Biofilm and dental caries: application of the constant depth film fermentor
Deng, D.M.

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Chapter 2

Demineralization of Dentin by *Streptococcus mutans* Biofilms Grown in the Constant Depth Film Fermentor

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Abstract

To develop a bacterial demineralization model, we studied the growth of *Streptococcus mutans* biofilms in a Constant Depth Film Fermentor (CDFF) and studied the effects of sucrose pulsing frequency (SPF) in time on dentin demineralization. *S. mutans* biofilms were grown in dentin specimens with grooves and on dentin surface specimens for 20 days. During the experiments, 2% sucrose was pulsed either 4 or 8 times per day for periods of 30 min. Diluted brain-heart infusion medium containing 25 mM PIPES buffer and 1.5 mM CaCl$_2$ was pulsed as the alternative growth medium. Specimens with intact biofilms were taken out on days 5, 12 and 20. The model was assessed by viable counts of the biofilm, mineral loss and lesion depth in the dentin specimens (by transversal microradiography) and pH measurements in the groove (by pH microelectrode). The results showed that biofilms formed on the dentin surface specimens were constant in viable counts for the low SPF, while this parameter tended to increase with time under the high SPF. Lesions with intact surfaces were formed and the lesion size increased significantly over time and increased significantly with increasing SPF. Typical Stephan curves were found after sucrose pulsing. The pH inside the groove returned to neutral under low SPF, but remained below 6.5 under high SPF. With the CDFF *S. mutans* biofilm model, lesions can be created in dentin within reasonable experimental time periods, as a result of the presence of a biofilm and in response to carbohydrate challenges.

Introduction

Dental plaque contains numerous bacteria that ferment carbohydrates to various organic acids, which dissolve the dental hard tissues. In alternate time periods this loss of tissue may be counteracted by a deposition of minerals from saliva (‘remineralization’). The relative magnitude of these two processes determines whether destruction (caries) or repair occurs. To interfere with the onset or progression of dental caries, various agents have been developed. These either shift the calcium phosphate balance (the de- and remineralization), or interfere with microbial growth or metabolism in the dental plaque. Some (*e.g.* fluorides) have a dual mode of action.
Chapter 2

In the past caries-preventive agents have been tested for their effects on the de-and re-mineralization balance in bacteria-free models [ten Cate, 1990]. Alternatively, antimicrobials have been screened for their effects on viability and metabolism of bacteria being present either in a planktonic metabolic state [Järvinen et al., 1993] or recently in biofilm models [Shapiro et al., 2002]. In addition to the aforementioned tests, there is a need for a bacterial-hard tissue model that includes all steps of the caries process (bacterial acid formation, demineralization and remineralization). Such a model should also take into account diffusion processes, retention by the bacterial biomass and the biofilm nature of dental plaque. It should be realistic in terms of de- and re-mineralization cycles per day and allow an analysis of various output parameters, which reflect aspects of the caries process.

For this purpose we chose the Constant Depth Film Fermentor (CDFF) for growing biofilms. The CDFF was developed by Coombe et al. [1981; 1984] and improved by Peters and Wimpenny [1988]. In the CDFF, the thickness of biofilms can be controlled and after the initial period of its formation the properties of the biofilm are relatively constant over time [Peters and Wimpenny, 1988]. The removal of superficial layers of the biofilm by a scraper blade simulates conditions in the mouth where chewing and tongue movement continuously remove the outermost layers of supragingival plaque. Also, the growth conditions in the CDFF can be chosen to match those of the oral cavity, making the CDFF suitable for oral biofilm studies. Unlike in other systems, in the CDFF, large numbers of similar biofilms can be grown simultaneously and then subjected to various types of assessment or to subsequent experiments.

So far, CDFF biofilm studies on oral topics have been included: (1) the properties of single or multiple species biofilms grown in the CDFF, in particular the effect of different growth conditions on the structure of biofilms [Pratten et al., 2000a]; (2) the properties of biofilms grown on different substrata and the effect of the surface roughness on the biofilms [Morgan and Wilson, 2001]; (3) the effects of different antimicrobial and anticaries agents on biofilms [Wilson et al., 1996]; and (4) microbial microleakage around dental restorations [Matharu et al., 2001]. These studies demonstrated that CDFF biofilms reflect properties that have been published for dental plaque [Bowden and Li, 1997; Wood et al., 2000] and that the CDFF can be used for the initial screening of new antimicrobials under controlled conditions [Kinniment et al., 1996b; Pratten et al., 2000b].
To date no study has shown the potential of CDFF as a biofilm caries model. To this end, *Streptococcus mutans* biofilms were grown on dentin in CDFF and were studied the effects of sucrose pulsing frequency (SPF) in time on the demineralization process. We used dentin smooth surface specimens and dentin groove specimens as substrata. The dentin smooth surface specimen represents sites in the mouth with unhindered access by food and saliva, for example buccal surfaces. The dentin groove specimens represent plaque retention sites, for example marginal gaps, pits and fissures. Dentin rather than enamel was used as substratum because of its higher solubility, which leads to reduced experimentation times.

**Materials and Methods**

**Constant Depth Film Fermentor**

A CDFF (University of Wales, Cardiff), described by Wilson [1999], was used to grow biofilms. The rotating turntable in the CDFF containing 15 polytetrafluoroethylene (PTFE) pans, rotated under PTFE scraper bars that smeared the incoming medium over the 15 pans. Each sampling pan had 5 cylindrical holes (5.0 mm in diameter), which contained dentin specimens placed on top of PTFE plugs.

**Preparation of Specimens**

Dentin discs (1.5 mm thick and 5 mm diameter) were prepared from freshly obtained bovine incisors. One group (n = 28) of dentin discs was first covered with bonding agent (Scotchbond, multipurpose, 3M), then three parallel grooves were sawn perpendicular to the outer surface with a diamond-coated wire (well type A3-4; W. Ebner, Mannheim, Germany). All grooves were about 330 μm wide and 650 μm deep [Lagerweij et al., 1996a]. The dentin discs with grooves were placed on top of PTFE plugs. The surfaces of these discs were at the same level as the surface of sampling pans. The remaining dentin discs, as dentin smooth surface specimens (n = 18), were also placed on top of the PTFE plugs, but recessed to a depth of 300 μm. The positioning of the specimens is shown in figure 1.

**Inoculum**

The bacterium used in this study was *Streptococcus mutans* C180-2 [De Stoppelaar et al., 1967]. To prepare the inoculums, *S. mutans* was first grown on brain-heart
infusion (BHI) agar plates for 3 days, then single colonies were inoculated into 10-ml broths in triplicate and incubated anaerobically at 37°C overnight. The broth contained the same components as the growth medium (described below).

![Diagram](image)

**Fig. 1.** Positioning of the dentin specimens in a PTFE pan.

**Processed Saliva**

A total of 100 ml stimulated whole saliva was obtained from 2 volunteers at least 2 h after eating, drinking or tooth cleaning. At all times, saliva samples were kept on ice. The samples were centrifuged (30 min, 4°C, 27,000 g), and the supernatant was pasteurized (60°C, 30 min), re-centrifuged in sterile bottles and stored at -20°C [Guggenheim et al., 2001]. The efficacy of pasteurization was assessed by plating processed saliva samples onto blood agar plates. After 72 h at 37°C, no colony-forming units (CFUs) were observed on either aerobically or anaerobically incubated plates.

**Growth Medium**

The growth medium contained 3.7 g/l BHI medium (Difco, Laboratories, Detroit, Mich., USA), 25 mM PIPES buffer (acid form) and 1.5 mM CaCl₂; pH was adjusted to 7.0 by addition of 5 M KOH.
Inoculation and Operation of the CDFF

First, 100 ml sterile saliva was pumped into the CDFF for 3.5 h to form pellicle on the specimen surfaces. Then, 15 ml *S. mutans* overnight culture was mixed with 500 ml growth medium and inoculated into the CDFF over a period of 8 h. After 8 h, the inoculum flask was disconnected and sterile growth medium was dripped into the system by a peristaltic pump (type MS-4/6-100, Ismatec, Zürich, Switzerland) at a rate of 0.5 ml/min, according to the resting flow rate of saliva in healthy individuals [Dawes, 1996]. During the growth period of *S. mutans* biofilms, 2% (w/v) aqueous solution of sucrose was pumped into the CDFF for periods of 30 min at the same speed via a second peristaltic pump, while the supply of growth medium was stopped. Sucrose solution was added either 4 or 8 times per day in separate experiments. On days 5, 12 and 20, PTFE sample pans were taken out with a sterile stainless steel tool. The dentin specimens were removed aseptically for the determination of biofilm viability, dentin mineral loss and lesion depth. On day 20, the pH inside the grooves at different time points was measured. Contamination was checked by plating the effluent medium on blood agar plates during the experiments.

Assessment of CDFF Biofilm Viability

Only *S. mutans* biofilms grown on smooth surface dentin specimens were checked for viable counts because of sampling difficulties in groove specimens. After PTFE sample pans were collected from the CDFF, the plugs were carefully pushed out of the pan with biofilms intact on the surface. Then the specimens were placed into sterile vials containing transport medium (cysteine peptone water). Biofilms were removed from the surface and dispersed by sonicating on ice for 30 s at an amplitude of 40 W (Vibra cell™, Sonics & Materials INC, USA) and vortex-mixing for 30 s. After serial dilution, aliquots were plated out on the BHI agar plates. The plates were incubated anaerobically at 37°C for 3 days.

Assessment of Mineral Loss and Lesion Depth

After the biofilms were removed, both surface and groove dentin specimens were sectioned and three sections per specimen were radiographed and analysed by transversal microradiography (TMR) [Lagerweij *et al.*, 1996a]. The mineral loss, expressed as integrated mineral loss (IML), and the lesion depth (LD) were calculated (TMR software 1.25e, Inspektor Research Systems, Amsterdam, The Netherlands).
For the smooth ‘surface’ specimens, three scans, perpendicular to the surface, were made at random positions. IML and LD values for each specimen were obtained by averaging corresponding values of scans and sections. For the groove specimens, scans were made of both walls of each groove, at 50 µm (‘entrance’) and 300 µm (‘middle’) from the outer dentin surface and at the deepest part (‘bottom’) of the groove. IML and LD values at various positions of the groove per specimen were averaged from corresponding values of scans and sections.

**pH Measurements**

On day 20, dentin groove specimens were taken out at different time points: 15 min, 30 min after sucrose pulsing, 45 min, 4.5 h and 5.5 h after growth medium pulsing (sucrose pulse 4 times/day) or 45 min, 1.5 h and 2.5 h after growth medium pulsing (sucrose pulse 8 times/day).

The pH inside the groove was measured by an H⁺-selective microelectrode, which is a potentiometric liquid membrane microsensor. The electrode was made of borosilicate glass microcapillaries with a tip of 7-15 µm diameter. The tip was filled with combined liquid and polyvinylchloride-gelled membrane with H⁺ ionophore II (Fluka Chemi, Zwijndrecht, The Netherlands).

The pH measurements were performed in a flowcell containing a dentin specimen. The microelectrode was positioned inside the groove by XYZ computer-controlled microtranslators (850G; Newport Corp., France). Fifty millilitres of 1 mM KH₂PO₄ buffer (pH 7.0) was pumped through the system at a flow rate of 10 ml/min. The Ag/AgCl reference electrode was placed in the buffer. Both pH microelectrode and reference electrode were connected to an amplifier and a Data Acquisition Card (National Instruments). Software developed in Labview (National Instruments, Austin, TX, USA) was used to acquire data and position the microelectrode. The equipment for pH measurements was described in detail by Zaura *et al.* [2002].

To start the measurements the pH microelectrode was first positioned at the bottom of the groove, then the pH was measured at 50-µm intervals from groove bottom to buffer. The pH of the buffer was 7.0, which served as an indicator of the reliability of the microelectrode. The averaged pH values within 150 µm from the bottom and within 200 µm from the entrance represented the groove bottom pH and groove entrance pH, respectively. The measurements were repeated three times.
Statistical Analysis

All statistical analyses were performed with SPSS (version 9.0). The effects of time and sucrose pulsing on S. mutans biofilm viability were assessed by two-way analysis of variance (ANOVA). The effects of time, sucrose pulsing and scan position in dentin groove specimens on IML and LD were assessed by repeated-measurement ANOVA. IML and LD of dentin surface specimens were compared with those at the entrance of the grooves by one-way ANOVA.

Results

Viability of S. mutans Biofilms

Figure 2 shows viable counts (after log conversion) of S. mutans biofilms on dentin surface specimens at different time points under different SPF. The numbers of S. mutans were about $10^8$ CFU/cm$^2$ on day 5 after inoculation. This value remained constant till the end of the experiment when sucrose was pulsed 4 times per day. However, the viable counts tended to increase when sucrose was pulsed 8 times per day. Compared to SPF 4 times per day, on day 20 viable counts were significantly higher under SPF 8 times per day.

Mineral Loss and Lesion Depth

Five days after inoculation, lesions had developed along the dentin groove wall and in the dentin surface. The lesions did not show surface loss or erosion. Figure 3 shows lesions formed after various time periods in groove and surface specimens under different SPF. Figure 4A gives the IML values at various time points and under different SPF. The corresponding data for LD are given in figure 4B.

Irrespective of the age of the biofilm and the SPF, IML values generally decreased significantly from the entrance of the groove towards the bottom. A similar finding was made for LD. IML and LD at the entrance of the grooves were always significantly higher than in the surface specimens.

IML and LD increased significantly over time both in grooves and in surface specimens. Repeated-measurement ANOVA showed a significant interaction between the lesion positions in the grooves and time ($p < 0.001$). This indicated that IML and LD at various depths increased at different speeds.
Fig. 2. *S. mutans* viable counts (log conversion) on the dentin surface at different time points and under different SPFs. Significant difference between the two groups: *p < 0.05 (n = 3)*

Fig. 3. Microradiograms of subsurface lesions in the wall of the grooves (top row) and on the surface (bottom row) on day 5 and day 20 under different SPFs. For the groove, the wall lesions were scanned at three depths: 50 μm and 300 μm from the outer dentin surface, and at the base of the groove.
Fig. 4. Integrated mineral loss (A) and lesion depth (B) over time and with increasing SPF. Groove entrance: 50 μm from dentin surface; groove middle: 300 μm from dentin surface; groove bottom: the deepest part of the groove. Groove specimens: day 5, day 12, n = 4 per SPF; day 20, n = 6 per SPF; Surface specimens: day 5, 12, 20, n = 3 per SPF.

All values in the grooves except those marked # were significantly different among the entrance, middle and bottom.

IML and LD were also shown to increase significantly with increasing SPF in both specimens. But significant interactions between the lesion position in the groove and SPF was only found for the IML value.

To further analyse this finding the rates of change of IML and LD per day (denoted as ΔIML and ΔLD) were calculated for the four scan positions and the two SPF s (table 1). These data showed no significant deviation from a linear pattern of ΔIML with time for the entrance and middle of the groove position. In the bottom of the groove the value of ΔIML decreased with time for both SPF. This indicates that, unlike at the entrance and middle of the groove, lesion size does not increase linearly
with time for the bottom of the groove. In the surface specimens a lag phase in ΔIML was observed, which would indicate that in the early phase of the experiment the surface biofilm caused less demineralization than later. The ΔLD generally decreased with time, except for the surface specimens. Doubling the SPF resulted in a two- to threefold increase in both ΔIML and ΔLD.

**Table 1.** Change in integrated mineral loss (ΔIML) and in lesion depth (ΔLD) for the experimental conditions studied.

<table>
<thead>
<tr>
<th>Sucrose times/day</th>
<th>Days</th>
<th>Groove specimen entrance</th>
<th>Groove specimen middle</th>
<th>Groove specimen bottom</th>
<th>Surface specimen</th>
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<tr>
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</table>

**pH Measurements**

Figure 5 shows pH at the entrance and at the bottom of the grooves at various time points during the cycles of sucrose pulsing followed by medium pulsing; 2% sucrose pulsing resulted in typical, though prolonged ‘Stephan curves’. We note that the medium pulsing lasted 150 and 330 min for the 8 and 4 times sucrose pulse per day schemes, respectively. Under the low sucrose frequency, pH at the bottom of the groove returned to 7 at least 1 h before the new cycle started; while under the high
sucrose frequency, pH throughout the groove was still below 6.5 when the new sucrose pulse started.

Fig. 5. pH inside dentin grooves over time under different SPFs on day 20. A 4 times sucrose /day. B 8 times sucrose /day (n = 2). Blank bar refers to the periods of sucrose pulsing. Dashed lines are interpolations.

Discussion

Previous in vitro biofilm studies, using enamel or dentin smooth surface specimens as substrata, showed the formation of typical subsurface lesions [Hodgson et al., 2001; Noorda et al., 1986]. An increase in enamel lesion depth during a 3-week experiment period was reported, when S. mutans was inoculated into an artificial mouth system [Noorda et al., 1986]. In our study, similar phenomena were found both in dentin smooth surfaces and in dentin groove specimens. Moreover, we found that the lesion size decreased from the entrance towards the bottom in dentin grooves, which was similar to what was found in a non-bacterial in vitro demineralization groove model [Lagerweij et al., 1996a] and to the demineralization patterns observed in fissures in
The reason why bigger lesions were always found at the entrance of the groove might be that there were fewer vital bacteria at the bottom of the groove. Also, densely packed cells and extracellular polysaccharides might form a barrier. This could slow down diffusion of nutrients and acid into the deeper parts or diffusion of mineral ions out of the groove [Dibdin et al., 1983]. Alternatively, nutrients may be consumed by bacteria in the upper layers and not reach the bottom of the groove. In a parallel study, we examined the biofilm structure inside the groove by confocal laser scanning microscopy and observed that biofilms initially formed mainly in the upper part of the groove, but gradually filled the entire groove (data not shown).

Another interesting finding from this study is that IML and LD values of dentin surface specimens were always lower than the corresponding values at the groove entrance. We assume that this is caused by differences between the respective sites in biofilm formation and access to sugars and the growth medium, while the clearance of acids formed at the sites might also differ.

Regarding the mechanism of lesion formation, Arends et al. [1992] concluded that enamel demineralization in situ followed first order kinetics of mineral loss (IML), in contrast to demineralization in vitro, which was reported to follow second or third order kinetics [Christoffersen and Arends, 1982; Featherstone and Mellberg, 1981]. Data from the current study (table 1) essentially also showed a linear relationship between mineral loss and time, which would indicate that demineralization kinetics in this biofilm model are similar to those in vivo.

Comparing the rates of dentin mineral loss from the current study with data from in situ studies [Øgaard et al., 1988], we found that for dentin surface specimens, with 4 sucrose pulses per day, the mineral loss in this biofilm model was of the same magnitude as for dentin in situ. This would imply that also in terms of severity of caries attack rate the conditions of this biofilm model mimic in vivo caries.

Clinical and animal studies have demonstrated a positive correlation between the frequency of carbohydrate consumption and the development of dental caries [König, 1969]. Most of these findings were from the pre-fluoride era. Recently, Duggal et al. [2001] found that the mineral loss in an in situ study significantly increased with increased sucrose frequency, both when subjects used fluoride or non-fluoride toothpaste. Hodgson et al. [2001] studied the effects of sucrose pulsing on enamel demineralization in an in vitro biofilm model. Significantly higher IML values and lesion depths were found in the 50 mM sucrose treatment group, compared to the
water and the 10 mM sucrose treatment groups. In our study, we tested the effects of two different SPF on demineralization, and a similar positive correlation was found. So, the CDFF biofilm model, when carefully designed regarding SPF, is a sensitive model to study demineralization induced by bacteria.

Time/pH measurements on the last day of the experiment indicated that during the experimental period, pH inside the groove was below 6.5 most of the time under high SPF. However, it reached pH 7 at least 4 h per day under low SPF. During this period of neutral pH, remineralization could take place if the medium is well formulated in terms of its mineral constituents. In the current study this was achieved by the addition of buffer and calcium to the growth medium. On this point we note that in most artificial mouth systems or biofilm models the pH was either in the pH 4-5 range continuously or cycling between pH values well below the critical pH for dissolution [Hodgson et al., 2001], or never fell below 6.5 [Guggenheim et al., 2001]. This would make such models unrealistic compared with the in vivo plaque simulation.

In this study, single-species S. mutans biofilms were grown in CDFF. When growing multiple-species biofilms in the CDFF, Kinniment et al. [1996a] reported significant variations among experiments even though the bacterial compositions reached steady state. They concluded that it was difficult to replicate the biofilm community exactly. It was our purpose to test the system as a suitable and reliable caries model. We therefore chose single-species inoculation to reduce the variation. Moreover, Shu et al. [2000] showed dentin lesions formed under a single-species and consortium biofilm to be essentially comparable.

In conclusion, the current CDFF biofilm model may be used as a de- and remineralization model involving bacteria, in which the effects of anticaries agents, the efficacy of which could be pH-dependent, may be studied. Considering the demonstrated effect of SPF on caries formation it could also be used for studies of other cariogenic substances.