An active attachment biofilm model to develop anti-caries strategies

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Chapter 2

Different Response to Amine Fluoride by *Streptococcus mutans* and Polymicrobial Biofilms in a Novel High-throughput Active Attachment Model.

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Abstract

Background/Aims
Anti-microbial resistance of micro-organisms in biofilms and polymicrobial interactions in these biofilms that modulate resistance require novel strategies to evaluate the efficacy of caries preventive compounds. The current study aimed at evaluating effects of a caries preventive agent in Streptococcus mutans and polymicrobial biofilms.

Methods
We developed a novel high through-put active attachment model. The model consisted of a custom designed lid containing glass discs that fitted on top of standard 24-well plates. Biofilms were formed using either S. mutans C180-2 or saliva. At the end of biofilm formation, up to 96 h, biofilms were treated with amine fluoride (AmF) solutions. Viability of the biofilms was determined by CFU counts, metabolic activity was measured via lactate production.

Results
The effect of AmF on the viability of polymicrobial biofilms was significantly less than that on S. mutans biofilms indicating a higher resistance in the complex biofilms. Both types of biofilms became more resistant to AmF with age.

The higher resistance of polymicrobial biofilms was not reflected in metabolic activity: in dose-response experiments AmF reduced lactate production in both types of biofilm to the same extent. Also the age induced increased resistance in polymicrobial biofilms was less pronounced in inhibition of metabolic activity.

Conclusions
This study clearly shows that when evaluating the efficacy of caries preventive compounds it is essential to use appropriate polymicrobial biofilm models and more importantly that efficacy needs to be judged on reduction of acid formation (i.e. cariogenic potential) as well as on bacterial viability.
Introduction

The primary cariogenic pathogen *Streptococcus mutans* thrives 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 is now well documented that bacteria in biofilms, including *S. mutans*, are considerably more resistant to treatment with anti-microbials than their planktonic counterparts (Tenover, 2006). This implies that the efficacy of caries preventive compounds should be evaluated in biofilms and not in the traditional liquid cultures. Moreover, it has become clear that in multispecies biofilms polymicrobial interactions contribute to resistance to anti-microbials and to host immunity (Kara et al., 2006; Burmølle et al., 2006; Luppens et al., 2008; Ramsey et al., 2009).

In order to study bacteria in biofilms a number of in-vitro models have been developed that all aimed to mimick the complexity of dental plaque. These models range from simple microtiter plate models (Ceri et al., 1999; Guggenheim et al., 2001; Guggenheim et al., 2004; Filoche et al., 2007) to more complex models based upon continuous culture systems (Bradshaw et al., 1996; Hodgson et al., 2001). Models like the constant depth film fermentor (CDFF; Peters and Wimpenny, 1988; Wilson et al., 1998; McBain et al., 2003) or an artificial mouth (Sissons et al., 2007) used inoculation with mixtures of bacteria or saliva. Results with the more complex models showed the potential of in-vitro biofilms to simulate polymicrobial complexity comparable to dental plaque. While in the latter models the substratum could be varied, they generally lack the possibility for high-throughput (HTP) screening and/or active attachment of bacteria. In more simple HTP models generally biofilms are grown that consist of single species or mixtures of a few species. The only HTP model that allows active attachment of bacteria (Ceri et al., 1999), a parameter essential to guarantee actual biofilms instead of layers of sedimented cells, lacks the possibility for substratum variation. Alternatively, the only HTP model that describes complex biofilms on variable substrata, lacks the active attachment option (Filoche et al., 2007).
Various of these considerations have recently been reviewed in detail (McBain, 2009).

The primary aim of this study was to develop and use a HTP model for screening and testing the efficacy of (potential) anti-microbial compounds that combines all 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 substrata and (iv) it should be possible to compare multiple compounds, concentrations and treatment times in a single experiment.

A secondary aim was to use this HTP model to compare the anti-cariogenic activity of an aminefluoride (AmF) on single species, actively attached \textit{S. mutans} biofilms and actively attached complex polymicrobial biofilms.

\textbf{Materials & Methods}

\textit{The biofilm model}

The biofilm model (fig. 1) consisted of a custom-made stainless steel lid with 24 clamps that can contain different substrata. In the current study glass cover slips (Ø 12mm, Menzel, Braunschweig, Germany) were used as substrata to grow biofilms. After assembling the lid and substrata the model was autoclaved. The lid fits onto standard polystyrene 24-well plates (multiwell plates, Greiner Bio-One, Alphen a/d Rijn, The Netherlands).

\textit{Saliva collection}

Saliva was collected on ice from a single donor. The saliva was filtered over sterile glass-wool to remove any debris. The saliva was diluted 2 fold with 60\% sterile glycerol to protect the bacterial cells from cryo damage. Saliva was either used immediately for an experiment or stored at -80\degree C.
Figure 1.

Pictures of the biofilm model used in this study. (A) Custom-made stainless steel lid on which 24 clamps are fixed. Substrata glass cover slips (on the left) or HA disks (on the right) are shown. (B) Position of the substrata in the 24-well plate at the time of biofilm growth.
Production of biofilms

The model was inoculated with either *S. mutans* C180-2 or saliva. The inoculation medium for *S. mutans* biofilms was a 10-fold diluted overnight culture of *S. mutans* C180-2 in a medium that consisted of 18.5 g/l BHI, 0.2% sucrose and 50 mmol/l PIPES at pH 7.0. The inoculation medium for the polymicrobial biofilm was a 50-fold diluted saliva in semi defined medium consisting of 2.5 g/l Mucin (Sigma M-2378), 2.0 g/l BactoPeptone (Difco 0118-01-8), 2.0 g/l Trypticase Peptone (BBL 211921), 1.0 g/l Yeast Extract (Bacto 212750), 0.35 g/l NaCl, 0.2 g/l KCl, 0.2 g/l CaCl$_2$, 0.001 g/l Haemin (Sigma H-1652), 0.0002 g/l Vitamin K1 (Mc Bain, 2005), with 0.2% sucrose and 50 mmol/L PIPES at pH 7.0.

Biofilms were produced by adding 1.5 ml of the inoculation medium to each well. The model was subsequently incubated anaerobically (10% CO$_2$, 10% H$_2$ and 80% N$_2$) for 8 h at 37 °C. After this initial inoculation period the lid was transferred to a new plate containing fresh medium (without bacteria) and incubated for another 16 h. When biofilms were grown for longer than 24 h the medium was refreshed twice daily in a schedule of 8 and 16 h growth. In pilot experiments it was shown that biofilms derived from saliva resulted in slower biofilm formation: Only after 48 h CFU counts were obtained that were comparable to 24 h single species *S. mutans* biofilms. For this reason *S. mutans* biofilms were grown for 24, 48 and 72 h, and polymicrobial biofilms for 48, 72 and 96 h.

Acid production assay

At the end of the biofilm formation period, and when applicable after the subsequent treatment, the lid was placed on a new plate containing 1.5 ml/well BPW (buffered peptone water) with 0.2% sucrose. The model was incubated anaerobically for 3 h at 37 °C. The amount of lactic acid formed during this period was analyzed using a colorimetric assay (van Loveren et al., 2006).
**Determination of CFU**

The glass discs with the biofilms were removed from the lid and transferred into 2 ml CPW (cysteine peptone water). The biofilms were dispersed using a sonicator and a series of dilutions were made. *S. mutans* C180-2 was plated on BHI agar plates and the polymicrobial bacteria were plated on Tryptic Soy Agar Blood plates for total counts. Plates were incubated for 48 h at 37°C under anaerobic conditions (10% CO₂, 10% H₂ and 80% N₂).

**Testing for reproducibility**

In a series of experiments biofilms were made with either fresh (collected on 4 different days) or frozen saliva (in 7 independent experiments) as inoculum. The outcome parameters were acid production and total CFU. Also biofilms that were produced with *S. mutans* C180-2 (in 10 independent experiments) were analysed to quantify the variation between experiments.

**Testing of anti-microbials**

Biofilms were treated with a range of concentrations of an amine fluoride (AmF, Olafur, GABA, Switzerland) using the following procedure: At the end of the biofilm growth period the lid was transferred to a new plate containing 1.5 ml/well of the test solutions and incubated for 5 minutes at room temperature. After treatment the lid was transferred to a new plate containing CPW and rinsed by moving the lid up and down. The lid fits precisely on the 24-well plate, therefore movement is restricted to the vertical direction. The lid was moved upwards until the glass discs were above the CPW solution and then moved back into solution 10 times. This procedure was repeated three times with fresh CPW to ensure removal of excess treatment solution.

The group size for each experiment was 4, allowing for 6 experimental groups to be tested within one lid (24 positions in total). In each lid a water-treated control group was included. Each experiment was repeated at least in triplicate.
**Statistics**

CFU counts to determine the reproducibility of the biofilms were compared using one-way ANOVA with Games-Howell post hoc testing (SPSS release 16.0). CFU-counts and lactic acid production data from experiments with anti-microbials were recalculated to compensate for inter-experimental variation: The data from a single 24-well plate were expressed as the percentage of the averaged values of the water treated controls in that plate. Experimental groups were compared using one-way ANOVA with Games-Howell post hoc testing (SPSS release 16.0).

**Results**

When overnight cultures of *S. mutans* C180-2 were used to inoculate the model the overall average CFU counts of 24 h biofilms were $1.5 \times 10^9$ (fig. 2). This figure also shows the inter-experiment variation in CFU counts. Saliva induced polymicrobial biofilms (48 h) showed slightly lower CFU counts than the *S. mutans* biofilms. Inoculating the model with frozen saliva resulted in somewhat lower CFU counts compared to inoculation with fresh saliva. This difference was not statistically significant. Based on these data it was decided to use one batch of frozen saliva as inoculum for all experiments.

Despite the buffered media, the pH dropped during sucrose metabolism, but not below pH 5.7 for the *S. mutans* biofilms and not below 6.5 for the polymicrobial biofilms.

When short term biofilms (*S. mutans*, 24 h and saliva, 48 h) were treated with AmF both types of biofilms showed a decrease in relative CFU counts with increasing AmF treatment concentration (fig. 3). The absolute CFU counts for the water treated controls were $1.3 \times 10^9$ and $5.3 \times 10^8$ for *S. mutans* and polymicrobial biofilms, respectively.
Comparison of total CFU counts from polymicrobial (48 h) and S. mutans C180-2 biofilms (24 h). Each bar represents the data from a single experiment (n=4). The different bars represent the different experiments. The last bar of each group of bars depicts the overall average. Saliva was collected on 4 different days and used immediately to produce biofilms (Fresh, n=4). The remaining saliva was stored at -80 °C and thawed later to produce polymicrobial biofilms (Frozen, n=4). Data are compared with biofilms made from fresh overnight cultures of S. mutans C180-2 (n=4).

The S. mutans biofilms showed a steeper dose-response decrease when compared with the polymicrobial biofilms. The differences in relative CFU counts between the S. mutans and polymicrobial biofilms were statistically significant at all AmF concentrations tested: When treated with 1000 ppm AmF the polymicrobial biofilms showed 52% survival compared to 3% survival for the S. mutans biofilms.

Considering its application in caries prevention also the effects of AmF on acid production were tested. Lactic acid formation for the water treated controls was 13.5 mmol/l and 10.7 mmol/l for S. mutans and polymicrobial biofilms, respectively. As for CFU counts the lactic acid formation data showed a dose response for both types of biofilm: with increasing AmF concentration the lactic acid production was reduced (fig. 4). Unlike the CFU counts no differences were observed between the two biofilm types at each of the tested AmF concentrations (p>0.05).
Figure 3.
Effects of a single treatment with AmF on the CFU counts of S. mutans C180-2 (24 h, ▲) and polymicrobial biofilms (48 h, ■). CFU counts are expressed as percentage of the respective water-treated control. Concentration of AmF is expressed as mg/l in the treatment solution. S. mutans C180-2 biofilms and polymicrobial biofilms were significantly different at each concentration of AmF.

Figure 4.
Effects of a single treatment with AmF on the lactic acid production by S. mutans C180-2 (24 h, ▲) and polymicrobial biofilms (48 h, ■). Lactic acid production is expressed as percentage of the respective water treated control. Concentration of AmF are expressed as mg/l in the treatment solution. Differences between S. mutans C180-2 and polymicrobial biofilms were not significant at any concentration. When comparing the treatment concentrations all differences reached statistical significance except for the 0 vs 10 ppm AmF and the 10 vs 50 ppm AmF.
In subsequent experiments “older” biofilms were studied and treated with AmF solutions. In these biofilms the CFU counts for *S. mutans* biofilms increased marginally from $1.5 \times 10^9$ to $1.9 \times 10^9$ and $2.9 \times 10^9$ for 24, 48 and 72 h biofilm formation, respectively. The total CFU counts for the polymicrobial biofilms were $5.3 \times 10^8$, $5.2 \times 10^8$ and $7.8 \times 10^8$ for 48, 72 and 96 h biofilms, respectively. The corresponding lactic acid production values were 14.2, 12.0 and 15.9 mmol/l for the *S. mutans* biofilms and 10.7, 11.4 and 8.9 mmol/l for the polymicrobial biofilms.

In Figure 5 the normalized CFU counts for biofilms of increasing age are shown as a function of AmF treatment concentration. Generally significant differences were found between young and old biofilms (p<0.05), with older biofilms being more resistant to AmF at all concentrations tested. For both types of biofilms no statistically significant increase could be observed between the data points representing the older biofilms (72 h vs 48 h for *S. mutans* and 96 h vs 72 h for polymicrobial biofilms).

![Figure 5](image)

*Figure 5.* Effects of a single treatment with AmF on the CFU counts of *S. mutans C180-2* (closed symbols) and polymicrobial (open symbols) biofilms of different age (◆ ◆=24 h / 48 h; ■ ■=48 h / 72 h; ▲ ▲=72 h / 96 h). CFU counts are expressed as percentage of the respective water-treated control. Concentration of AmF is expressed as mg/l in the treatment solution. Data points at the same concentration labeled with the same character are not statistically different.
The lactic acid production (fig. 6) shows a similar increase in resistance against AmF treatment with increasing age of *S. mutans* biofilms, at all AmF concentrations tested. This is also observed for the polymicrobial biofilms, except for the 1000 ppm AmF treatment. At this concentration the differences between the oldest and youngest biofilm did not reach statistical significance (p>0.05).

*Figure 6.* Effects of a single treatment with AmF on the lactic acid production by *S. mutans* C180-2 (closed symbols) and polymicrobial (open symbols) biofilms of different age (◆ ◇=24 h / 48 h; □ ▲=48 h / 72 h; ▲ ▲=72 h/ 96 h). Lactic acid production is expressed as percentage of the respective water-treated control. Concentration of AmF is expressed as mg/l in the treatment solution. Data points at the same concentration labeled with the same character are not statistically different.
Discussion

We developed a novel HTP biofilm model to form either single species biofilms, with \textit{S. mutans} C180-2, or more complex biofilms after inoculation with saliva. This model with either biofilm type was first tested for reproducibility and then evaluated regarding applicability in studying the efficacy of AmF on biofilms.

From the collective data (e.g. fig. 2) we concluded that reproducible biofilms could be produced with the developed hardware and the chosen experimental design. The coefficients of variation were 15\% and 36\% for intra- and inter-experimental variation, respectively. Normalizing against water control groups proved a reliable method to improve the discriminative power between experiments. For polymicrobial biofilms both fresh and frozen saliva were used. Using frozen saliva, rather than working with separate saliva batches, presumably adds to the compositional reproducibility between experiments, in particular when these are carried out over an extended period. Biofilms formed with frozen saliva resulted in a 19\% reduction of CFU counts after 24 h (fig. 2). The lactic acid data provides additional information showing different responses to AmF compared to the CFU viability data and therefore of obvious relevance when compounds are tested for caries preventive potential.

With this model, biofilm formation could be extended for periods substantially beyond 24 h. In these cases CFU counts increased only marginally, while the overall biomass did (by visual inspection). Studying older plaque biofilms is of clinical relevance as these occur at stagnation sites prone to caries development. Preventive compounds should affect both growth and metabolism of such biofilms. It is interesting to note that CFU counts and lactic acid formation plateau, both at very similar values, irrespective biofilm age and biofilm type. Presumably the compositions will show considerable differences, e.g. in \textit{S. mutans} levels. The level of acid formed in polymicrobial biofilms being very similar to the \textit{S. mutans} biofilms, hints at either large numbers of other
acid forming bacteria in polymicrobial biofilms (Beighton, 2005), or that not all acid forming capacity is expressed in the S. mutans biofilms. This could also explain why, in the experiments with AmF discussed below, S. mutans biofilms that had experience almost 2-log killing still produced substantial amounts of lactic acid (fig. 3 and 4).

As indicated, drawbacks of existing models are that these are generally based on bacterial sedimentation rather than attachment, and that treatments can typically not be given in a clinically realistic way (e.g. with respect to duration, clearance). Our model avoids most of these disadvantages: exposure time and concentration of the compound can be controlled easily and by attaching the substrates to a custom designed lid initial biofilm formation is based upon active attachment to the substrate used. In the current study glass slides were used as substratum, but these can easily be replaced by hydroxyapatite, enamel or dentin discs (see fig. 1). An additional advantage is that all specimens from the same lid undergo exactly the same procedures throughout the biofilm growth and treatment procedures, which guarantees reproducible experiments (fig. 2). Our model also lacks some of the features that the more complex flow-through models do have. For example, the option to pulse nutrients and thereby influence the growth rate of the biofilms is lacking. In principle this could be added, but the model would then no longer be very versatile, and neither a HTP model. We note that our model does not necessarily replace existing complex models. It is designed as initial screening device for potentially effective compounds, which could then be studied in the more advanced models.

The experiments dealing with 5 min AmF treatments on both S. mutans and polymicrobial biofilms, showed that biofilm type, biofilm age and treatment concentrations are all parameters that determine the reduction in both CFU counts and acid formation capacity, and also that there are differences in numerical values between the latter two. The small difference in pH of the biofilms after biofilm growth is not thought to influence the AmF efficacy.
For the treatment biofilms were submerged in 1.5 ml AmF solution and were brought to the same pH by this procedure.

We observed that AmF affects the viability of young polymicrobial biofilms significantly less than that of young *S. mutans* biofilms (fig. 3), indicating a higher resistance in the complex biofilms. Our group and others have reported that polymicrobial interactions in biofilms induce resistance to antimicrobials (Kara et al., 2006; Burmølle et al., 2006; Luppens et al., 2008). The reduced sensitivity towards AmF of polymicrobial biofilms, in terms of CFU counts, was not reflected to the same extent in lactic acid production. In this case an almost parallel dose response relationship for inhibition of acid formation was observed in the *S. mutans* and polymicrobial biofilms (fig. 4). This finding indicates that the acid forming capacity of both types of biofilm is affected similarly by the AmF treatment, in spite of the observed difference in AmF induced killing. This observation requires additional experimentation to be fully understood.

Both types of biofilms became more resistant to AmF with age. When viability of older biofilms was tested (fig. 5) it was shown that increasing age of the biofilm resulted in higher survival percentages for both types of biofilm. A similar observation has been reported for other oral pathogenic bacteria (Walker and Sedlacek, 2007). The age induced increased resistance in polymicrobial biofilms was less pronounced in inhibition of metabolic activity (fig. 6).

An explanation for the higher resistance could not be found in an increased number of viable cells with increasing biofilm age. However, it was visually observed that biofilms became thicker with increasing age. As this increased biomass is not reflected in increased CFU counts, it is probably accounted for by the extracellular matrix and dead cells. Biofilm resilience to anti-biotics and anti-microbial treatments has been attributed to a number of factors, including slow diffusion of compounds through the biofilm, their binding to the EPS matrix (Chambless et al., 2006), and slower bacterial growth rates in
biofilms (Gilbert et al., 1997). These hypotheses would also explain our observed differences between young and old biofilms.

The AmF concentrations tested comprises the range that is relevant for oral care products. Typically a toothpaste contains up to 1500 ppm fluoride and a mouth rinse up to 250-400 ppm fluoride. In the 200-500 ppm concentration range a 40-60% reduction in acid production was observed. In our set up this was found after a single short treatment followed by extensive rinsing. In vivo clearance of AmF is probably slower so that our data may be an underestimation of efficacy.

In summary, we conclude that our new biofilm model allows for rapid screening of potential anti-microbial compounds. The model is sensitive enough to determine dose response relations and offers multiple options to study biofilm parameters and processes in a relatively short time. The remarkable AmF effect, i.e. a marginal effect on the viability of polymicrobial biofilms, but a clear inhibitory effect on metabolic activity clearly shows that when evaluating the efficacy of caries preventive compounds it is essential to use appropriate polymicrobial biofilm models. Biofilms grown from single species may be underestimating the complexity of dental plaque biofilms and may result in misleading conclusions. Moreover, the efficacy of caries preventive agents needs to be judged on cell viability effects but probably equally important also on the reduction of acid formation.

Declaration of interests

The study was financially supported by GABA International (Therwil, Switzerland). The tested amine fluoride (Olaflur) was supplied by GABA. There were no restrictions with respect to publication of the results.
References


