Biofilm and dental caries: application of the constant depth film fermentor
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Chapter 3

Caries-Preventive Agents Induce Remineralization of Dentin in a Biofilm Model

This chapter was submitted for publication.
Abstract

The aim was to study remineralization in dentin underneath a biofilm. This was done in a Constant Depth Film Fermentor (CDFF) which was modified so that two treatments can be applied simultaneously in one experiment. Forty-five Streptococcus mutans biofilms were grown in grooves in dentin. Growth medium (3.7 g/L BHI, 1.5 mM calcium and 25 mM PIPES) was administrated alternately with 2% sucrose pulsing 4 x 30 min/day. Fluoride at 135 ppm as NaF only or in a mixture with 0.2% chlorhexidine was applied 2 x 5 min/day. The treatments started 5 days after inoculation and lasted 15 days. Five specimens per group were removed at various time points. The biofilms were checked for viability (by plating) and acid content (by capillary electrophoresis). The dentin specimens were analyzed for mineral loss and lesion depth by transversal microradiography. Fluoride treatment had no effect on the viability, but reduced lactic acid production by 75%. The mixture treatment reduced the viability by 80% and the lactic acid content by 93% in the first day and later reduced the two parameters to below the detection limit. Significant differences in changes in mineral loss and lesion depth were observed between the treatment groups. Partial remineralization but deeper lesions were observed in fluoride group, while nearly complete remineralization was seen in mixture group. In conclusion, the CDFF S. mutans biofilm model can be used as a de- and remineralization biofilm model and the split model is particularly suitable for testing caries-preventive agents.

Introduction

It has been shown that bacterial cells in biofilms are more resistant to antimicrobials than bacterial cells in suspensions [Costerton et al., 1999; Wilson et al., 1996]. A number of biofilm models has been developed to assess the killing of caries pathogenic bacteria by caries-preventive agents [Pratten and Wilson, 1999; Shapiro et al., 2002]. Equally important it is to simultaneously study the resulting effect on the underlying dental tissues, in which caries forms. Until now no biofilm model has convincingly addressed the effects of caries-preventive agents on demineralization and remineralization of dental hard tissue.
Chapter 3

The Constant Depth Film Fermentor (CDFF) has been used to study the efficacy of antimicrobials on biofilm cells [Kinniment et al., 1996b; Pratten et al., 2000b]. Demineralization was also studied in this model [Deng and ten Cate, 2004], but remineralization has not been investigated in a CDFF biofilm model yet.

Remineralization of caries lesions has been studied over decades, but primarily in purely inorganic solutions, supersaturated with respect to hydroxyapatite [Arends et al., 1989; ten Cate et al., 1995]. In those studies fluoride enhanced remineralization. The inclusion of salivary components or bacterial metabolites gave contradictory results on enamel remineralization. On one hand, microbial deposits on enamel were shown to promote remineralization [Hardie et al., 1971]. On the other hand, salivary pellicle proteins or plaque could block the surface zone of enamel and restricted the diffusion of remineralising ions [Robinson et al., 1990]. Therefore, it is of interest to study remineralization underneath a biofilm.

The purpose of this investigation was to test the CDFF biofilm model for remineralization when the growth medium contained calcium and phosphate as in traditional remineralization solutions. More specifically, it was studied whether fluoride or a combination fluoride and chlorhexidine treatment shifts the de- and remineralization balance in a biofilm caries model. To this end, Streptococcus mutans biofilms were grown in grooves in bovine dentin specimens in the CDFF. In order to test the treatment of fluoride only and the combination of fluoride and chlorhexidine under similar conditions, the CDFF apparatus was modified into a split model to allow two treatments to be tested in the same experiment.

Materials and Methods

Split Constant Depth Film Fermentor

A Constant Depth Film Fermentor (CDFF) (University of Wales, Cardiff), described by Wilson [1999] was used to grow biofilms after modification. The drive shaft of the CDFF was controlled by micro-switches to allow the turntable not only to rotate 360° (as originally designed), but also to oscillate over 180°, splitting the CDFF into two independent sides. Each side has its own delivery ports and own polytetrafluoroethylene (PTFE) scraper bar to scrape the liquid over the specimens. In this way, different treatments can be applied to the two sides. When it rotates 360°, the rotating turntable can contain 15 PTFE pans. However, when it oscillates over 180°, only 11
pans, 5 in one and 6 in the other side, can be included in order to avoid carry-over. The position of the pans in the turntable is shown in figure 1. Each sampling pan has 5 cylindrical holes (5.0 mm in diameter), where dentin specimens were placed on top of PTFE plugs. The split CDFF modification was evaluated beforehand. It was concluded that similar biofilms were formed at either side when identical treatments were given and that there was no carry-over between the two sides.

**Preparation of Specimens**

Forty-five coronal dentin discs (1.5 mm thick and 5 mm diameter) were prepared from freshly obtained bovine incisors. The discs were first covered with bonding agent (Scotchbond, multi purpose, 3M), then three parallel grooves (330 µm wide and 650 µm deep) were sawn perpendicular to the outer surface with a diamond coated wire (Well type A3-4; W. Ebner, Mannheim, Germany) [Lagerweij et al., 1996a]. This cutting resulted in a random orientation of dentin tubules with respect to the groove. Previously our group demonstrated that the direction of the tubules has no effect on demineralization [Lagerweij et al., 1996b; 1997]. The surfaces of the dentin discs were placed flush with the surface of PTFE pans. During the experiment, biofilms were grown in the grooves. Per specimen, the biofilms in one groove were subjected to viability assessment and the biofilms in the other two grooves were pooled for acid measurements.

**Inoculation and Operation of the CDFF**

Before the start of the experiment, the CDFF was assembled with all dentin specimens and autoclaved at 121°C for 30 min.

To inoculate the CDFF, 100 ml sterile human saliva was pumped into the CDFF for 3.5 h to form pellicle on the specimen surfaces. Then, 15 ml of an overnight culture of *Streptococcus mutans* C180-2 [De Stoppelaar et al., 1967] was mixed with 500 ml growth medium and inoculated into the CDFF for 8 h. After 8 h, the inoculum flask
was disconnected. The sterile growth medium or 2% (w/v) sucrose solution was dripped into the system alternately by a peristaltic pump (Type MS-4/6-100, Ismatec, Zürich, Switzerland) at a rate of 0.5 ml/min. The sucrose was applied 4 times per day for periods of 30 min. The growth medium contained 3.7 g/L BHI medium (Difco, Laboratories, Detroit, Michigan, USA) containing 0.9 mM phosphate, 1.5 mM CaCl₂ and 25 mM PIPES buffer. pH was adjusted to 7.0 by the addition of 5 M KOH. The overall procedure in this section was described in detail by Deng and ten Cate [2004].

The Regime of the Treatments

During the first 5 days after inoculation, the turntable rotated 360° and no treatments were given.

The treatments, 135 ppm fluoride solution (as NaF) or a mixed solution of 135 ppm fluoride (as NaF) and 0.2% chlorhexidine digluconate, was pulsed from day 6 until day 20 at each side, respectively, while the turntable oscillated over 180°. The treatments were applied twice daily for 5 minutes at a rate of 1 ml/min, while the supply of growth medium (or sucrose) were stopped. These applications were always given immediately after a sucrose pulse. At days 5, 7, 9, 12 and 20, one sample pan from each group was removed from the CDFF immediately after a 30-min sucrose pulse, which was 360 min after treatments. The diagram of the regime of the treatments is given in figure 2.

All sample pans were taken out with a sterile stainless steel tool. The dentin specimens were removed aseptically from the pans for the determination of viability and acid content in the biofilm and mineral loss and lesion depth in the underlying dentin specimens.

![Diagram](image)

**Fig. 2.** A diagram of the treatment regime. F refers to 135 ppm F and F + CHX refers to 135 ppm F + 0.2% chlorhexidine. ⋆ refers to sampling days.
Assessment of CDFF Biofilm Viability

Biofilms were removed by repeated scraping with 3 consecutive sterile paper points (size #20, QDENT; NDO Leeflang, the Netherlands). This method was discussed in detail by Deng et al. [2004a]. All three paper points were placed into a sterile vial containing 1ml Cysteine Peptone Water solution (5 g yeast extract, 1 g peptone, 8.5 g NaCl, 0.5 g L-cysteine hydrochloride and 100 ml glycerol per liter, adjusted to pH 7.3). Samples were dispersed by sonication on ice for 30 s at the amplitude of 40 W (Vibra cell\textsuperscript{TM}, Sonics & Materials INC, USA). Serially diluted samples were plated onto BHI agar. The plates were incubated anaerobically at 37°C for 3 days.

Assessment of Acid Content

For the acid content analysis the biofilms were removed by paper points in the abovementioned way and transferred into vials containing 50 μl Milli-Q water on ice. The vials were placed in water bath at 80°C for 5 minutes and centrifuged for 15 min at 13,000 rpm at 4°C (Heraeus centrifuge, Dijkstra bv, the Netherlands). The supernatants were analyzed for organic acids by capillary electrophoresis. The pellets were determined for protein content by Bradford’s method. Details were described by Damen et al. [2002].

Assessment of Integrated Mineral Loss and Lesion Depth

After the biofilms were removed, dentin specimens were sectioned and three sections per specimen were radiographed moist and analyzed with the transversal microradiography (TMR) procedure [Lagerweij et al., 1996a]. Scans were made of both walls of each groove at 50 μm (‘entrance’) from the surface and 150 μm from the deepest part (‘bottom’) of the groove. The mineral loss, expressed as integrated mineral loss (IML) and lesion depth (LD) were calculated (TMR software 1.25e, Inspektor Research Systems, Amsterdam, The Netherlands).

Statistic Analysis

All statistical analyses were performed with SPSS (Version 9.0). The effects of various treatments and the time points of sampling on viability, acid content, IML and LD were analyzed by two-way ANOVA. When the position of the scan in the groove wall was included as parameter, the data were analyzed by repeated-measurement ANOVA. In addition, IML and LD values during the treatments were compared to the
values on day 5, within respective scan position and treatment group, by one-way ANOVA. The post hoc test was Student-Newman-Keuls if the data were equally distributed and Tukey if the data were not equally distributed.

Results

Viability of Biofilms under Different Treatments

Figure 3 shows the number of viable cells in the dentin grooves. Five days after inoculation, the number of viable cells reached $7.9 \pm 4.8 \times 10^7$ (s.d) CFU/groove. The viability of the cells was not affected by twice daily pulsing with 135 ppm F$^-$ and remained constant throughout the experimental period. However, the mixture treatment significantly reduced the number of viable cells by 80% after the first day and to under the detection limit (0.01% of the original values) from the third day till the end of the experiment.

![Figure 3](image)

**Fig. 3.** The effect of 135 ppm F$^-$ (×) or the combination of 135 ppm F$^-$ and 0.2% chlorhexidine (□) on the viability of *S. mutans* biofilms grown in dentin grooves. N = 5 for each data point. The dark line shows the detection limit.

Acid Production of Biofilms under Different Treatments

After 30-min sucrose pulse in the CDFF, various organic acids were produced in the biofilms. Traces of acetic acid, formic acid and propionic acid were found. Only the data of the predominant lactic acid are presented here (Figure 4). Five days after
Remineralization of Dentin Underneath the Biofilms

inoculation, the lactic acid produced after sucrose pulse was $11.9 \pm 2.2$ nmol acid/µg protein. After three days of fluoride treatment, lactic acid content was reduced by 75% and then remained at this level until the end of the experiment. In the mixture group, the reduction was already up to 93% after one day treatment. The amount of lactic acid was further decreased to below the detection limit in the next days. The detection limit was 0.21 nmol acid/µg protein.

**Fig. 4.** The effect of 135 ppm F⁻ (×) or the combination of 135 ppm F⁻ and 0.2% chlorhexidine (□) on the lactic acid production of *S. mutans* biofilms grown in dentin grooves. The acid contents were measured after 30-min 2% sucrose pulse. N = 5 for each data point. The detection limit was 0.21 nmol acid/µg protein.

Lesion Formation in Dentin Grooves under Different Treatments

In figure 5, images of lesions from representative specimens in each group are shown. Figure 6 gives the average mineral profiles of the dentin samples in each group on days 5, 12 and 20. The profiles at the entrance and at the bottom of the dentin grooves are presented separately.

In a separate experiment, *S. mutans* biofilms were grown under the same condition as in current study, except sterilized Milli-Q water instead of treatment solutions was applied twice daily. Lesion formation in dentin grooves was measured on days 5, 12 and 20. The data from the water group are included in the figures for comparison.

On day 5, subsurface lesions had been formed along the walls of the grooves. In the water group, the lesion size increased significantly throughout the groove over time. The increase at the entrance was significantly greater than at the bottom. In the fluoride group, laminations were found in the lesion on day 12. The original lesion was partly remineralized but in depth followed by a second lesion body. On day 20...
both remineralization and this second lesion body were more pronounced. The second lesion body was significantly larger at the bottom of the groove than at the entrance, irrespective of the time of assessment. In the mixture group, there was partial remineralization in the body of the lesion and slight demineralization at the front of the lesion on day 12. Again, the newly demineralized zone was bigger at the bottom than at the entrance. On day 20, complete remineralization was seen throughout the dentin grooves.

IML and LD values were calculated from the profiles (Table 1). The values on day 20 were compared to those on day 5. In water group, both IML and LD value increased significantly. After fluoride treatment, the IML value did not change at the entrance, but increased significantly at the bottom of the grooves. The LD values increased irrespective of the scan position in the grooves. After the mixture treatment, the IML values decreased throughout the grooves; the LD values tended to decrease at the entrance but did not change at the bottom of the grooves.

Fig. 5. Microradiograms of lesions formed in the wall of the dentin grooves in different treatment groups on days 5, 12 and 20. A: water group; B: 135 ppm F group; C: 135 ppm F + 0.2% chlorhexidine group.
Fig. 6. The average mineral profiles of dentin on days 5, 12, 20. A: water group; B: 135 ppm F\textsuperscript{−} group; C: 135 ppm F\textsuperscript{−} + 0.2% chlorhexidine group. All treatments started on day 6. Figures in the left column are from the groove entrance; figures in the right column are from the groove bottom. Groove entrance refers to the position at 50 μm from the surface of the groove; groove bottom refers to 150 μm from the deepest part of the groove (N = 5).
Table 1: Integrated Mineral Loss (IML) and Lesion Depth (LD) values in time after different treatments

<table>
<thead>
<tr>
<th>Day</th>
<th>Water</th>
<th>F</th>
<th>F + CHX</th>
<th>Water</th>
<th>F</th>
<th>F + CHX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1043(115)</td>
<td>969 (160)</td>
<td>969 (160)</td>
<td>39.0(2.8)</td>
<td>32.0 (4.2)</td>
<td>32.0 (4.2)</td>
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<tr>
<td>7</td>
<td>1117 (159)</td>
<td>1011(217)</td>
<td></td>
<td>45.6 (10.3)</td>
<td>38.2 (7.6) *</td>
<td></td>
</tr>
<tr>
<td>Entrance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1033 (263)</td>
<td>864 (121)</td>
<td></td>
<td>57.3 (9.9) *</td>
<td>36.3 (1.9) *</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2495(247) *</td>
<td>1123 (180)</td>
<td>793 (51)</td>
<td>80.5(4.5) *</td>
<td>68.9 (17.2) *</td>
<td>40.0 (1.2) *</td>
</tr>
<tr>
<td>20</td>
<td>3383(253) *</td>
<td>1087 (112)</td>
<td>454 (63) *</td>
<td>116.4(8.0) *</td>
<td>85.7 (14.8) *</td>
<td>26.2 (7.6)</td>
</tr>
<tr>
<td>5</td>
<td>723(36)</td>
<td>660 (63)</td>
<td>660 (63)</td>
<td>34.5(3.0)</td>
<td>31.7 (4.1)</td>
<td>31.7 (4.1)</td>
</tr>
<tr>
<td>7</td>
<td>896 (197)</td>
<td>889 (202)</td>
<td></td>
<td>48.2 (8.1) *</td>
<td>46.3 (6.4) *</td>
<td></td>
</tr>
<tr>
<td>Bottom</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1045 (131)*</td>
<td>704 (183)</td>
<td></td>
<td>63.4 (6.2) *</td>
<td>46.9 (8.8) *</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1757(255) *</td>
<td>1270 (62)*</td>
<td>714 (175)</td>
<td>76.7(7.4) *</td>
<td>84.0 (15.0) *</td>
<td>48.4 (7.6) *</td>
</tr>
<tr>
<td>20</td>
<td>2417(431) *</td>
<td>1620 (190)*</td>
<td>329 (72)*</td>
<td>104.4(10.1) *</td>
<td>119.8 (15.3) *</td>
<td>31.6 (14.7)</td>
</tr>
</tbody>
</table>

Data are presented as mean (s.d).

F refers to 135 ppm F and F + CHX refers to 135 ppm F + 0.2% chlorhexidine.

N = 5 for each data point.

* indicates significant differences compared with the values on day 5 (p < 0.05).
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Discussion

One of the advantages of the CDFF is that it gives good reproducibility in the properties of the 75 biofilms grown within one CDFF experiment [Peters and Wimpenny, 1988]. This reproducibility is achieved by growing the biofilm at a constant depth. However, the reproducibility among CDFF experiments has been questioned. Kinnement et al. [1996a] reported a significant variation in bacterial composition among experiments when multi-species biofilms were grown in the CDFF. In the two experiments reported in our study, we observed a good reproducibility in the viability when single-species biofilms were grown under identical conditions. A small, though significant, difference in LD value was found. We suggest that biofilm parameters should be studied after at least a five days lead-in period under constant conditions when separate experiments are to be compared.

To achieve a better comparison, we modified the CDFF into a split model to allow two different treatments be given during one experiment. Various tests were carried out to validate the split model design (data not shown). Firstly, similar lesions were formed at different locations of the turntable run in full rotation mode, which ensured similar baseline values at both sides when the oscillating mode was started. Secondly, similar lesions were formed on both sides when the biofilms at the two sides were grown under the same condition. Thirdly, the oscillating mode did not show carry-over between the two sides. Therefore the split model seems preferable for studying antimicrobial treatments in a CDFF biofilm model.

With the split CDFF model, the effects of 0.2% chlorhexidine, combined with fluoride, on S. mutans biofilms and dentin were demonstrated. The majority of biofilm cells was killed by the chlorhexidine treatment and acid production was stopped. As a result the pre-formed lesions were completely remineralized in two weeks. Fluoride only did not stop the acid production and the lesions progressed further into the dentin while the original lesion was only partly remineralized. A lamination of the lesion appearing under fluoride treatment was also observed in in vitro [Mukai et al., 2001] and in in situ studies [Nyvad et al., 1997].

To our knowledge, remineralization has not been shown in any in vitro biofilm model. It had been suggested that proteins from salivary pellicle or plaque prevent the deposition of mineral on the surface of the teeth [Moreno and Zähradnik, 1979] or block the surface zone of enamel and restrict the ingress of remineralising ions [Robinson et al., 1990]. Our data showed that it was possible to remineralize a lesion
formed after salivary coating and underneath the biofilms. Zahradnik et al. [1978] found that prolonged salivary pellicle formation influenced the process of enamel demineralization. In our study, saliva was pumped into the CDFF for 3.5 h. It would be interesting to study a time dependency of pellicle formation on remineralization in this biofilm model.

In this study, the second lesion body after fluoride treatment was considerably larger at the bottom than at the entrance of the groove. We assume that most of fluoride might be entrapped in the dentinal crystallites at the entrance of the groove, which might result in less fluoride diffusing to the bottom. Several pH-cycling experiments carried out in our lab showed that when the acid attack was constant, the size of the second lesion body increased with decreasing amounts of fluoride (data unpublished). It is also possible that fluoride induced inhibition of demineralization at the entrance led to less acid being neutralized at the entrance. Consequently acids would diffuse deeper into the groove and higher amounts of acid would be present at the bottom of the groove. When the biofilms were treated by the mixture of fluoride and chlorhexidine, no second lesion body was observed. However, during the first week of the treatment, further lesion progression was found at the bottom of the groove. This finding implies that chlorhexidine is less effective at bottom, possibly because it is absorbed by the biofilm cells or matrix at the groove entrance. Moreover, more cells might be killed by chlorhexidine or fluoride at the entrance than at the bottom of the groove. This change of viability distribution might also influence the diffusion of sugar and its conversion to acids.

Fluoride is known for its antimicrobial activity. A pre-experiment showed that the minimum inhibitory concentration of sodium fluoride against S. mutans C180-2 in suspension was 80 ppm F⁻. Since bacterial cells in biofilm have been shown to be more resistant to antimicrobials than those in suspension [Costerton et al., 1999; Wilson et al., 1996], a higher amount of NaF (135 ppm F⁻) was used for this study. Under this concentration, no effect of fluoride on viability was observed. However, a decrease in acid formation was found after fluoride treatment, which was not predicted from the viability data. A similar phenomenon was reported by McDermid et al. [1985]. In their study, the small change in viability also could not account for the observed large inhibition of acid production after fluoride treatment.

In conclusion, our study shows that the CDFF biofilm model can be used as a demand remineralization biofilm model and that the split mode of running the CDFF is feasible for studying caries-preventive agents.