CHAPTER 7

General Discussion
In this thesis we forward the hypothesis that the viscoelasticity of a biofilm relates to antimicrobial penetration. This hypothesis has been confirmed by the results presented in this thesis. In this chapter, we will discuss the following aspects of the different studies presented in this thesis:

- Relation of biofilm viscoelasticity and antimicrobial penetration
- *In vivo* biofilm models
- Future studies.

**Relation of biofilm viscoelasticity and antimicrobial penetration**

Viscoelasticity is introduced to study biofilm structure and composition in an indirect but quantitative way. Stress relaxation of a biofilm was analysed in terms of three different underlying processes with characteristic time constants of 0-5 s, 5-100 s and > 100 s, called the fast, intermediate and slow response, respectively. Water and other smaller molecules of the biofilm matrix are expected to constitute the fast response; higher molecular weight components of the extracellular matrix are assumed to cause the intermediate response; bacteria themselves represent the slow response. Although intuitive, this sectioning of time is arbitrary. Nevertheless, principal component analysis of the stress relaxation behavior of different model biofilms has confirmed that three principal components suffice to describe the viscoelastic relaxation of biofilms (Peterson *et al.*, 2013), while moreover the meaning attributed to these biofilm components based on the known compositions of the biofilms used roughly corresponds with our intuitive ones.

It has been discussed that antimicrobial penetration into a biofilm is a process of antimicrobial diffusion (Stewart, 2003), but the structural and
compositional heterogeneity of a biofilm greatly influence the diffusion process (Corbin et al., 2011) in a way that is not easy to model. Biofilms develop their chemical, biological and mechanical properties during formation. Taking oral biofilms as an example, oral biofilms experience the challenge of daily mechanical removal and chemical exposure. Studies reveal that most chemicals undergo an instant and reversible binding to oral biofilms (Lieleg et al., 2011; Brindle et al., 2011), although oral biofilms exist not only as a target of antimicrobials, but also as a reservoir for those antimicrobials (Otten et al., 2012). Interestingly, we demonstrated in chapter 5 that the viscoelastic properties of oral biofilms after brushing had been changed in a direction that, in line with the hypothesis underlying this thesis, enhanced antimicrobial uptake in biofilm left-behind after brushing.

Chlorhexidine (CHX), used as an indicator molecule of antimicrobial penetration in most chapters because of its high killing efficacy, is positively charged and binds to negatively charged biofilm components. In chapter 2 and 3, we found that CHX penetration was negatively correlated to $E_1$, the fastest relaxation element. Interestingly, Wilson et al. (1998) described that killing by CHX of bacteria in biofilms formed in the presence of sucrose was twice higher than in biofilms formed without sucrose. Sucrose promotes the formation of extracellular matrix in biofilms, which might impede penetration of CHX. On the other hand, the sucrose-rich nutrition promotes the metabolic activity of bacteria, which may make bacteria in a biofilm more susceptible to antimicrobials. Hence, the authors considered their experimental results as inexplicable (Wilson et al., 1998). Based on the hypothesis underlying this thesis, we can now explain these results using the differences in viscoelasticity of biofilms grown in absence
or presence of sucrose: biofilms grown in absence of sucrose have a three times higher fast component $E_1$ than biofilms grown in presence of sucrose (see Table 1, unpublished data). Our hypothesis, as confirmed in this thesis, now yields a clear explanation of higher killing of bacteria by CHX in biofilms formed in the presence of sucrose. This is merely one of the many pathways opened by our hypothesis for improved understanding of the recalcitrance to antimicrobials of biofilms in general. Besides improved understanding of the mechanism of antimicrobial recalcitrance, our study also points to a pathway of fine-tuning the frequency and the energy output of powered toothbrushes to optimize the reservoir function of oral biofilm-left-behind after brushing.

**Table 1** The viscoelasticity$^1$ of *S. mutans* and the concentration of sucrose in growth medium$^2$.

<table>
<thead>
<tr>
<th>Sucrose (%)</th>
<th>$E_1$ (%)</th>
<th>$E_2$ (%)</th>
<th>$E_3$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.6 ± 4.2</td>
<td>17.5 ± 7.6</td>
<td>61.6 ± 10.1</td>
</tr>
<tr>
<td>3</td>
<td>6.8 ± 0.9</td>
<td>7.0 ± 1.3</td>
<td>86.2 ± 0.4</td>
</tr>
</tbody>
</table>

$^1$data refer to average ± SE over triplicate experiments

$^2$unpublished data

**In vivo biofilm models**

Structure and composition of biofilms are influenced by the hydrodynamic environment during formation (Paramonova *et al.*, 2009). Especially viscoelasticity has been demonstrated to correlate with biofilm structure (Lau *et al.*, 2009). In chapters 2 and 4, growth of single strain biofilms in different *in vitro* systems indicated that the viscoelastic properties and antimicrobial penetration of single strain biofilms differed when grown in a parallel plate flow chamber *versus* a constant depth film fermenter *versus* a
well plate system, although experiments in all different systems, including in an \textit{in vivo} biofilm collection device (chapter 3), confirmed our initial hypothesis.

Conventionally, \textit{in vivo} biofilm was collected from substrata (Fig. 1), like extracted teeth (Zijinge et al., 2010), modified molar bands (Amrberg et al., 1984) or fixed and removable enamel/dentin chip (Macpherson et al., 1990; Lagerweij et al., 1996; Shore et al., 2001; Hara et al., 2003). Removal of biofilms in either type of collection device however, will violate the integrity of the biofilm. In this thesis, for the first time we introduced a fixed stainless steel device to grow an undisturbed \textit{in vivo} oral biofilm. The device design enables repetitive sampling from the same volunteer without damage to the enamel surface or disturbing the biofilm. During the experiment, there was no excessive biofilm accumulation around the device or teeth. Additionally, the \textit{in vivo} biofilm grown on a stainless steel plate guarantees the integrity of the biofilm during harvest and facilitates the manipulation of the biofilm during measurement, which makes the \textit{ex vivo} measurement feasible and coherent.

\textbf{Future studies}

In this thesis, we discovered a relation between the viscoelasticity of biofilms and antimicrobial penetration. Based on this discovery, we proposed to consider viscoelasticity a virulence factor of biofilms. Three possible directions for future studies are suggested:

- Verification of our hypothesis for biofilms and antimicrobial penetration for biofilms relevant to other, non-oral infections in the human body and development of means to direct their viscoelastic
Figure 1 Devices used in the literature to collect *in vivo* biofilms. (A) Modified band mounted on a premolar (occlusal view). *In vivo* biofilm is formed between the band and tooth (Arneberg *et al*., 1984). (B) Intra-oral device mounted on upper maxilla (diagram), containing dentin chips. *In vivo* oral biofilm was collected from the chips (Hara *et al*., 2003). (C) Dentin chip mounted on protheses (diagram). *In vivo* oral biofilm forms in the grooves (Lagerweij *et al*., 1996). (D) Enamel chips bonded on teeth (diagram). *In vivo* oral biofilm forms in the space created by the nylon ring (Shore *et al*., 2001). (E-F) Stainless steel device bonded on a molar (buccal view), as being used in this thesis. *In vivo* oral biofilm was collected from the inner side of the plate (occlusal view) (He *et al*., 2013). All the pictures are reproduced with permission.
properties in a direction making the biofilms more susceptible to antimicrobial penetration;
- Identification of the chemical composition and detailed structure responsible for the stress relaxation and different Maxwell elements of biofilms on a non-intuitive level;
- To explore and establish a synergy between the parameters of powered toothbrushes and the penetration of different antimicrobials to make the optimal use of the known reservoir function of oral biofilm left-behind after brushing.
REFERENCES


Shore RC, Kirkham J, Devine D, Marsh P, Nattress B, Robinson C (2001). Investigation to evaluate and validate the Leeds in situ device for the study of


