Viscoelasticity of oral biofilms and antimicrobial penetration - an in vitro and in vivo study -
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Summary
Biofilms are communities of microorganisms embedded in a matrix of extracellular polymeric substances that play an important role in conveying viscoelastic properties to a biofilm. Viscoelasticity is one of the features that a biofilm possesses to relieve itself from mechanical stresses causing its removal and is governed by an interplay of structure and composition of the biofilm. Structure and composition of biofilms also determine the penetration of antimicrobials into a biofilm. However, hitherto biofilm structure and composition have only been visually assessed using microscopic techniques and no quantitative means exist to evaluate these features to provide a better basis for understanding antimicrobial penetration or lack of penetration into biofilms. Because of the relation between viscoelasticity, structure and composition, we hypothesise in Chapter 1 that the viscoelasticity of a biofilm relates to antimicrobial penetration since both relate to biofilm structure. The verification of this hypothesis constitutes the general aim of this thesis.

In Chapter 2, we aimed to gain in vitro evidence to support our hypothesis that viscoelastic properties of oral biofilms relate with chlorhexidine (CHX) penetration into biofilms. In vitro biofilms with different viscoelastic properties of coccal and rod-shaped microorganisms were grown to the same thickness in a parallel plate flow chamber (PPFC) and constant depth film fermenter (CDFF) and subjected to deformation. Stress relaxation analysis identified a fast, intermediate and slow response of a biofilm to an induced deformation, presumably corresponding with the outflow of water, extracellular polymeric substances and re-arrangement of the bacteria. The penetration of CHX into biofilms increased with increasing importance of the slow relaxation response and decreasing importance of the fast
response. Involvement of the slow relaxation component suggests that biofilm structures allowing extensive bacterial re-arrangement after deformation are more open for better antimicrobial penetration. Assuming the fast relaxation component represents the presence of water, its involvement suggests that a high amount of water dilutes the CHX upon penetration to an ineffective concentration in deeper layers of a biofilm.

In Chapter 3, we designed a device for \textit{in vivo} oral biofilm collection to evaluate up to what extent \textit{in vivo} oral biofilms are comparable with respect to their viscoelastic properties and CHX penetration to \textit{in vitro} oral biofilms formed in a PPFC or CDFF. The \textit{in vivo} oral biofilms were grown in absence of mechanical perturbation. Volunteers wore the biofilm collecting device, fixed on the buccal surface of the maxillary first molar for approximately eight weeks. Biofilms formed on a rectangular, replaceable stainless steel plate, bonded onto the device. Every two weeks, the stainless plate with \textit{in vivo} biofilm formed on its inner surface was removed for evaluation of the viscoelastic properties of the biofilms and penetration of CHX. Two weeks undisturbed biofilm formation yielded \textit{in vivo} biofilm thicknesses that were similar to the ones obtained \textit{in vitro} after 48 h (PPFC) or 96 h (CDFF) of growth. \textit{In vivo} formed oral biofilms showed relaxation characteristics upon 10 and 20\% induced deformation that were most comparable to the ones observed for \textit{in vitro} biofilms formed in the PPFC. Upon 50\% induced deformation, the base of the biofilms became invoked in the relaxation process, and no significant differences were observed between stress relaxation of \textit{in vivo} and \textit{in vitro} biofilms. Nevertheless, \textit{ex situ} CHX penetration into \textit{in vivo} formed oral biofilms followed a similar dependence on the prevalence of the fast and slow relaxation components as observed
for *in vitro* biofilms. This study demonstrates that quantitative relations can be obtained between biofilm properties and CHX penetration, which are not only valid for *in vitro* formed biofilms but also for *in vivo* formed ones. In this respect, it may be important to use *in vitro* biofilms formed in different systems in order to mimic biofilms formed in different regions of the oral cavity. Biofilms grown in the PPFC better resemble the outside of *in vivo* grown biofilms, while biofilms grown under compaction in the CDFF better mimic the base properties of *in vivo* grown biofilms.

CHX, though effective, possesses several drawbacks for extended clinical use. It is known that other commercially available mouthrinses also contain a variety of different antimicrobial compounds. In *Chapter 4*, we confirmed that commercially available mouthrinses contain antimicrobials that follow the relation found before between viscoelasticity and antimicrobial penetration. Importantly, in this chapter we applied oral biofilms grown statically in a well system, but not in a CDFF or PPFC. Incidentally, we noticed that the biofilms grown statically have relatively high fast relaxation components, attesting to a large water content of the biofilms.

In *Chapter 5*, we evaluated the influence of non-contact brushing on antimicrobial penetration in biofilm left-behind after brushing. Dual-species oral biofilms were subjected to 20% mechanical deformation before and after non-contact brushing at 4 mm distance, causing no decrease in biofilm thickness. Stress relaxation was measured and analyzed according to a three element Maxwell model. Also antimicrobial penetration from commercial mouthrinses was evaluated for biofilms before and after brushing. We found that non-contact brushing decreased the prevalence of
the fast and increased the prevalence of the slow relaxation element, which were accompanied by deeper penetration of CHX and cetylpyridinium chloride. A decrease in the prevalence of the fast element indicated less water retained in the biofilms to prevent the dilution of antimicrobials to an ineffective concentration upon deeper penetration. An increased prevalence of the slow element was due to a more open structure, facilitating deeper penetration.

In Chapter 6, we reviewed advances made since the 17th century in our qualitative understanding of the recalcitrance of oral biofilm toward antimicrobial penetration. It is demonstrated that biofilm viscoelasticity may serve as a key parameter describing biofilm structure and composition into a quantifiable parameter. Considering its relation with antimicrobial penetration, viscoelasticity of biofilms is proposed to be a virulence factor.

Summarizing, this thesis not only provides a better, more quantitatively based understanding of the recalcitrance of biofilm towards antimicrobial penetration, but also points to a new pathway for the development of powered toothbrushes that should aim to leave biofilm behind in a viscoelastic state of optimal antimicrobial-penetration to form a reservoir that contributes to the substantivity of antimicrobials.