CHAPTER 3

Viscoelasticity of Oral Biofilms *In Vivo* and the Penetration of Chlorhexidine - a Comparison with *In Vitro* Biofilms

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ABSTRACT
The aim of this chapter is to evaluate up to what extent in vivo oral biofilms are comparable with respect to their viscoelastic properties and chlorhexidine penetration to in vitro oral biofilms formed in a parallel plate flow chamber (PPFC) or constant depth film fermenter (CDFF). To this end, an intra-oral device was developed to grow oral biofilm in vivo, 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, inserted in the device. Every two weeks, the stainless plate with in vivo biofilm was removed for evaluation of the viscoelastic properties of the biofilms and penetration of chlorhexidine. Two weeks undisturbed biofilm formation yielded in vivo biofilm thicknesses that were similar to the ones obtained in vitro after 48 h (PPFC) or 96 h (CDFF) of growth. In vivo formed oral biofilms showed relaxation characteristics upon 10 and 20% induced deformation that were most comparable to the ones observed for in vitro biofilms formed in the PPFC. Upon 50% induced deformation, the base of the biofilms became involved in the relaxation process, no significant differences were observed between stress relaxation of in vivo and in vitro biofilms. Nevertheless, ex situ chlorhexidine penetration into 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 through the relation between biofilm structure and relaxation, quantitative relations can be obtained between biofilm properties and antimicrobial 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. 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.
INTRODUCTION

Viscoelastic properties of oral biofilms are governed by their structure and composition and are determinant for their detachment during brushing (Busscher et al., 2010). Previously, we demonstrated that in vitro biofilms may have different viscoelastic properties, depending on the bacterial strain involved, the presence or absence of fluid shear and compaction during growth. Moreover, it was found that chlorhexidine penetration increased with increasing prevalence of the slow relaxation component and with decreasing prevalence of the fast relaxation component, as obtained from a Maxwell analysis of stress relaxation after an induced deformation (see chapter 2). This suggests a structure-relaxation relation that may help to understand the mechanism of antimicrobial resistance of biofilms on a more quantitative basis than can be obtained from microscopic images. Assuming that the fast relaxation component represents the presence of water, its involvement suggests that increasing amounts of water will dilute the antimicrobial upon penetration to an ineffective concentration in deeper layers of a biofilm. Involvement of the slow relaxation component suggests that biofilm structures, allowing extensive bacterial re-arrangement after deformation, are more open for better antimicrobial penetration.

It is unknown, however, whether the viscoelastic properties of in vitro biofilms relate in any way with those of in vivo biofilms grown in the human oral cavity. Unlike in vitro biofilms, in vivo biofilms encounter fluctuating environmental conditions, like absence or presence of flow and compaction, temperature, redox potential and pH. Small changes in temperature for instance, can significantly alter the gene expression and
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competitiveness of microorganisms (Marsh and Devine, 2011). Moreover, oral biofilms in vivo are comprised of a much larger number of strains and species than can ever be achieved under in vitro conditions.

The aim of this chapter is to evaluate up to what extent in vivo oral biofilms are comparable with respect to their viscoelastic properties and chlorhexidine penetration to in vitro oral biofilms formed in a parallel plate flow chamber (PPFC) or constant depth film fermenter (CDFF). To this end, an intra-oral device was developed to grow oral biofilms in vivo, in absence of mechanical perturbation.

MATERIALS & METHODS

Oral biofilm collection in vivo

The intra-oral biofilm collecting device (Fig. 1) was made of medical grade stainless steel 316, and is composed of two parts: a base (5×3×2 mm) that is fixed to the middle of the buccal surface of the upper first molar and a replaceable cover plate (4 × 3 × 0.2 mm). Biofilms formed on the inner side of the replaceable cover plate in the absence of mechanical perturbations, were collected for this study.

Five volunteers (aged 26 to 29 years) were included in this study. Volunteers all had a complete and healthy dentition, no bleeding upon probing and were not using any medication. Each volunteer was assigned a random number between 1 and 5 as registration for later data processing. The study was performed according to the guidelines of the Medical Ethics Committee of the University Medical Center Groningen, Groningen, The
Netherlands (letter 28-9-2011), including the signed informed consent by the volunteers and the tenets of the Declaration of Helsinki.

An autoclaved base of the device was fixed to the buccal surface of the upper first molars of the volunteers (see also Fig. 1) after mild etching of the tooth surface using light cure adhesive paste (Transbond™ XT, 3M Unitek, USA), a procedure similar to the one used for the bonding of orthodontic brackets. The base of the device was brushed using rubber cup and cleaner paste (Zircate® Prophy Paste, Densply, Caulk, USA) at low speed, (less than 2,500 rpm/min). Subsequently, the base surface was coated with a thin layer of primer and bond (CLEARFIL SE BOND, Kurary Medical Inc., Japan) and the autoclaved stainless steel cover plate was put in place using a pair of tweezers and bonded onto the base device using light cure adhesive paste. Volunteers were asked to wear the device for a total of eight weeks during which they were requested to perform manual brushing with a standard fluoridated toothpaste (Prodent Softmint, Sara Lee Household & Bodycare, Exton, USA) according to their habitual oral hygiene but to refrain from the use of an additional mouthrinse.

The cover plate could be removed with a dental explorer, after which the cover plate with biofilm was placed in a moisturized petri dish for transport from the dental clinic to the laboratory. In a separate pilot study, it was established that two weeks of intra-oral biofilm formation in the device yielded biofilm thicknesses that were similar to the ones obtained in vitro. Therewith, in vivo biofilms could be collected four times from each volunteer. After each experiment, cover plates were sanded to remove biofilm and other residuals, prior to autoclaving.
After the experiments, the base of the device was removed from the tooth surface with a debracketing plier and residual adhesive was grinded off the tooth surface with a low speed hand piece. The tooth surface was polished and cleaned with rubber cup and cleaner paste. No signs of gingival inflammation were observed in any volunteer after removal of the base device. A base device was only used once in each volunteer.

Figure 1 Intra-oral pictures of the biofilm collecting device. (A) The base and cover plate of the stainless steel, intra-oral biofilm collection device. (B) The base of the intra-oral biofilm collection device fixed to the center of buccal surface of a maxillary first molar. (C) Side view of the intra-oral biofilm collection device, showing the open spacing in which undisturbed biofilm growth on the cover plate occurred. (D) Top view of the closed intra-oral biofilm collection device in situ, showing the hole in the cover plate used for removal with a dental explorer.
Viscoelastic properties of oral biofilm

Low load compression testing and analysis of stress relaxation was carried out as described in chapter 2.

Penetration of chlorhexidine into in vivo oral biofilm

Penetration of chlorhexidine and analysis of confocal laser scanning microscopic images were done as described in chapter 2. Note that chlorhexidine treatment was done ex vivo with the same method explained in chapter 2, immediately after removing the biofilm collection device from the oral cavity.

Statistical analysis

Statistical analysis was performed with SigmaPlot software (version 11.0, systat software, Inc., California, USA), comparing in vivo data from this study and in vitro data taken from chapter 2. Prior to statistical comparison, sequential tests of normal distribution and equal variance of the data were carried out. If the data failed one of these tests, a Mann-Whitney Rank Sum test was used to determine statistical significance of possible differences; otherwise a Student t-test would be applied. Secondly, the parameters mentioned above were grouped according to volunteers. A Pearson Product Moment Correlation test was used to disclose the relation between the penetration of chlorhexidine and the relaxation of in vivo biofilms.

RESULTS

The average thickness of the oral biofilms formed in vivo was 121 ± 86 μm, ranging from 13 μm to 274 μm, which is comparable to the thickness of in vitro biofilms (p > 0.05, Mann-Whitney Rank Sum test), as can be seen in Table 1.
Table 1 The thickness, penetration ratio, total stress relaxation and the prevalence of the three Maxwell elements of *in vivo* oral biofilms of all volunteers, as determined in duplicate in each volunteer, compared with the properties of *in vitro* single species oral biofilms taken from chapter 2, as averaged over all data obtained in a parallel plate flow chamber (PPFC) and constant depth film fermenter (CDFF). Additionally, *in vitro* data obtained in the PPFC and CDFF, as averaged over single-species biofilms of *Streptococcus orinis* J22 and *Actinomyces naeslundii* T14V-J1 are presented.

<table>
<thead>
<tr>
<th>Thickness (µm)</th>
<th>Penetration ratio</th>
<th>10% deformation (%)</th>
<th>20% deformation (%)</th>
<th>50% deformation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relaxation</td>
<td>E₁</td>
<td>E₂</td>
</tr>
<tr>
<td><em>In vivo</em></td>
<td>121±86†</td>
<td>0.20±0.1*</td>
<td>60±14*</td>
<td>21±16*</td>
</tr>
<tr>
<td><em>In vitro</em></td>
<td>120±52</td>
<td>0.46±0.1</td>
<td>82±14</td>
<td>44±20</td>
</tr>
<tr>
<td>PPFC</td>
<td>109-131†</td>
<td>0.33³-0.56</td>
<td>64-97³</td>
<td>17-60³</td>
</tr>
<tr>
<td>CDFF</td>
<td>11⁴-125</td>
<td>0.39-0.48³</td>
<td>83³-83</td>
<td>47³-49</td>
</tr>
</tbody>
</table>

* indicates *p* < 0.05,
† indicates the comparison was carried out by Mann-Whitney Rank Sum test,
§ indicates data for *S. oralis* J22.
The total stress relaxation of \textit{in vivo} biofilms upon 10 and 20\% deformation were more comparable to the stress relaxation observed for \textit{in vitro} biofilms grown in the PPFC than in the CDFF. No significant differences were observed between the total stress relaxation of \textit{in vivo} and \textit{in vitro} biofilms upon inducing a deformation of 50\% (see Table 1). On average, \textit{in vitro} biofilms showed higher total stress relaxation than \textit{in vivo} formed biofilms, although this difference was only significant ($p < 0.05$, Student $t$-test) for 10 and 20\% induced deformations (see Fig. 2).

\textit{In vivo} formed biofilms furthermore distinguished themselves significantly from \textit{in vitro} ones by lower prevalence of the fast component ($E_1$) and higher prevalence of the slow component ($E_3$) ($p < 0.05$, Student $t$-test; see Table 1) for induced deformations of 10 and 20\%. At 50\% induced deformation, however, differences in the prevalence of the different relaxation parameters had disappeared (see also Fig. 2). The prevalence of the intermediate component ($E_2$) was relatively similar across the different biofilms (see Table 1).

The chlorhexidine penetration ratio for \textit{in vivo} formed biofilms (Fig. 3) was smaller than the average penetration for the \textit{in vitro} biofilms ($p < 0.05$, Student $t$-test; see Table 1). Similar however, as observed for \textit{in vitro} biofilms, penetration ratio decreased with increasing prevalence of the fast ($E_1$) component and with decreasing prevalence of the slow component ($E_3$) (see Fig. 4). No relation was observed with the intermediate component ($E_2$), as was also lacking for \textit{in vitro} biofilms.
Figure 2 Relaxation properties of *in vivo* oral biofilms, obtained in different volunteers as indicated by different colors in comparison with the average relaxation properties of different single species biofilms formed in a PPFC and CDFF, falling with the black rectangles.

Figure 3 Cross sectional view of a two weeks old, *in vivo* formed oral biofilm after *ex vivo* exposure to a 0.2 %wt chlorhexidine-containing mouthrinse for 30 s, showing dead (red fluorescent) and live bacteria (green fluorescent). The bar represents 75 µm.
DISCUSSION

In this study we used an intra-oral biofilm collection device to grow oral biofilms in vivo in absence of mechanical perturbations. The duration of in vivo biofilm growth (two weeks) was adjusted to yield a biofilm thickness that corresponded with the ones observed under flow for 48 h in vitro in a PPFC or imposed by compaction for biofilms grown for 96 h in the CDFF (chapter 2). From the differences between growth times in the different systems, it can be concluded that the environmental conditions have a major influence on biofilm formation.

Figure 4 Penetration ratio of chlorhexidine as a function of the prevalence of the fast, intermediate and slow Maxwell elements $E_1$, $E_2$ and $E_3$ for in vivo biofilms formed in different volunteers after 10, 20 and 50% induced deformation. All data points refer to single experiment in one volunteer. Different volunteers are indicated by the same color codes as used in Fig. 2. The dotted lines represent the 95% confidence intervals.
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10 and 20% induced deformations yield information on the relaxation-structure of the outermost surface of the biofilms, opposite to data derived upon inducing 50% deformation that invokes the deeper layers of the biofilms. Relaxation characteristic after 10 and 20% deformation of biofilms formed in the PPFC more closely resemble those of in vivo formed biofilms than biofilms formed in the CDFF, although cross section of biofilms formed in vivo appear slightly more “fluffy” when examined with CLSM (compare Fig. 3 with Fig. 2 in chapter 2). This being true for the images selected, it must be realized that it is difficult if not impossible by human nature to obtain CLSM images of biofilms in an unbiased, observer-independent way. This is why conclusions on biofilm structure from quantitative, observer-independent stress relaxation analysis of larger sections of a biofilm than can ever be obtained microscopically, are to be preferred. Interestingly, upon increasing the induced deformation to 50%, a better resemblance between in vivo and in vitro grown biofilms appears. This is probably because biofilms formed in vivo (even in the absence of external compaction or mechanical perturbations) are compacted more than when formed in a PPFC through the presence of multiple strains and species that can arrange themselves through their differences in size and shape in a highly compact manner. In a CDFF, this compaction is achieved intentionally by continuously scraping off the biofilm by a rotating blade. Therefore it can be expected that oral biofilm in fissures and interproximal spaces, left behind multiple times after brushing, will be compacted and better resemble biofilm formed in the CDFF than oral biofilms freshly formed, for which the PPFC may be the preferred model system.
Comparison of Fig. 4 from chapter 2 with the current in vivo results as presented in Fig. 4 confirm the relation between chlorhexidine penetration and the prevalence of the fast and slow relaxation components, each representing structural features of the biofilm, i.e. presence of water (channels) and bacterial compaction reducing open space, respectively. The in vivo relations between relaxation characteristics and chlorhexidine penetration have larger 95% confidence intervals than the in vitro ones, partly due to the limited power of the study that was confined to five volunteers. More importantly however, it can be argued that it is intrinsically impossible to obtain the same narrow confidence intervals for in vivo biofilms as found for in vitro biofilms, which were all single species. In our analyses, we employed chlorhexidine killing as an indicator of its penetration. In vivo formed biofilms contain a large number of different strains and species that all have their own susceptibility to chlorhexidine not only within one volunteer, but also among volunteers. This inevitably affects the penetration as indicated by bacterial killing of chlorhexidine, making the in vivo relation less significant than the one obtained for in vitro biofilms.

This study demonstrates that through the relation between biofilm structure and relaxation, quantitative relations can be obtained between biofilm properties and antimicrobial penetration, which are not only valid for in vitro formed biofilms but also for in vivo formed ones.

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REFERENCES

