:

$$\Gamma = \frac{Df_s}{2}$$

The mechanical equivalent circuit of the complex frequency is a system, consisting of a spring and a dashpot in parallel (Figure 10).

![Mechanical equivalent circuit](image)

**Figure 10.** Mechanical equivalent circuit of the coupled resonance model with energy losses. A spherical particle of mass $m$ is connected through a spring of stiffness $k$ and a dashpot of drag coefficient $\xi$ in parallel to the QCM-D sensor, which is characterized by its effective mass $M$ and internal stiffness $K$.

Thus the frequency shift due to attachment of spherical particles to the QCM-D sensor surface is given by:

$$\frac{\Delta f^*}{f_F} = \frac{\Delta f + i\Delta \Gamma}{f_F} = \frac{N_p \cdot m \omega_n (\omega_p^2 + i \omega_n \gamma)}{\pi Z_q \omega_n^2 (\omega_p^2 + i \omega_n \gamma)}$$

(20)
where $N_P$ is the number of particles per unit area and $Z_q$ is the acoustic impedance of AT-cut quartz ($Z_q = 8.8 \times 10^6$ kg m$^{-2}$ s$^{-1}$). We have also introduced the damping rate, $\gamma$, as the ratio of the drag coefficient, $\zeta$, and the mass of the particle. It is instructive to plot the frequency and bandwidth change as a function of the ratio of the resonant frequency of the QCM-D sensor to the resonance frequency of the particle (Figure 11).

![Figure 11. Frequency and bandwidth shift for particles adhering to the QCM-D sensor surface according to the coupled resonance model. When the particle resonates slower than the QCM-D sensor ($f_S/f_P > 1$), positive frequency shifts are detected upon the addition of mass. The frequency at which the particle and the QCM-D sensor resonate at the same rate is accompanied by a maximum in bandwidth shift. As was discusses earlier, when the particle resonates faster than the QCM-D sensor ($f_S/f_P < 1$), the instrument records negative frequency shifts and vice versa when the particle resonates slower ($f_S/f_P > 1$). The transition from negative to positive frequency shifts occurs at the point where the particle and the sensor resonate at the same frequency. This point is termed the zero-crossing](image-url)
frequency, \( f_{ZC} \), and is also accompanied by a maximum in bandwidth. This frequency can be used to calculate the stiffness of the bond between the particle and the surface:

\[
  f_{ZC} = f_p = \frac{1}{2\pi} \sqrt{\frac{k}{m}}
\]

(21)

Zero-crossing points occurring at higher frequencies will indicate a stiffer contact than such occurring at lower frequencies.

The coupled resonance theory applies with several limitations in QCM-D. While Figure 11 was plotted for all frequencies, the QCM-D can only probe discrete frequencies equal to one of the overtones of the fundamental frequency of the crystal (5 – 65 MHz). Moreover, the exact determination of the spring constant of the bond between the particle and the surface requires knowledge of the mass of the particle. In liquids, this parameter is even harder to estimate as it represents the inertial mass of the particle. However, this does not preclude the qualitative comparison of bond stiffness for adhering particles in liquid.

1.5 Coupled resonance analysis of the adhesive bond stiffness of streptococci

The QCM-D coupled resonance theory was applied by Olsson el al. to study the adhesive bond stiffness of two Streptococcus salivarius strains with different surface appendages at different ionic strengths.\(^{27}\) Strains *S. salivarius* HB7 and HBV51 do not produce extracellular polymeric substances and possess a hydrophilic surface with fibrillar surface appendages of different length and density.\(^{28}\) Strain HB7 expresses a dense layer of 91 nm long fibrils, measured in deionized water by electron microscopy after ruthenium red staining, while strain HBV51 possesses a sparse layer of 63 nm long fibrils. The attachment of the two strains to a gold-plated QCM-D crystal was studied at 57 mM, 12.5 mM, and 5.7 mM adhesion buffer (50 mM potassium
chloride, 2 mM potassium phosphate, and 1 mM calcium chloride, pH 6.8). Both strains caused positive frequency shifts at all ionic strengths over all overtones upon attachment to the sensor, suggesting that the adhesion proceeded according to the coupled resonance model (Figure 12).

Figure 12. Shifts in frequency and bandwidth as a function of sensor frequency and ionic strength for *S. salivarius* HB7 and *S. salivarius* HVB51. Both strains cause positive frequency shifts at all ionic strengths, which indicates that they adhere to the QCM-D sensor according to the coupled resonance model.

The available frequency range in QCM-D, however, did not allow for the precise determination of the zero-crossing frequency for either of the strains. Several extrema in frequency and bandwidth were observed. These, however, are accompanied by large standard deviation, which precludes their identification as second zero-crossing frequencies, which could result from a subpopulation of bacteria with different bond characteristics. The general
appearance of the curves in Figure 12 suggests that both strains have zero-crossing frequencies below 5 MHz at the ionic strengths tested. The data can be extrapolated using Equation 20 in order to estimate these values (Figure 13).

![Graph showing the zero-crossing frequency for S. salivarius HB7 and S. salivarius HBV51 at different ionic strengths.](image)

**Figure 13. Estimation of the zero-crossing frequency for S. salivarius HB7 and S. salivarius HBV51 at different ionic strengths using data extrapolation.** Decrease in ionic strength leads to decrease in the zero-crossing frequency for both strains. Extrapolations too long to be considered reliable are presented as dashed lines.

Extrapolation suggests that the zero-crossing frequency of strain HB7 decreases from approximately 1.1 MHz to 0.7 MHz with a decrease in buffer ionic strength from 57 mM to 5.7 mM. Strain HBV51 experiences a more pronounced decrease in zero-crossing frequency, from roughly 1.1 MHz to below 100 kHz at 5.7 mM adhesion buffer. It should be noted that the dashed lines in Figure 13 indicate extrapolations that may not be reliable. The change in zero-crossing frequency is correlated to change in the stiffness of the bond between the bacterial body and the QCM-D sensor surface according to Equation 21. Thus for both strains, decreasing the buffer ionic strength decreases the stiffness of the bond between the cell and the surface.
The group also quantified the change in distance between the bacterial cell body and the substratum with changes in ionic strength using Total Internal Reflection Microscopy (TIRM). Decreasing the ionic strength from 57 mM to 5.7 mM increased the distance between the substratum surface and the cell body by 90 nm and 43 nm for strain HB7 and strain HBV51, respectively. This indicates that at 57 mM buffer, the surface appendages of strain HB7 are completely collapsed on the cell surface.

1.6 Aim and significance of this study

QCM-D allows for the study of the bond stiffness between the bacterial cell body and the substratum surface under non-forced contact. It is, however, limited by the range of discrete frequencies under which this bond can be probed. Recent developments in AFM have allowed for the convenient mapping of sample mechanical properties, including surface elasticity.\textsuperscript{29, 30} The aim of this study was to use AFM Quantitative Nanomechanical Imaging to measure the Young’s modulus of bacterial cell surface appendages in order to validate the predicted adhesive bond stiffness between the bacterial cell body and the QCM-D sensor surface at different ionic strengths of the bulk solution. The development of the QCM-D as a quick and convenient tool to study bacterial bond stiffness under different environmental conditions will expedite the development of agents to influence bacterial surface attachment and detachment. Preventing bacterial adhesion and the dissemination of biofilms will reduce infection rates and alleviate chronic conditions.
2. MATERIALS AND METHODS

2.1 Bacterial strains, culture conditions, and harvesting

*Streptococcus salivarius* HB7 and HBV51\textsuperscript{28} were short-term stored on blood agar at 4 °C. The strains were cultured aerobically at 37 °C in Todd Hewitt broth (THB; Oxoid, Basingstoke, UK). For experiments, a 24 h preculture was diluted 1:20 and cultured for 16 h before harvesting. Bacterial cells were harvested by centrifugation for 5 min at 5000 g and washed twice with 10 mL of deionized (DI) water. Adhesion buffer (50 mM potassium chloride, 2 mM potassium phosphate, and 1 mM calcium chloride, pH 6.8) was prepared daily from 0.5 M stock solutions.

2.2 Bacterial immobilization for AFM experiments

Glass slides were washed by sonication for 3 min in 2% RBS 35 (Omnilabo International BV, The Netherlands) before every experiment. Slides were rinsed thoroughly with tap water, ethanol, tap water, and finally demineralized water before being air dried. Bacteria, which have a negatively charged membrane, were attached to the glass slides through electrostatic interactions with positively charged poly-L-lysine (PLL; Sigma, Poole, UK). A droplet of PLL was spread over a cleaned glass slide and allowed to air dry. The slide was rinsed with DI water and a droplet of bacterial suspension was placed on the functionalized glass surface. After allowing the cells to adhere for 30 min, unbound cells were rinsed with DI water. The so-formed bacterial lawn was allowed to incubate in buffer for 1 h before initiation of AFM experiments in order to allow the fibrils to restore their natural conformation.

2.3 Atomic Force Microscopy procedure

Experiments were conducted on a BioScope Catalyst AFM (Bruker AXS, Santa Barbara, CA). The spring constants and radii of SCANASYST-FLUID ($f_0 \approx 150$ kHz, $k \approx 1.2$ N/m, $R \approx 18$
nm; Bruker AFM Probes, Camarillo, CA) AFM tips were measured before every experiment. A thermal method\(^{31}\) was used for the spring constant determination and the tip radius was estimated by scanning a standard titanium rough sample (Appendix). Bacteria were imaged in the PeakForce QNM mode. Areas of 100 \(\mu m^2\) were scanned at 0.5 Hz with a contact PeakForce setpoint of 2 nN in order to obtain information about the mechanical properties of the cell surface appendages only. For extracting the Young’s modulus of the fibrils, it was assumed that the AFM tip is infinitely hard and that the sample Poisson’s ratio is equal to 0.5.\(^{32, 33}\) Experiments were performed in triplicates with at least three areas scanned per experiment.

### 2.4 Data processing and analysis

AFM data were analyzed using Gwyddion 2.26.\(^{34}\) An image mask was calculated based on the height data using particle detection and watershed algorithms and applied to the channel of interest. The mask was edited manually if necessary (Figure 14).

![Image Mask](image.png)

**Figure 14.** Image mask created for AFM data analysis. The mask was created based on the height profile and applied to the modulus channel in order to determine the mean stiffness of the sample.
The mean channel value for each particle was calculated and a Gaussian fit was applied to the distribution of the data (Figure 15).

![Graph showing Gaussian fit with mean value of 1.08 MPa](image)

**Figure 15.** Gaussian fit applied to the distribution of the mean channel values for the particles detected per image. The mean of the distribution was used to represent the respective sample property (e.g. Young’s modulus)

The mean of the distribution was recorded.
3. RESULTS AND DISCUSSION

3.1 Adhesion of bacterial cells in liquid

Adhesion of particles in air and liquid may differ significantly due to the difference in strength of the physical interactions in each medium (Chapter 1.2). Most notably, electrostatic forces decay with an increase in the salt concentration in the bulk liquid and van der Waals forces are also affected by the intermediate medium between the two bodies.\(^{35}\) Thus adhering particles in liquid often do not experience long-range attraction (snap into contact) and may not require significant additional work to separate.

High Speed Data Capture (HSDC) of cantilever deflection as a function of time during PeakForce QNM imaging in buffer reveals the adhesion dynamics between the AFM probe and the bacterial cell surface (Figure 16). During HSDC, the cantilever deflection is recorded with microsecond temporal resolution (Figure 16A) over one line of the image (Figure 16C). Force curves at each pixel of the image can be extracted by separating the approach and retraction cycles and using the spring constant of the cantilever to convert the deflection signal to force (Figure 16B).
Figure 16. High Speed Data Capture of bacterial cells. Cantilever deflection was recorded with high temporal resolution (Panel A) over one line of the image (Panel C). The approach and retraction cycles have been separated in Panel B to form force curves.

No snap into contact between the AFM tip and the cell can be observed and no strong adhesion forces are evident (Figure 16B). In the region of the force curve where the tip and the cell are not in contact, noise of about 0.5 nN is arises. This is partly due to cantilever instabilities resulting from the high speed of force curve acquisition (~1 kHz) and the increased viscosity of water as compared to air. The high frequency of force curve acquisition also decreases the contact time between the cell and the AFM tip. This, in turn, also lowers the magnitude of the adhesion force,
which has been reported to depend on the probe-sample residence time. These artifacts, however, do not significantly influence the contact region of the force curves, which is used for fitting of the contact mechanics models. Upon contact, increasing the penetration depth increases the repulsive forces in a power-law dependence, which suggests the applicability of such models. Contact between the AFM tip and the glass substratum results in the abrupt increase of the applied force to the user-specified value (Figure 17).

![Graph](image1)

**Figure 17.** High Speed Data Capture of glass substrate. Cantilever deflection was recorded with high temporal resolution (Panel A) over one line of the image (Panel C). The approach and retraction cycles have been separated in Panel B to form force curves.
This would result in infinite measured stiffness, as the AFM tip is not able to indent the glass slide (Appendix).

No surface forces (less than 0.2 nN) were detected during contact with the cell surface, indicating the applicability of the Hertz, rather than the DMT model, which is the only one available in the native AFM software. However, since the DMT model is an extension of the Hertz model to include the effect of long-range attractive forces upon contact, the DMT model naturally converges to the Hertz model as the adhesion work, $\sigma$, approaches zero. This justifies the use of the Hertz theory of contact mechanics for data analysis in this study.

3.2 Conformation of bacterial surface appendages at different ionic strength buffers

The conformation of the fibrillar surface appendages of *S. salivarius* HB7 and HBV51 is affected by a number of environmental factors, including ionic strength of the bulk solution. To understand the behavior of the surface appendages at solutions of different total salt concentration, the fibrils can be examined as proteinaceous polymers composed of amino acids of different charge and hydrophobicity. A polymer composed of uncharged hydrophobic residues will fold in a polar solvent (e.g. water) in order to exclude solvent molecules from its structure and decrease the total free energy of the system. On the other hand, a polymer of uncharged residues in a solvent of like polarity will adopt a random conformation, similar to the self-avoiding three-dimensional random walk. If the polymer is composed of monomers of like charges, the chain will extend in solution due to Coulomb repulsion. An increase in the salt concentration of the bulk solution will cause screening out of the repelling charges and the polyelectrolyte chain will again collapse.37
The surface appendages of *S. salivarius* HB7 and HBV51 are characterized by net negative charge and low hydrophobicity. Strain HB7 is slightly less hydrophobic (surface free energy $\gamma = 117 \text{ mJ m}^{-2}$) and less negatively charged (zeta potential $\zeta = -18 \text{ mV}$) than strain HBV51 ($\gamma = 113 \text{ mJ m}^{-2}$, $\zeta = -22 \text{ mV}$). Thus, at high ionic strength the fibrils of both strains will likely be folded on the cell surface. Decreasing the salt concentration of the buffer will cause the fibrils to extend into the solution in order to decrease the electrostatic repulsion between the negatively charged amino acids. Measurements by TIRM of the decrease in the length of the cell surface appendages upon increasing the buffer ionic strength indicate that in 57 mM adhesion buffer the fibrils of strain HB7 completely collapse on the cell surface, while those of HBV51 fold to approximately one third of their length in DI water (Figure 18).

![Figure 18](image)

*Figure 18. Effect of increasing ionic strength on the conformation of *S. salivarius* cell surface appendages. High salt concentration reduces the electrostatic repulsion within the polymer chains, which allows them to collapse on the cell surface thus reducing the total free energy of the system.*

### 3.3 Stiffness of bacterial surface appendages at different ionic strength buffers

The stiffness of bacterial surface appendages is largely affected by their conformation on the cell surface, which is influenced by the ionic strength of the bulk solution. The Young’s
moduli of *S. salivarius* HB7 and HBV51 as characterized by PeakForce QNM imaging is presented in Figure 19.

![Figure 19](image)

**Figure 19. Stiffness of bacterial surface appendages at different ionic strengths as characterized by PeakForce QNM imaging.** Decreasing ionic strength decreased the Young’s modulus of *S. salivarius* strain HB7 and HBV51

It was observed that decreasing the buffer ionic strength decreased the Young’s moduli for both strains. The fibril stiffness of HB7 dropped from 1200 kPa at 57 mM adhesion buffer to 850 kPa at 5.7 mM buffer. The Young’s modulus of HBV51 decreased from 860 kPa to 330 kPa with the decrease in ionic strength. Thus the surface appendages of strain HB7 exhibited higher stiffness at both ionic strengths. It was also observed that strain HBV51 experienced a more pronounced decrease in stiffness with decrease in ionic strength.

### 3.4 Deformation of bacterial surface appendages under constant load

The stiffness of bacterial fibrils is directly proportional to the sample penetration depth as is given by the Hertz model (Equation 5). Thus under a constant load, the penetration depth can
be used to compare the variation in Young’s moduli between samples. The surface deformation of *S. salivarius* mutants at different buffer ionic strengths was extracted from the PeakForce QNM maps (Figure 20).

![Graph showing deformation of bacterial surface appendages layer at different ionic strengths as characterized by PeakForce QNM imaging](image)

**Figure 20.** Deformation of bacterial surface appendages layer at different ionic strengths as characterized by PeakForce QNM imaging. Decreasing ionic strength increased the fibril layer deformation of *S. salivarius* strain HB7 and HBV51. Decrease in ionic strength increased the deformation of fibrils layer for both strains. Strain HB7 underwent an increase in penetration depth from 28 nm to 41 nm with the change in ionic strength, while the same caused an increase from 33 nm to 54 nm for strain HBV51. This indicates that strain HBV51 exhibited a higher deformation at both ionic strengths under the same load.

The sample penetration depth and consequently stiffness depend on changes in the conformation of the surface appendages at the tested buffer ionic strengths. The densely
populated long fibrils on strain HB7 collapse on the cell surface at high ionic strength. As the AFM tip compresses the fibril layer, multiple molecules come in contact with the tip and high steric repulsive forces arise as the atoms are brought closer together and their electron clouds begin repelling. Thus at the constant applied force, the AFM tip does not cause large deformation of the fibril layer and it appears stiff. Conversely, the sparsely populated surface appendages of HBV51 extend far into solution at low ionic strength. As the AFM tip compresses the layer, fewer polymers come in contact with the tip and thus the steric hindrance is reduced. This results in a large penetration depth, which translates to low Young’s modulus of the fibril layer. Decreasing the ionic strength caused the fibrils of strain HB7 to extend into solution. This decreases the steric hindrance between the molecules upon compression. The AFM tip penetrates further into the layer, which results in a decrease of its Young’s modulus. Similarly, increasing the buffer ionic strength contracts the surface appendages of strain HBV51, increases the steric repulsion forces, and leads to a stiffer fibril layer. It should be noted that at 57 mM adhesion buffer, the AFM tip may also be compressing the bacterial cell membrane. This can be deduced from the measured penetration depth and the change in fibril length upon changing the buffer concentration as quantified by TIRM.

3.5 Comparison of the adhesive bond stiffness of streptococci as characterized by AFM and QCM-D

Bacterial surface appendages are not the only factor responsible for microbial adhesion, but they can significantly influence the process and ultimately alter its outcome. The adhesive bond stiffness of streptococci measured acoustically by QCM-D depends on the Young’s modulus of the cell surface appendages, which can be probed mechanically using AFM. Instrument limitations did not allow for the exact determination of the zero-crossing frequency
upon bacterial adhesion to the QCM-D sensor, which can be used to calculate the bond stiffness according to Equation 21. Instead, the data was extrapolated using the coupled resonance model (Equation 20) in order to qualitatively characterize the bond properties. The increase in zero-crossing frequency with increase in ionic strength suggested the formation of stiffer contact upon buffer change for both strains. Strain HB7 was predicted to have higher adhesive bond stiffness at all ionic strengths than strain HBV51, which also experienced a more pronounced change in contact stiffness between the different buffers (Figure 13).

These qualitative predictions were confirmed in this study by measurements of the Young’s moduli of the bacterial surface appendages using AFM. It was observed that increase in buffer ionic strength leads to increase in the stiffness of the fibrils layer. Strain HB7 exhibited higher Young’s moduli at both ionic strengths. Furthermore, strain HBV51 experienced double the percent change in Young’s modulus compared to strain HB7 at the different buffers tested.
4. CONCLUSIONS

Quantitative Nanomechanical AFM imaging was used to characterize the influence of ionic strength on the stiffness of bacterial surface appendages. *S. salivarius* HB7 and HBV51 demonstrated decreases in the Young’s modulus and increases in the deformation of the surface appendages layer when the buffer ionic strength decreased. *S. salivarius* HB7, which expresses a dense layer of 91 nm long fibrils, exhibited higher stiffness and lower deformation of the surface appendages layer than *S. salivarius* HBV51 possessing a sparse layer of 63 nm long fibrils at both ionic strengths tested. Additionally, strain HBV51 experienced a more pronounced decrease in stiffness with the change in buffer ionic strength.

These results confirm predictions of the adhesive bond stiffness of the two strains to the surface of a QCM-D sensor. This demonstrates that AFM can be used in conjugation with QCM-D to study bacterial bond stiffness. Furthermore, this study validates the use of the QCM-D as a convenient tool to investigate bacterial bond stiffness under different environmental and experimental conditions. The combination of AFM and QCM-D studies has the potential to facilitate the development of methods to influence microbial surface attachment and dissemination, in order to reduce clinical infection rates and industrial spending on equipment maintenance.
APPENDIX: PeakForce QNM Calibration

Quantitative Nanomechanical AFM imaging requires proper calibration in order to obtain the true values of the measured parameters. The solution of Equations 3, 6, and 7 requires specification of the AFM tip radius, $R$, and the sample Young’s modulus, $\mu$. Furthermore, determination of the applied force from the measure of the tip deflection necessitates knowledge of the spring constant of the tip and the optical sensitivity of the instrument. The latter is measured by compressing an infinitely hard sample (e.g. clean glass slide or freshly cleaved mica) and recording the change in detector signal with the extension of the piezo tube. The spring constant of the cantilever is usually measured by a thermal method for soft probes.\(^{38}\) The cantilever amplitude is monitored as a function of frequency and a thermal model, based on the equipartition theorem, is applied to the data in order to extract the spring constant. Calibration of the optical sensitivity and the spring constant of the AFM cantilever is straightforward and does not require additional instrumentation. Measurement of the tip radius, however, requires additional experimentation and is often less precise. It can be achieved by either direct imaging using scanning electron microscopy or indirectly, by AFM imaging of a specially designed sample with sharp peaks, which results in the acquisition of image artifacts, from which the tip radius can be extracted. Finally, calculation of the sample Young’s modulus, $E$, requires the knowledge of its Poisson’s ratio. This parameter is difficult to measure experimentally, which necessitates the assumption of an estimated value. Alternatively, the reduced modulus, $K$, may be reported.

PeakForce QNM calibration can either be performed directly, by measuring the calibration parameters as discussed above, or indirectly, by imaging a sample of known stiffness and adjusting the calibration parameters to match the sample Young’s modulus. The indirect
(relative) method avoids accumulation of error by measurement of separate calibration parameters, but required the use of a reference sample of well characterized stiffness. The direct (absolute) method avoids the use of a of a reference sample, but requires accurate measurement of the tip radius and cantilever spring constant.

In this study, calibration of the PeakForce QNM imaging parameters was performed using the direct method before every experiment. The AFM tip radius was measured by scanning a titanium rough sample (RS-15M, Bruker AFM Probes, Camarillo, CA) and analyzing the data using the built-in function of the Nanoscope Analysis 1.40 software (Figure A1).

![Image of AFM tip radius measurement](image)

**Figure 21.** Estimating the tip radius from image of tip quantification sample. Images of the tip (upper right) are automatically detected from a scan of the tip quantification sample (left) and the radius of the tip ($R = 18$ nm) is measured at 5 nm from its end (lower right).

The validity of the direct calibration method was confirmed by imaging a polydimethylsiloxane (PDMS) sample of known Young’s modulus (Figure A2).
Figure 22. Quantitative Nanomechanical imaging of PDMS reference sample. The measured elasticity of the sample is in good agreement with the manufacturer’s specification.

The measured stiffness of the sample was $E = 3.74 \pm 0.15$ MPa, under the assumption that its Poisson’s ratio is equal to 0.5. This is in perfect agreement of the $3.5 \pm 0.5$ MPa stiffness specified by the manufacturer.

The stiffness (spring constant) of the AFM probe must also be considered for correct determination of sample mechanical properties. The AFM cantilever needs to be stiff enough in
order to cause of minimum of 2 nm indentation of the sample, but still flexible enough, so that to provide a high level of sensitivity. SCANASYST-FLUID tips are appropriate for samples of Young’s moduli in the range of 0.1 – 10 MPa. A HSDC of the PDMS sample reveals that the tip causes deformation of approximately 3 nm under a load of ~1 nN (Figure A3).

Figure 23. High Speed Data Capture of PDMS sample. The AFM tip causes sample indentation of approximately 3 nm under applied load of ~1 nN

Thus SCANASYST-FLUID tips are appropriate for imaging bacterial cells, which typically have Young’s moduli in the 0.1 – 3 MPa range. These probes, however, cannot be used to measure the
stiffness of glass (~70 GPa). Thus even though the glass substratum was imaged as part of the sample characterization in this report, the glass stiffness values evident from the presented figures are not correct due to calibration and instrument limitations issues.
REFERENCES