An active attachment biofilm model to develop anti-caries strategies
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Chapter 1

Introduction
Caries remains to be one of the most widespread diseases in the world (Bagramian et al., 2009). Caries is caused by bacteria fermenting foods (mostly sugars) and producing weak organic acids like lactic acid and acetic acid. These acids demineralize the dental apatite structures. In the past 40 years a sharp decline in caries prevalence has occurred which is most probably associated with the introduction of fluoride in products used in daily oral care. Fluoride is considered the most effective anti-caries product (Zero, 2006; Ten Cate, 2013). Fluoride protects the dental hard tissues from being demineralized and enhances remineralization of these tissues. The effect of fluoride on bacterial metabolism is limited, mainly because high concentrations of fluoride are required to effectively reduce acid production by bacteria and such concentrations are limited to short periods after treatments. It is now generally accepted that optimal fluoride protection is obtained by providing a continuous low level of fluoride in the oral cavity. Despite the widespread use of fluoridated products only a small percentage of the population remains caries free. Even in a population that was exposed to fluoridated water for more than 90% of the time, caries did still develop (Mahoney et al., 2008).

The efficacy of fluoride relies partly in its capacity to enhance dental hard tissue repair during periods of near neutral pH. The total time that this near neutral pH exists in the oral cavity depends strongly on the number of acid challenges, and thereby on the number of food-intake moments per day. With an increased frequency of food consumption (so called “grazing”) the efficacy of fluoride in the remineralization of apatite structures is reduced. This could be, in part, an explanation for the stagnation, or even reversal, of the caries decline (Birkeland et al., 2002).

From the above it is apparent that there is still need for additional caries preventive agents. Some of these agents might focus on interfering with the bacteria causing caries. Among these bacteria are mutans streptococci, lactobacilli and bifidobacteria (Marsh, 2012). These all thrive in oral biofilms, particularly under caries inducing conditions. Biofilms, by definition, are matrix-enclosed microbial communities in which cells adhere to each other and/or to surfaces or interfaces (Costerton et al., 1995). It has been shown that bacteria in biofilms are more resistant to antimicrobial therapies than their planktonic counterparts (Donlan et al., 2002;
Bjarnsholt et al., 2005; Tenover, 2006; Ceri et al., 1999). Moreover, it has become clear that in complex, multispecies biofilms polymicrobial interactions enhance the resistance to anti-microbials even further (Kara et al., 2006; Burmølle et al., 2006; Luppens et al., 2008; Ramsey et al., 2009).

Therefore, it is now acknowledged that studying new compounds aimed at interfering with bacteria requires polymicrobial biofilm models instead of traditional bacterial cell cultures.

In order to study bacteria in biofilms a range of in vitro models has been developed. All these models were aimed at mimicking the complexity of the biofilm structure and/or complexity of dental plaque.

**Multiwell and microtiter plates**

Relatively simple models consist of multiwell plates that are inoculated with combinations of selected species (fig 1).

Guggenheim et al. (2001) developed a model based upon pellicle-coated sintered HAP discs that were placed flat on the bottom of 24-well plates. The model was inoculated with a mixture of 6 selected species to mimic the composition of supragingival plaque. The model was evaluated for its use by assessing the treatment efficacy of Triclosan and CHX. The same model was used to measure remineralization of previously demineralized enamel discs (Guggenheim, 2004). Filoche et al. (2007) used Thermanox-cover slips on the bottom of 24-well plates to grow saliva-derived biofilms for periods up to 10 days. Checkerboard DNA-DNA hybridization was used to
determine the composition of the biofilms that were formed. It was shown that the composition of the biofilms varied with donor, growth medium and sucrose supply.

Walker (2007) used HAP-discs at the bottom of six-well or twelve-well plates. The model was inoculated with samples of subgingival plaque from eight periodontally healthy and eight subjects having non-aggressive periodontitis. The samples were collected using paperpoints. Biofilms were grown on trypticase soy broth for up to 10 days with changes to fresh medium every 48 h. Checkerboard DNA-DNA hybridization was used to determine the composition of both the biofilms formed and the inocula. The authors showed compositional differences in the samples from healthy and periodontitis subjects and also in the biofilms that were formed using these inocula.

In the above mentioned models the substratum was placed at the bottom of the wells. This means that biofilm formation was not depending on the initial binding of the bacteria to the substratum, but started with aggregation of bacteria on top of the substratum.

Duarte et al. (2008) and Koo et al. (2010) used individual disc holders to position HAP discs perpendicular to the bottom of 24 well plates. This way the bacteria were forced to actively attach to the HAP discs. The model was used (amongst others) to monitor the effects of starch and sucrose on the growth of *Streptococcus mutans* (*S. mutans*) biofilms. Ceri et al. (1999) introduced the Calgary Biofilm Device. This model consists of a lid having 96 pegs that are placed on top of a 96-well microtiter plate. Biofilms were allowed to grow on these pegs and were harvested by breaking the pegs from the lid. The model was evaluated by measuring the susceptibility of *P. aeruginosa* and *E. coli* to a range of antibiotics. This model has the advantage that all biofilms could be moved to either new growth medium or treatment solutions at the same time, by simply moving the lid to a new 96-well plate.

*Models with continuous flow*

All of the models described above use biofilms that are grown in batch culture with periodic medium refreshments. A more sophisticated model would involve the continuous or intermittent flow of nutrients and or treatment solutions. The modified Robbins device (MRD) used by Honraet et al. (2006) allows for biofilms to
grow on 6 HAP-discs arranged in a linear array. With the HAP-discs in place the device has a rectangular flow chamber that can be connected to a peristaltic pump to allow for the flow of medium. As the six HAP discs in a single MRD are all in the same flow chamber only one condition can be tested in a single MRD, and the discs are not independent from each other. In order to test different conditions multiple MRD’s or multiple runs are required. The model showed reproducible biofilm growth from *S. mutans* as established by a fluorescence assay. And it was possible to differentiate between biofilms that were grown with different plant extracts in the growth medium. The same model was used by Sliepen et al. (2010) to measure the susceptibility of biofilms, formed from two *Aggregatibacter actinomycetemcomitans* (*Aa*) strains, to three commercially available oral rinses (Perioaid®, Meridol® and Listerine®). To that end a series of MRD’s was connected in parallel to a vessel with a continuous culture of *Aa* and biofilms were grown for 4 days in total. The biofilms were treated with the respective oral rinses by replacing the content of the flow chambers with the oral rinse. After 30 s the contents of the flow cells were replaced with fresh medium and then normal flow was re-established. The authors presented differences in treatment efficacy between the three oral rinses. Bercy and Laserre (2007) also used the MRD to measure the effect of 0.2% chlorhexidine, 1% povidone-iodine and Listerine® on dual-species biofilms consisting of *S. gordonii* (ATCC 10558) and *P. gingivalis* W83. In contrast to Sliepen these authors used a short inoculation time. One hour for *S. gordonii* followed by 1 day for *P. gingivalis*. Subsequent biofilm formation with fresh broth (without bacteria) lasted for 7 days. The treatment efficacy was tested by pumping the respective agents through the MRD. Exposure times were 15 and 30 min. Killing efficacy was above 90% for all three agents after 15 min. Blanc and co-workers (2014) used the MRD to test the efficacy of oral rinses (0.12% chlorhexidine plus 0.05% cetlypyridinium chloride (CHX+CPC); 0.12% CHX; and 0.12% CHX plus sodium fluoride (CHX+NaF)) on multispecies biofilms. They also connected the MRD’s to a continuous culture system with six strains from which a continuous flow was run through the MRD’s. Biofilms were allowed to grow up to 4 days and then treatments of the biofilms were performed outside of the MRD. Differences were shown in overall killing efficacy of the undiluted, 2x diluted and 5x diluted oral rinses.
McBain (2005) used a multiple Sorbarod® device to grow saliva derived biofilms. A Sorbarod is a 10mm diameter and 20 mm long paper filter. Filters were fitted in a custom made holder and were perfused with a modified artificial saliva medium. The Sorbarods were inoculated with saliva from individual donors on two occasions 14 h apart. Biofilms were allowed to grow inside the Sorbarods for up to 5 days. Composition of the biofilms was measured using both selective media and checkerboard DNA-DNA hybridization. It was shown that composition of the content of the Sorbarod differed between inocula and also between different Sorbarods.

*Advanced models allowing for intermittent flow and pulsing of nutrients and/or actives*

The above mentioned models allow for a flow of nutrients through the holders containing the biofilms. But these models still lack the potential to pulse nutrients and/or treatment solutions into the system.

Hodgson (2001) used a so-called weir system to grow multispecies biofilms. The system consists of a vessel with a continuous culture of seven species. A second vessel contains fresh medium. A feed line from both vessels was connected to a second stage that contained the surfaces (either HAP-discs or enamel slices) for biofilm growth. The continuous culture and the fresh medium were mixed and pumped into the second stage. The model was used to monitor the effect of sucrose pulses on biofilm composition and on the pH of the planktonic phase in the second stage. This model did allow for pulsing of nutrients (and possibly treatment agents). But a weir system has a serious drawback. Any nutrient or agent that is pulsed into the model will be slowly diluted out of the model, thereby lacking control of exposure time of either the nutrient or the treatment solution.
Two other models were used in the past decades to allow for pulsing. Peters and Wimpenny (1988) introduced the constant depth film fermentor (CDFF, fig 2).

![Figure 2](image)

*Figure 2. Overview of the CDFF, with a detail of the disc containing the sample pans (top right) and the sample plugs that can be recessed below the sample pan (bottom right).*

It consists of a disc that can hold 15 sample pans which each contain 5 sample plugs. The sample plugs are recessed below the surface of the sample pan. The sample pan is set flush to the PTFE disc. The PTFE disc rotates below a spring loaded scraper that removes any excess medium and/or biofilm. The disc is placed in a jar that has a central opening for the addition of nutrients and a large opening to allow for the individual pans to be removed from the CDFF. Kinniment et al. (1996) used this model to grow multispecies biofilms. The CDFF was inoculated during 8 h from a continuous culture containing nine species, after which the CDFF was supplied with only fresh medium. The biofilms were grown for several periods of time, even as long as 5 weeks (up to 840 h). Data showed that all nine species could be identified in the biofilm but the time needed for a single species to obtain a steady state varied between species. Three CDFF runs were carried out, but the results of each run were different, indicating that replication of biofilms was difficult to obtain. The model was used by Pratten et al. (1999) to grow saliva derived biofilms. Besides a continuous flow of artificial saliva the model was pulsed three times a day for 30 min with 10% sucrose. After 5 days the CDFF was additionally pulsed twice daily with 10 ml of 0.2% CHX. The composition of the untreated biofilms after 4 days and shifts in the composition up to 9 days were monitored using selective plates. Shifts in the
biofilm composition resulting from the sucrose pulsing were observed. Pulsing with chlorhexidine did not affect the total viable counts, but did affect biofilm composition. The model was used to study the effects of amine-fluorides on selected species (Embleton et al., 1998) and urease on a 10 species biofilm (Shu et al., 2003). Hope et al. (2006) used the model to grow biofilms from pooled subgingival plaque samples. Deng et al. (2004) used the CDFD to measure dentin demineralization in grooves in dentin as a function of the number of sucrose pulses per day.

Sissons and co-workers (1991) introduced a multi-station artificial mouth (MAM, fig.3) to study plaque growth, metabolism and pH. The system originally consisted of a culture chamber with five plaque growth stations, and was later expanded to eight stations.

Figure 3. Overview of a MAM (photographs courtesy of E. Zaura).

Biofilms are grown on top of LuxThermanox plastic 25mm tissue culture cover slips. Each plaque growth station has its own supply lines for the addition of nutrients and experimental treatments. Three lines are available per station. This allows not only for continuous feeding of nutrients, but also for pulsing with additional sucrose. Using additional ports one can access the plaque growth support for measuring pH or sampling the plaques that are grown. The model was inoculated with samples of supragingival plaque from a single donor. The model was (re)inoculated on day 0, 2 and 5 with fresh plaque samples. Plaques were grown up to 6 weeks in this system. This model has been used in a number of experiments aimed at measuring calcification of plaques (Sissons et al., 1991; Pearce et al., 2002; Wong et al., 2007), effect of medium (Wong et al., 2001), the effect of fluoride-supplemented sucrose
(Cutress et al., 1995) and to grow defined-species consortium biofilms (Shu et al., 2000).

Taken together a wide range of biofilm models has been used in the past decades. These range from relatively simple models, to models that are impressively sophisticated. A review on available models was written by McBain (2009) and on artificial mouth model systems by Tang et al. (2003).

Each of the models has pro’s and con’s that are listed in table 1. The simple models lack the possibility of flow and/or pulsing of nutrients. But they do allow for the formation of a high number of biofilms at the same time. But some of these models do not include active attachment to the substratum at the onset of the biofilm formation. They do allow a proper control on exposure time/concentration of the treatments that are to be tested, which is generally more complicated in the advanced models. In those models the active ingredient is washed out of the system by the flow of the applied growth medium.

Along with the need for additional caries preventive agents comes the need to have or develop models that allow for reliable high-throughput testing of such agents. Such a model should meet the following prerequisites: (i) biofilm formation should be reproducible and based on active attachment of bacteria to a substratum (ii) besides single species biofilms it should allow screening of complex polymicrobial biofilms, (iii) it should be possible to vary substratum and (iv) it should be possible to compare multiple compounds, concentrations and treatment times in a single experiment.

These prerequisites could not be fully met in the existing models. Therefore it was thought there was need for an additional model that would combine the advantageous features of existing models.
<table>
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<th>substratum in individual holder in multiwell plates</th>
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<th>Modified Robins device</th>
<th>CDFF</th>
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*Table 1. Schematic overview of available models*
Outline of the thesis

This thesis describes a number of experiments that were performed to develop, test and utilize a biofilm model suitable for screening agents that target the oral microflora.

In chapter 2 the model is introduced and its potential to reproducibly grow single species or polymicrobial biofilms is shown. Furthermore, the suitability of the model to determine dose response relationships with anti-microbial compounds is determined.

Chapter 3 describes the potential of the model to measure the effects of arginine additions to the growth medium on a number of selected output parameters.

Mild anti-microbials could cause a shift in the microbial composition of polymicrobial biofilms. While DNA-based technologies may be more appropriate for the assessment of bacterial composition than culturing, these techniques amplify DNA from both live and dead cells. This could mean that after an anti-microbial treatment shifts in composition are partly masked by the presence of DNA from cells that were killed by the treatment. A treatment with propidium monoazide (PMA) has been suggested to overcome this problem. In chapter 4 the effect of a PMA treatment on the measured composition of in vitro grown biofilms after a single chlorhexidine treatment was assessed.

The finding that a PMA treatment improved the detection of shifts in polymicrobial biofilms after a single chlorhexidine treatment in vitro resulted in a clinical study. In chapter 5 the concept that DNA of dead bacterial cells would influence the measured composition of clinical samples was tested. To that end a small group of subjects was sampled twice before and twice after a 2-weeks use of an oral rinse (Meridol, GABA). On all time points half the samples were treated with PMA and the other half were left untreated. The bacterial composition of buccal plaque, tongue scrapings and saliva before and after the use of the oral rinse were determined. Also the effect that the PMA treatment had on the measured composition of the samples was determined.
In chapter 6 the results are summarized and an outline is given of future experiments that are thought to contribute to the further development of the model. A summary of the results and future perspectives in Dutch is presented in chapter 7.

References


