Antibacterial measures for biofilm control
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Summary
In this thesis different antibacterial measures for oral biofilm control were investigated. **Chapter 1** identifies problems related to oral biofilm infections with emphasis on orthodontics and describes different approaches for antibacterial measures.

In selecting research topics for (biomedical) research the involvement of end-users becomes increasingly important, particularly in a field as orthodontics with which a major part of the population has become in contact with. A case example of the participation of orthodontic end-users in selecting research topics is presented in **Chapter 2**, in which patients, parents and care providers are involved in the set-up and topic selection of a part of this thesis, using a structured questionnaire. The questionnaire addressed different aspect of oral biofilm control in orthodontic patients and asked what aspects and new developments would be valued most by them as end-users. All respondents, including patients, parents of patients, orthodontists and paramedics scored highest for 'non-compliance' bacterial-killing adhesives with lasting killing effect. The results demonstrate that end-users can make a valuable contribution for scientists in the selection for societally-relevant research topics, when the main purpose of the research work is to reach its potential end-users and provide benefit for their health and wellbeing. Moreover, public opinion can help scientists to better understand the needs of end-users.

In daily life, manual or powered brushing are still by far the most effective measure for oral hygiene maintenance in orthodontic patients. In **Chapter 3** orthodontic, multi-strand retention-wires are used as a generalized model for oral retention sites to investigate whether biofilm left-behind after powered toothbrushing *in-vivo* enabled better penetration of antibacterials as compared with manual brushing. 2-cm multi-strand, stainless-steel retention-wires were placed in brackets bonded bilaterally in the upper arches of 10-volunteers. Volunteers used a NaF-sodium-lauryl-sulphate-containing toothpaste and antibacterial, triclosan-containing toothpaste supplemented or not with an essential-oils containing mouthrinse. Opposite sides of the dentition including the retention-wires, were brushed manually or with a powered toothbrush. Health-care-regimens were maintained for 1-week, after which wires were removed and oral biofilm was collected. When powered toothbrushing was applied, slightly less bacteria were collected than after manual brushing, regardless whether an antibacterial-regimen was used or not. Powered-toothbrushing combined with antibacterial-regimens yielded lower biofilm viability
than manual brushing, indicating better antibacterial penetration into biofilm left-behind after powered brushing. Major shifts in biofilm composition, with a decrease in prevalence of both cariogenic species and periodontopathogens, were induced after powered brushing using an antibacterial-regimen. Oral biofilm left-behind after powered brushing *in-vivo* enabled better penetration of antibacterials than after manual brushing.

Oral healthcare products with antibacterial components may be effective for oral biofilm control, but at the same time bear the threat of developing bacterial resistance. Clinically, strains such as *Staphylococcus aureus* have been found resistant to chlorhexidine, while in dental practice oral bacterial strains, including *Streptococcus mutans* have remained largely susceptible to chlorhexidine. The aim of Chapter 4 is to speculate on the mechanisms through which *S. aureus* adapts resistance against chlorhexidine *versus* *S. mutans* remaining susceptible.

Chlorhexidine exposure of adhering bacteria to (sub)-MIC concentrations of chlorhexidine yielded reversible, nanoscopic cell wall deformation in *S. mutans*, but not in *S. aureus*, indicative of loss of intracellular, cytoplasmic pressure in *S. aureus*. Although overall cell surface properties of both strains did not significantly change, propidium iodine staining demonstrated that the *S. aureus* cell membrane was indeed more easily damaged than the *S. mutans* cell membrane. Significantly, metabolic activity of *S. mutans* changed little upon exposure to chlorhexidine, while *S. aureus* metabolic activity became much higher. Concurrently, repeated culturing in presence of chlorhexidine demonstrated that chlorhexidine resistance was easy to induce in *S. aureus*, but not in *S. mutans*. Exact interpretation of these data is difficult. *S. aureus* may adapt a high metabolic activity to survive chlorhexidine attack, e.g. by activating efflux pumps or opening of membrane channels to decrease the intracellular chlorhexidine concentration. This may cause loss of intracellular pressure yielding cell wall deformation, and at the same time stimulate development of chlorhexidine resistance. In *S. mutans*, cell wall deformation was reversible within 15 min after exposure to chlorhexidine, suggesting spontaneous, strong cell wall self-repair. Due to cell wall self-repair, *S. mutans* may be unable to effectively reduce the chlorhexidine concentration in its interior, preventing its survival and development of a resistant progeny.

Like resistance to antimicrobials can develop differently in different strains, antibiofilm activity of an antimicrobial may be achieved by different mechanisms of
action: preventing bacterial adhesion, limiting bacterial growth, disrupting an already established biofilm or altering the composition and/or pathogenicity of the biofilm. One strategy of particular interest in dentistry is modifying dental materials to equip them with antimicrobial properties. Cationic surfaces with alkylated quaternary-ammonium groups kill adhering bacteria upon contact by membrane disruption and are considered increasingly promising as a non-antibiotic based way to eradicate bacteria adhering to surfaces. However, reliable in-vitro evaluation methods for bacterial contact-killing surfaces do not yet exist. More importantly, results of different evaluation methods are often conflicting. Therefore, we compared in **Chapter 5** five methods to evaluate contact-killing surfaces. To this end, we have copolymerized quaternary-ammonium groups into diurethane dimethacrylate/glycerol dimethacrylate (UDMA/GDMA) and determined contact-killing efficacies against five different Gram-positive and Gram-negative bacterial strains. Spray-coating bacteria from an aerosol onto contact-killing surfaces followed by air-drying as well as ASTM E2149-13a (American Society for Testing and Materials) were found unsuitable, while the Petrifilm® system and JIS Z 2801 (Japanese Industrial Standards) were found to be excellent methods to evaluate bacterial contact-killing surfaces. It is recommended however, that these methods be used in combination with a zone of inhibition on agar assay to exclude that leakage of antimicrobials from the material interferes with the contact-killing ability of the surface.

For clinical applications, it would be advantageous to incorporate contact-killing properties in a material with other unique features, e.g. 3D printability and mechanical versatility. 3D printing is seen as a game-changing manufacturing process in many domains, including general medicine and dentistry, but the integration of more complex functions into 3D-printed materials remains lacking. In **Chapter 6-I**, we demonstrated the development of a 3D-printable antibacterial material. Monomers containing antimicrobial, positively charged quaternary ammonium groups with an appended alkyl chain are either directly copolymerized with conventional diurethane dimethacrylate/glycerol dimethacrylate (UDMA/GDMA) resin components by photocuring or prepolymerized as a linear chain for incorporation into a semi-interpenetrating polymer network by light-induced polymerization. For both strategies, dental 3D-printed objects fabricated by a stereolithography process kill bacteria on contact when positively charged quaternary
ammonium groups are incorporated into the photocurable UDMA/GDMA resins. Leaching of quaternary ammonium monomers copolymerized with UDMA/GDMA resins is limited and without biological consequences within 4–6 d, while biological consequences could be confined to 1 d when prepolymerized quaternary ammonium group containing chains are incorporated in a semi-interpenetrating polymer network. Routine clinical handling and mechanical properties of the pristine polymer matrix are maintained upon incorporation of quaternary ammonium groups, qualifying the antimicrobially functionalized, 3D-printable composite resins for clinical use.

Our manuscript on the development of a 3D printable antimicrobial composite resin, within the first weeks after being published in Advanced Functional Materials, immediately received tremendous attention from both national and international mainstream media. Even though our research project received enormous attention and positive recognition at the social media platforms, the impact of these reports is a double-edged sword, as discussed in Chapter 6-II. The public attention of this research project could be largely attributed to recognition by the general public of its potential societal impact that the research outcome might generate but was not based on its true scientifically proven content. Therefore, it remains a challenge for researchers to reach the public with their research project, and properly guide this process so that the right information and interpretation are spread.

In the general discussion in Chapter 7 the results of the studies are discussed from a clinical perspective and suggestions for future research opportunities are made.