Chapter 7

General discussion
Introduction

Along with the development of new materials and changing societal views, such as an increasing desire for social competition, more and more people want orthodontic treatment to obtain a beautiful and healthy smile to enhance their quality of life, self-image and competitiveness in job interviews\(^1\)\(^-\)\(^3\). In North America alone, there are five million patients receiving orthodontic treatment. However, like other forms of medical and dental treatment, orthodontic treatment is also accompanied by risks\(^4\). The placement of orthodontic appliances on teeth not only impedes the maintenance of a proper oral hygiene\(^5\)\(^,\)\(^6\) but also increases the level of cariogenic bacteria in the oral cavity\(^7\)\(^,\)\(^9\), leading to serious biofilm-related side-effects such as white spot lesions and gingival inflammation\(^10\)\(^-\)\(^12\), compromising facial esthetics after an often lengthy and costly course of orthodontic treatment.

Clinical observation indicates that the most common site for bacterial adhesion and biofilm formation is at the bracket-adhesive-enamel junction, an area that is difficult to clean by daily brushing\(^12\),\(^13\). Oral biofilms at this junction not only cause damage to oral hard and soft tissues but also weaken the bond strength of adhesives\(^14\)\(^-\)\(^16\). Excessive adhesive around brackets especially provide a site for the rapid adhesion and growth of bacteria\(^17\). Furthermore, the surface of an orthodontic adhesive is often rough, with a gap of around 10 \(\mu\)m at the adhesive-enamel interface due to polymerization shrinkage. This provides adhering bacteria with a protected site against oral cleansing forces\(^13\),\(^18\),\(^19\). Consequently, the bracket-adhesive-enamel junction is a critical site for bacterial adhesion and biofilm formation in orthodontic patients.

Adhesion forces determine the ability of adhering oral bacteria to withstand oral detachment forces, i.e. forces exerted by tooth-brushing, mastication, salivary flow or tongue movement. These adhesion forces can be directly measured using atomic force microscopy (AFM), either by immobilizing bacteria on a substratum surface and probing the cell surface with the AFM tip, or by attaching bacteria to a cantilever to constitute a bacterial probe to examine interactions with orthodontic
materials\textsuperscript{20-22}. Bacterial adhesion forces can be further decoupled into hydrogen bonding contributions and nonspecific forces\textsuperscript{23-25} to provide insights into the mechanism and nature of bacterial adhesion to substrata.

The studies in this thesis measured and analyzed bacterial adhesion forces mediating biofilm formation to orthodontic materials, constituting the bracket-adhesive-enamel junction, and investigated the nature of bacterial bond strengthening on enamel and stainless steel. Moreover, the influence of surface roughness of adhesives on bacterial adhesion forces was investigated and an antimicrobially modified adhesive was developed by incorporation of a quaternary ammonium compound for the prevention of orthodontic biofilm formation.

**Bacterial adhesion forces to orthodontic materials**

For a better understanding of the mechanism and nature of bacterial adhesion to orthodontic materials, we firstly measured the adhesion forces of different oral bacterial strains to materials constituting the bracket-adhesive-enamel junction using AFM in the absence and presence of a salivary conditioning film. Secondly, we investigated bacterial adhesion forces in relation to physicochemical properties of the orthodontic materials and bacterial strains.

Bacterial adhesion involves an interplay of various physicochemical properties of substratum and bacterial cell surfaces as well as local environmental conditions\textsuperscript{26}. The three materials studied in this thesis, stainless steel, composite, and enamel, possess different roughnesses, hydrophobicities, and chemistries (Chapter 2). Composite, with the roughest and most hydrophobic surface, exerted the strongest adhesion forces. Saliva-coated materials, with smoother and more hydrophilic surfaces, showed weaker adhesion forces. We demonstrated that increased surface roughnesses of composites lead to increasing bacterial adhesion forces. This might be due to the fact that the rougher surfaces provide bacteria with more extensive contact areas to form bonds contributing to stronger adhesion...
forces$^{18,27-30}$. Moreover, water is more easily removed from the interface between bacteria and a hydrophobic material than between bacteria and a hydrophilic material$^{31-34}$, which increases the bond strength as the presence of water attenuates the attractive Lifshitz-Van der Waals forces. Similarly, increasing adhesion time will stimulate the formation of strong and irreversible bonds between bacteria and surfaces due to an increase in bacterial adhesion forces, as confirmed by our results. The influence of the materials chemistries on bacterial adhesion forces is reflected directly in the force values measured, and indirectly from the effects of salivary conditioning films on the adhesion forces found (Chapter 3 and Chapter 4).

The development of multi-species oral biofilms in vivo follows a well-sequenced spatio-temporal pattern$^{26,35-39}$, in which late colonizers, such as the more cariogenic strains used in this thesis, do not adhere directly to the substratum surface but to initial colonizers already adhering on the surface. In order to ensure successful and stable biofilm formation, the initial colonizers must adhere more strongly to the substratum surface than late colonizers. This may be a manifestation of the division of labor and the cooperation among bacteria in a microbial community, as illustrated also in previous studies$^{40,41}$. In fact, it may constitute the reason why adhesion forces of initial colonizers are significantly stronger than those of late colonizers.

**The nature of bacterial adhesion forces to orthodontic materials**

The nature of the adhesion force can be analyzed by Poisson analysis of the AFM retract force-distance curves of bacterial probes from surfaces. The adhesion force can be decoupled into a short-range hydrogen bonding contribution ($F_{H-bond}$) and a long-range non-specific force ($F_{Non-specific}$)$^{24,25,37}$. The $F_{Non-specific}$, including Lifshitz-Van der Waals and electrostatic forces, work instantaneously upon approach of the interacting surfaces. In contrast, the development of $F_{H-bond}$ is a time-dependent,
stereo-chemical interaction, requiring close approach and full removal of the interfacial water from the gap in between the interacting surfaces\textsuperscript{31}. Therefore, bacterial bond strengthening as reported in our studies, is governed by $F_{\text{H-bond}}$ on both non-conductive enamel surfaces and conductive stainless steel surfaces. This bond strengthening occurred in tens of seconds, leading to significantly stronger adhesion forces and explaining the transition from initially reversible to the more irreversible adhesion of bacteria to surfaces\textsuperscript{32,37}.

Due to the forced nature of AFM contact, the non-specific force contribution has hitherto turned out to be repulsive on non-conductive surfaces, such as glass and silicon nitride\textsuperscript{25,31}. Our findings, in line with this, indicated that $F_{\text{Non-specific}}$ was repulsive on the non-conductive surfaces of enamel and saliva-coated stainless steel. However, interestingly and for the first time, we found that the $F_{\text{Non-specific}}$ on conductive surfaces of stainless steel were attractive. Approach and retract force-distance curves deviated dramatically at close distance (20-40 nm) in contrast to the approach and retract curves on non-conductive enamel and saliva-coated stainless steel surfaces, which overlapped (Chapters 3 and Chapter 4). We contribute this difference to charge transfer between bacteria and conductive surfaces upon contact, and the attraction between the negatively charged streptococci and positive image charges in the conductive material. Our findings presented a new mechanism of bacterial adhesion to conductive materials, and indicated that special considerations may be needed for the development of preventive measures on metallic surfaces.

Previous studies reported slightly smaller $F_{\text{Non-specific}}$ and $F_{\text{H-bond}}$ values between \textit{Escherichia coli} and silicon nitride AFM tips\textsuperscript{25}. It is not clear whether the different results are due to the fact that those experiments were carried out with a small AFM tip on a bacterial cell surface, while we used a much larger streptococcal probe on a salivary conditioning film. Also different bacterial strains may adhere with completely different characteristics. Whether Poisson analysis truly yields a single $F_{\text{H-bond}}$ instead of a total $F_{\text{H-bond}}$ originating from a single characteristic
molecular moiety on the bacterial cell surface is currently not certain. These uncertainties provide a broad perspective for future research on the mechanisms of bacterial adhesion forces.

**Biofilm prevention by modification of orthodontic adhesives**

Development of orthodontic materials attracting less biofilms has been a goal for decades. Attempts have been made to develop effective antimicrobial adhesives to prevent orthodontic biofilms\(^{42,43}\). Yet, till date no commercial products have been available for orthodontic patients.

Different antimicrobial agents, including fluoride, chlorhexidine, cetylpyridinium chloride, benzalkonium chloride, 12-methacryloyloxydodecyl pyridinium bromide, and casein phosphopeptide-amorphous calcium phosphate, have been incorporated into orthodontic adhesives for biofilm prevention\(^{44-54}\). Most of these modifications largely depend on the release kinetics of the antimicrobial components in saliva. Due to the wash-out effect *in vivo*, a minimal inhibitory concentration preventing oral microbial growth often exists only for a short period of time in the oral cavity. For instance, the release of fluoride and chloride from adhesives showed a burst release during the first two weeks, followed by a much lower tail-release\(^{44,51}\). A relatively long-term antibacterial activity has been obtained by incorporating nanoparticles into adhesives, including silica, silver, polyethyleneimine, zinc oxide, and quaternary ammonium polyethylenimine nanoparticles\(^{54-57}\). However, the safety of nanoparticles for human use is still a matter of controversy\(^{54,58}\). Moreover, the orthodontic appliances remain in the oral cavity often for several years, exceeding the reservoir capacity that the small volume of adhesives could offer. The results in this thesis showed that a non-leaching contact-killing composite adhesive modified by a non-bactericidal monomer of quaternary ammonium compound (QAC, 3-(methacryloylamino)propyl trimethylammonium chloride) may provide a better solution for orthodontic
applications than modifications of adhesives based on release of antibacterial substances (Chapter 6).

QAC monomers can be polymerized within the composite resin to form a cationic surface. The mechanisms of action of polycations involve disruption of the integrity of the bacterial membrane. The most quoted theory hypothesizes that cationic polymers coated on a surface can penetrate bacterial membranes leading to contact-killing, depending on their molecular length. The cationic QAC monomer used in our study is not long enough to penetrate the bacterial membrane, resulting in non-bactericidal effects. After being chemically polymerized with the composite molecules, the cationic chains were bound chemically within the composite and were thereby prolonged and long enough to kill bacteria without leaching from the composites. This mechanism of contact-killing led to reduced bacterial growth, and although attenuated, was still significant after coating the modified composite with a salivary conditioning film. Another mechanism hypothesizes that the disruption of the integrity of the bacterial membrane is caused by a counterion exchange between a highly charged cationic surface and structurally critical mobile cations within the membrane. A minimum charge-density is necessary for optimum efficiency of QAC cationic surfaces and also depends on growth state and bacterial strain involved. In our study, at a low QAC concentration, the charge-density of the cationic surface was not high enough for bacterial killing, but showed a growth inhibition. To date, it is still unclear whether the contact-killing of QAC cationic surface is through penetration into the bacterial cells or an electrostatic mechanism based on the exchange of counterions between the functionalized cationic surface and the bacterial membrane, or both. Further insight into the mechanisms of contact-killing of QAC cationic surfaces is a promising field to explore.

Other QAC monomers such as methacryloyloxydodecyl pyridinium bromide (MDPB) incorporated in restorative composite also demonstrated antibacterial properties. The monomer of MDPB, however, was bactericidal with a potential
harm to cells \textit{in vivo} if not being thoroughly polymerized\textsuperscript{59,64}. The QAC monomer in our study is only bactericidal after polymerization, and therefore overcomes this weakness.

The polymerization between QAC monomers and composite molecules produces a crosslinked polymer network, resulting in a non-leaching contact-killing surface. On the other hands, crosslinking decreased the density of the adhesive, leading to a significant loss of its mechanical properties, although it is not clear what the clinically acceptable limit is. Research is ongoing in our team to improve the mechanical bonding strength of the QAC modified composites.

\textbf{Clinical implications and future research}

The findings of this thesis provide fundamental information for understanding the mechanism and prevention of bacterial adhesion on orthodontic materials, and may therewith be of considerable value to clinical practice.

Since our study shows that surface roughness increases the bacterial adhesion forces, it would be desirable that orthodontists minimize the adhesive surface roughness by smoothing, polishing, or varnishing after bonding. This is a simple yet efficient way to reduce bacterial adhesion at the bracket-adhesive-enamel junction. Orthodontic material manufacturers might also provide additional procedures to decrease the surface roughness of their products for clinical practice.

Although the hydrophobicities of stainless steel, adhesives, and enamel were different, the salivary conditioning film decreased this difference significantly and therewith also the bacterial adhesion forces. This indicates that the development of antibacterial modification of orthodontic materials should always take the effects of a salivary conditioning film into account. As the adhesion forces of initial colonizers were significantly stronger than those of the more cariogenic strains, while adhesion of initial colonizers is determinant for the strength of adhesion of the
overlaying biofilm structure, future research should be directed toward prevention of the adhesion of initial colonizers.

The long duration of orthodontic treatments and salivary flow in the oral cavity favor orthodontic materials with non-leaching, long lasting bactericidal properties. The modification of an orthodontic adhesive with a quaternary ammonium compound provided efficient contact-killing, with promising prospects for clinical application. Future research to enhance the mechanical strength by improving the processing conditions, *i.e.* curing the samples at a higher temperature, or adding a diacrylate to increase the density of crosslinking, would be approaches worth exploring.
References

Chapter 7


General discussion
