CHAPTER 2

Viscoelasticity of Oral Biofilms and the Penetration of Chlorhexidine
-an In Vitro Study-

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Y. He, B.W. Peterson, M.A. Jongsma, Y. Ren, P.K. Sharma, H.J. Busscher and H.C. van der Mei PLOS ONE 2013, 8:e63750.
ABSTRACT
Viscoelastic properties of oral biofilms are determinant for their detachment during brushing. Here, we aim to gain evidence to support our hypothesis that viscoelastic properties of oral biofilms also relate with the penetration of an antimicrobial into the biofilm through a relaxation-structure relationship. *In vitro* biofilms with different viscoelastic properties of a coccal and rod-shaped microorganism were grown to the same thickness in a parallel plate flow chamber and constant depth film fermenter 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 and extracellular polymeric substances and re-arrangement of the bacteria. The penetration of chlorhexidine 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 antimicrobial upon penetration to an ineffective concentration in deeper layers of a biofilm. Clinically, our results suggest that penetration of antimicrobials in biofilm-left-behind may be optimized by fine-tuning its viscoelastic deformation after tooth brushing.
INTRODUCTION
Caries and periodontitis are both diseases known to result from the presence of oral biofilm (Löe et al., 1965; Anerud et al., 1979) and despite advances in toothbrush design and formulation of oral care products, the maintenance of oral health is difficult for many people. Over the years, mechanical removal of oral biofilm has remained the preferred method to prevent biofilm-related oral diseases, but its efficacy is limited particularly in fissures, interproximal spaces, pockets and around orthodontic appliances (Parini and Pitt, 2006; Jackson et al., 2006; Garcez et al., 2011). This has led to the development of antimicrobial toothpaste and mouthrinse formulations. Although oral biofilms remaining after brushing have the ability to absorb small antimicrobials (Otten et al., 2012) and release them in bio-active amounts, the ability of most antimicrobials to penetrate into a biofilm is limited (Zaura-Arite et al., 2001). The biofilm mode of growth of bacteria on surfaces in general, including oral hard and soft surfaces, hampers antimicrobial penetration as microorganisms embed themselves in a matrix of extracellular polymeric substances (EPS) for self-protection (Flemming and Wingender, 2010). The biofilm mode of growth not only protects the microorganisms against antimicrobials, but also yields protection against mechanical disruption by contributing to the viscoelasticity of the biofilms (Rupp et al., 2005). Detachment of oral biofilm by powered brushing for instance, proceeds according to a viscoelastic failure model. In other words, biofilm detachment only occurs, if the brushing energy absorbed in a biofilm is sufficient and deformation is beyond the yield point; while if deformation remains low in the plastic range but below the yield point, biofilm is expanded after brushing but not removed (Busscher et al., 2010).
Viscoelastic properties of oral biofilms depend on the degree of compaction during formation (Paramonova et al., 2009), the absence or presence of flow during growth (Stoodley et al., 2002; Paramonova et al., 2009), its architecture and microbial composition (Purevdorj et al., 2002; Paramonova et al., 2009). The viscoelastic properties of oral biofilms can be determined by evaluating their relaxation after deformation during external loading. Stress relaxation during deformation is a time-dependent process and can be separated into a number of responses, each with a characteristic time-constant (Lau et al., 2009a). Although Maxwell analysis of stress-relaxation to derive the characteristic time-constants of the various relaxation processes that occur in a biofilm under external loading has been done before (Guelon et al., 2011), the details of the relaxation-structure relationship and physical processes associated with the different time-constants are unknown. Stress relaxation may involve a number of processes, like the outflow of water and EPS from the biofilm and rearrangement of the bacteria in the biofilm. Since penetration of an antimicrobial into an oral biofilm depends on diffusion (Stewart, 2003) and therewith with structural features, like the presence of water filled channels in the biofilm or EPS containing spaces, we here hypothesize that the penetration of an antimicrobial into a biofilm may relate with stress relaxation and its underlying processes.

The aim of this study is to gain evidence in support of this hypothesis. To this end, single-species biofilms of *Streptococcus oralis* J22 and *Actinomyces naeslundii* T14V-J1 were grown in a parallel plate flow chamber (Busscher and Van der Mei, 2006) and in a constant depth film fermenter (Hope and Wilson, 2006) and their viscoelastic properties as determined using a low
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load compression tester, will be related with the penetration of chlorhexidine into the biofilms.

MATERIALS & METHODS

Bacterial strains and growth conditions

*S. oralis* J22 and *A. naeslundii* T14V-J1 grown on blood agar plates, were used to inoculate 10 mL modified Brain Heart Infusion broth (BHI, Oxoid Ltd., Basingstoke, Hampshire, UK) (37.0 g/L BHI, 5.0 g/L yeast extract, 0.4 g/L NaOH, 1.0 g/L hemin, 0.04 g/L vitamin K1, 0.5 g/L L-cysteine, pH 7.3) and were cultured for 24 h at 37°C in ambient air for *S. oralis* J22 and anaerobically for *A. naeslundii* T14V-J1. These cultures were used to inoculate 200 mL modified BHI and grown for 16 h. Bacteria were harvested by centrifugation at 870 g, 10°C for 5 min and washed twice in sterile adhesion buffer (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride, pH 6.8). The bacterial pellet was suspended in 10 mL adhesion buffer and sonicated intermittently in an ice-water bath for 3 × 10 s at 30 W (Vibra cell model 375, Sonics and Materials Inc., Newtown, CT, USA) to break bacterial chains and clusters, after which bacteria were resuspended in adhesion buffer. A concentration of $3 \times 10^8$ bacteria/mL was used for parallel plate flow chamber (PPFC) experiments, while a concentration of $9 \times 10^8$ bacteria/mL was used in constant depth film fermenter (CDFF) experiments.

Biofilm formation in a PPFC and CDFF

Single strain biofilms of *S. oralis* J22 and *A. naeslundii* T14V-J1 were grown on glass slides (water contact angle 7 ± 3 degrees) and hydroxyapatite discs (water contact angle 34 ± 8 degrees) in a PPFC and a CDFF, respectively.
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after adsorption of a salivary conditioning film from reconstituted human whole saliva for 14 h at 4°C under static conditions. Reconstituted human whole saliva was obtained from a stock of human whole saliva from at least 20 healthy volunteers of both genders, collected into ice-cooled beakers after stimulation by chewing Parafilm®, pooled, centrifuged, dialyzed, and lyophilized for storage. Prior to lyophilization, phenylmethylsulfonylfluoride was added to a final concentration of 1 mM as a protease inhibitor in order to reduce protein breakdown. Freeze-dried saliva was dissolved in adhesion buffer (1.5 g/L). All volunteers gave their informed consent to saliva donation, in agreement with the guidelines set out by the Medical Ethical Committee at University Medical Center Groningen, Groningen, The Netherlands (letter 06-02-2009).

In a sterilized PPFC system (Paramonova et al., 2009), 200 mL bacterial suspension was circulated till a bacterial surface coverage of $2 \times 10^6 / \text{cm}^2$ on a salivary coated glass bottom plate and adhesion buffer was flushed for 30 min afterwards in order to rinse off non-adhering bacteria. Subsequently, growth medium (20% modified BHI and 80% adhesion buffer) was perfused through the system at 37°C for 48 h. All shear rates used in the flow chamber were $15 \text{ s}^{-1}$.

In the CDFF (Hope and Wilson, 2006; Pratten, 2007), 200 mL bacterial suspension was introduced droplet by droplet within 1 h, while the plate with the sample holders was turning with a speed of 1 rpm. Then the system was stopped for 30 min to allow bacteria to adhere before growth medium was introduced. The biofilm was grown for 96 h at 37°C under continuous supply of a mixture of adhesion buffer and modified BHI with a rate of 80 mL/h. The system was equipped with 15 sample holders and
each sample holder contained 5 saliva coated hydroxyapatite (HA) discs, recessed to a depth of 100 µm.

**Low load compression testing**

The thickness of the biofilms from the flow chamber on the salivary coated glass slides and biofilms from the CDFF on the salivary coated HA discs was measured with a low load compression tester (LLCT) as described before (Paramonova et al., 2009). Also the viscoelastic properties of the biofilms were measured by the LLCT. The biofilms were compressed to a deformation of 10, 20, and 50% in 1 s and the deformation was held constant for 100 s, while the deformation induced load in the biofilm, its relaxation was monitored over time (see Fig. 1A) and normalized over the cross-sectional area of the plunger to calculate the induced stress. The percentage change in induced stress occurring within 100 s from its initial value was termed the percentage stress relaxation. At each induced deformation, 3 measurements were performed at different locations within the same biofilm.

Measured relaxation curves for each biofilm were modeled using a generalized Maxwell model containing at most 3 elements (see Fig. 1B) according to

\[
E(t) = E_1 e^{-t/\tau_1} + E_2 e^{-t/\tau_2} + E_3 e^{-t/\tau_3}
\]

(1)

in which \(E(t)\) is the total stress of the biofilm expressed as the sum of three Maxwell elements with a spring constant \(E_i\), and characteristic decay time,
\( \tau_i \) (see also Fig. 1B). For calculating \( E(t) \), deformation was expressed in terms of strain, \( \varepsilon \), according to the large strain model using

\[
\varepsilon = \ln \left(1 + \frac{\Delta h}{h} \right)
\]  

(2)

where \( \Delta h \) is the decrease in height and \( h \) is the un-deformed height of the biofilm. The model fitting for \( E_i \) and \( \tau_i \) values of the three elements was done by minimizing the chi-squared value using the Solver tool in Microsoft Excel 2010. Fitting to three Maxwell elements yielded the lowest chi-squared values and increasing the number of Maxwell elements only yielded minor decreases in chi-squared values of less than 3%. The elements derived were rather arbitrarily named fast, intermediate or slow based on their \( \tau \) values i.e. \( \tau_1 < 5 \text{ s}, 5 \text{ s} < \tau_2 < 100 \text{ s}, \) and \( \tau_3 > 100 \text{ s} \) respectively (see also Fig. 1B). Relative importance of each element, based on the value of its spring constant \( E_i \), was expressed as the percentage of its spring constant to the sum of all elements’ spring constants at \( t = 0 \).

Each measurement was carried out on an uncompressed area of the biofilm. Three measurements were taken for each independently grown biofilm.

**Penetration of chlorhexidine into in vitro biofilms**

Biofilms grown in the PPFC or in the CDFF were exposed to a 0.2 wt% chlorhexidine-containing mouthrinse (Corsodyl®, SmithKline Beecham Consumer Brands B.V., Rijswick, The Netherlands) for 30 s and subsequently immersed in adhesion buffer for 5 min. After exposure to chlorhexidine and rinsing, biofilms were stained for 30 min with live/dead
stain (BacLight™, Invitrogen, Breda, The Netherlands) and confocal laser scanning microscopy (CLSM; Leica TCS-SP2, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) was used to record a stack of

**Figure 1** (A) Stress *versus* time diagram for relaxation of a compressed biofilm; (B) Schematics of a three element Maxwell model: $E_i$ represent the spring constants and $\eta_i$ the relaxation time constants, which are equal to $\eta_i/E_i$. 

![Stress relaxation diagram](image-url)
images of the biofilms with a 40× water objective lens. Images were analyzed with Leica confocal software to visualize live and dead bacteria in the biofilms (Fig. 2I).

The ratio of the intensity of red (dead bacteria) to green (live bacteria), R/G, was plotted versus the biofilm thickness (see Fig. 2II). The biofilm thickness where the ratio R/G became more than 1.5 was taken as the thickness of the dead band. Next, a penetration ratio was calculated according to

\[
\text{Penetration ratio} = \frac{\text{dead band thickness}}{\text{total biofilm thickness}}
\]  

Penetration ratios were calculated for three different, randomly chosen locations on each biofilm and the experiment was repeated three times.

Statistical analysis
Statistical analysis was performed with SigmaPlot software (version 11.0, systat software, Inc., California, USA). Differences in biofilm thickness and viscoelasticity were evaluated after testing for normal distribution and equal variance of the data. If the data failed one of those tests, a Mann-Whitney Rank Sum test was used to determine statistical significance; otherwise a Student t-test was applied. Pearson Product Moment Correlation test was used to disclose relations between the penetration of chlorhexidine and the relaxation of biofilms.
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Figure 2 I. Representative CLSM images (cross sectional view) of the penetration of chlorhexidine (0.2 wt%) during 30 s into biofilms grown in a PPFC or CDFF. (A) S. oralis J22 biofilm grown under flow in a PPFC; (B) S. oralis J22 biofilm grown under compaction in a CDFF; (C) A. naeslundii T14V-J1 biofilm grown under flow in a PPFC; (D) A. naeslundii T14V-J1 biofilm grown under compaction in a CDFF. Scale bar represents 75 µm. II. Ratio of the intensity of dead (red) to live (green) bacteria, R/G. R/G = 1.5 was taken as the cut-off for the thickness of the dead band.
RESULTS

Biofilm thickness and penetration ratio of chlorhexidine

The biofilm of the coccal-shaped *S. oralis* J22 in the PPFC reached a thickness of 131 ± 15 μm and the biofilm of the rod-shaped *A. naeslundii* T14V-J1 reached 109 ± 26 μm. The biofilm thickness in the CDFF was for *S. oralis* J22 119 ± 6 μm and for *A. naeslundii* T14V-J1 125 ± 9 μm. There were no significant differences (p > 0.05, Student t-test) in thickness between biofilms grown under flow and in the CDFF. Also differences in biofilms thickness across strains were not statistically significant (p > 0.05, Student t-test).

The penetration of chlorhexidine into biofilms grown in the PPFC was significantly different (p < 0.05, Mann-Whitney Rank Sum test) for *S. oralis* J22 and *A. naeslundii* T14V-J1, and the penetration ratio amounted to 0.33 ± 0.09 and 0.56 ± 0.08, respectively. On the other hand, there were no significant strain-dependent differences in penetration of chlorhexidine into biofilms grown in the CDFF, penetration ratios of 0.48 ± 0.04 and 0.39 ± 0.06 in biofilms of *S. oralis* J22 and *A. naeslundii* T14V-J1, respectively (p > 0.05, Mann-Whitney Rank Sum test) (Fig. 2). Interestingly, whereas biofilms offered a clear physical protection against chlorhexidine, bacteria dispersed from biofilms grown either in the PPFC or in the CDFF were highly susceptible to chlorhexidine (Fig. 3), confirming that the absence of bacterial killing in the deeper layers of the biofilms are not due to changes in inherent properties of the bacteria in their biofilm mode of growth, but solely to difficulties encountered by the antimicrobial in penetrating to the deeper layers. Note that a similar conclusion has been drawn for three days
old in vivo grown oral biofilms, after dispersal and exposure to chlorhexidine (Van der Mei et al., 2006).

**Figure 3** Fluorescence images of dispersed *S. oralis* J22 and *A. naeslundii* T14V-J1, treated with chlorhexidine for 30 s in their biofilm mode of growth prior to dispersal and treated immediately after dispersal. Live (green)–dead (red) staining was used to show the viability of bacteria. (A) *S. oralis* J22 grown in the PPFC and treated in its biofilm mode of growth. (B) *S. oralis* J22 grown in the PPFC and treated in its dispersed state. (C) *A. naeslundii* T14V-J1 grown in the CDFF and treated in its biofilm mode of growth. (D) *A. naeslundii* T14V-J1 grown in the CDFF and treated in its dispersed state. Scale bar represents 10 μm.
Viscoelastic properties

The stress relaxation of biofilms grown in the PPFC was different for both strains and S. oralis J22 biofilms showed significantly ($p < 0.05$, Mann-Whitney Rank Sum test) more stress relaxation than biofilms of A. naeslundii T14V-J1, especially after 10 and 20% induced deformation (Fig. 4). There were no significant differences ($p > 0.05$, Mann-Whitney Rank Sum test) in stress relaxation between biofilms of the cocal and rod-shaped microorganisms when grown in the CDFF. Interestingly, the penetration ratio of chlorhexidine decreased with increasing stress relaxation of the biofilms, regardless of the induced deformation (Fig. 4B).

Total stress relaxation was subsequently resolved in a fast, intermediate and slow component (Fig. 1B). The biofilms grown in the flow chamber showed significant difference in the Maxwell elements of all compression groups, e.g. $E_1$ of S. oralis J22 was $60 \pm 6\%$ and $E_1$ of A. naeslundii T14V-J1 was $17 \pm 2\%$ in 10% compression group (Fig. 5). In contrast, the biofilms grown in the CDFF showed no difference for the Maxwell elements of the S. oralis J22 and A. naeslundii T14V-J1 biofilms regardless of the compression groups (Fig. 5). Analysis of the stress relaxation according to a three element Maxwell model revealed that penetration increased with increasing relative importance of the slow relaxation component ($E_3$) and decreasing importance of the fast component ($E_1$) (Fig. 5). This confirms the existence of a relaxation-structure-composition relation that may facilitate a quantitative approach towards antimicrobial penetration in biofilms.
DISCUSSION

The recalcitrance of oral biofilm toward penetration of antimicrobials is known ever since Van Leeuwenhoek wrote in the 17th century that “the vinegar with which I washed my teeth killed only those animals which were on the outside of the scurf, but did not pass through the whole substance of it”. Over
in recent years, the limited penetration of antimicrobials into a biofilm has been attributed to reduced solute diffusion in water due to the presence of microbial cells, EPS, abiotic particles or gas bubbles trapped in a biofilm (Stewart, 2003). Interestingly, whereas the influence of the physics of diffusion on the chemistry and biology of biofilms has been amply described and reviewed (Stewart, 2003; Takenaka et al., 2008; Lau et al., 2009b), antimicrobial penetration has never been related with quantifiable, physical properties of a biofilm, like its viscoelasticity. This study demonstrates for the first time since Van Leeuwenhoek his observation of

Figure 5 Penetration ratio as a function of the prevalence of the three Maxwell elements $E_1$, $E_2$ and $E_3$, denoting the fast, intermediate and slow relaxation components, respectively for different biofilms after 10, 20 and 50% induced deformation. All data points refer to single experiments, while symbols are explained in Fig. 4. Dashed lines represent 95% confidence intervals.
the poor penetration of vinegar into an oral biofilm, through a relaxation-
structure-composition relation, that biofilm properties can be derived that
facilitate explanation of antimicrobial penetration into a biofilm on basis of
quantitative biofilm properties. Incidentally, not only antimicrobials have
difficulty penetrating a biofilm, but also nutrients may have difficulty
penetrating a biofilm, causing reduced viability of microorganisms residing
in deeper layers of biofilms (Sjollema et al., 2011).

It has been argued that the resistance of the biofilm mode of growth can
either be due to (I) limited antimicrobial penetration, (II) starvation of
microorganisms in deeper layers of a biofilm due to nutrient deprivation
bringing them in a less susceptible state or (III) accumulation of quorum-
sensing molecules in deeper layers triggering expression of protective
genes (Stewart, 2003). All of the above potential mechanisms rely on
diffusion. Diffusion into a biofilm thus critically requires water channels in
between the structure formed by the microbial cells themselves.

The bacteria in a biofilm constitute the heaviest masses, and their re-
arrangement responsible for stress relaxation upon an induced deformation
will thus be slow, which associates the prevalence of the slow Maxwell
element with the bacterial re-arrangement. Furthermore, the positive
correlation between penetration and the prevalence of the slow Maxwell
element confirms that microorganisms arranged in a more open structure
allow easier penetration of antimicrobials and are more readily amenable to
re-arrangement after deformation. Different from the role of water channels
in diffusion (Stewart, 2003), we found that water had a negative influence
on the efficacy of antimicrobials during penetration, as larger volumes of
water can dilute the antimicrobial below effective concentrations. Since water likely has the smallest viscosity in a biofilm, the fast Maxwell element may be associated with the outflow of water through biofilms. Consequently, dilution of antimicrobials after penetration into a biofilm to an ineffective concentration in deeper layers is evidenced by the negative correlation between the prevalence of the fastest Maxwell element and the penetration ratio. Accordingly, it must be emphasized at this point that in our study chlorhexidine might have penetrated beyond the dead bands visible in Fig. 2, but clearly to a concentration insufficient to yield bacterial killing.

In this study, we used two distinctly different systems to grow biofilms. In the CDFF, there was a constant turn-over of bacterial growth, death and biofilm removal by the scraper blades (Hope and Wilson, 2006) in addition to compaction by the scraper blades. Whereas similar turn-over, death and removal by fluid flow could be expected in a PPFC, compaction was definitely absent in a PPFC. In this respect, it is interesting that there was no difference in stress relaxation of biofilms formed by coccal or rod-shaped microorganisms in the CDFF, presumably because biofilms in the CDFF are mechanically compacted during formation (Fig. 4). In the absence of mechanical compaction like in the PPFC, rod-shaped microorganisms have more difficulties in forming a dense structure, as this requires them to take a favorable orientation with respect to one another. This becomes especially evident at the larger deformation induced of 50% and explains why biofilms formed by rod-shaped microorganisms in the PPFC had a different stress relaxation than coccal microorganisms, but not in the CDFF.
The two systems to grow biofilms used in this study represent two extreme situations that may occur in the oral cavity. Highly compacted biofilms may be expected in fissures due to mastication, while compaction occurs less on interproximal biofilms. In addition, biofilm left-behind in interproximal spaces inaccessible to contact brushing will be in a fluffed-up state, resembling biofilms grown in a PPFC. Since \textit{in vivo} the number of different strains and species is extremely large, it is hard to estimate up to what extent biofilms containing microorganisms with a large variability in shape, will affect the viscoelastic properties of biofilms on sites where compaction is absent or low.

In summary, this study is the first to demonstrate a role of viscoelastic properties of oral biofilm on antimicrobial penetration through a relaxation-structure relationship, next to the established role of biofilm viscoelasticity in non-contact removal by powered brushing. Herewith, biofilm viscoelasticity might become the most important quantifiable physical property of oral biofilm next to qualitative, observer dependent CLSM imaging of structure, with respect to the fine-tuning of brushing and antimicrobial strategies.

\textbf{ACKNOWLEDGMENT}

The China Scholarship Council and W.J. Kolff Institute, University Medical Center Groningen are gratefully acknowledged for scholarships, enabling this study. We thank Mr. Yun Chen for his help in data processing and Mrs. Jelly Atema-Smit for the help with CLSM.
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