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REVIEW ARTICLE

The use of *in vitro* model systems to study dental biofilms associated with caries: a short review

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A dental biofilm forms a distinct environment where microorganisms live in a matrix of extracellular polysaccharides. The biofilm favors certain bacteria and creates a habitat that functions differently compared to planktonic bacteria. Reproducible model systems which help to address various questions related to biofilm formation, the process of caries development, and its prevention are needed and are continuously developed. Recent research using both batch culture, continuous culture and flow cells in caries biofilm formation is presented. The development of new techniques and equipment has led to a deeper understanding of how caries biofilms function. Biofilm models have also been used in the development of materials inhibiting secondary caries. This short review summarizes available models to study these questions.

Keywords: *dental caries*; *batch culture*; *continuous culture*; *artificial mouth*; *flow cell*; *microcosm*

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The principle of dental caries may appear simple; however, when looking at the pathogenesis of caries, it all becomes much more complicated (1). It is a multifactorial disease with complex underlying biological processes. Caries is caused by low pH for a prolonged period of time within plaque, leading the enamel to dissolve (2). A simplified explanation of the clinical causes for caries includes: 1) the presence of plaque containing either an excessive amount of bacteria and/or an abundance of acid producing bacteria, 2) consumption of easily-fermentable carbohydrates on a frequent basis (e.g. sugar), 3) a low saliva production, or a decreased capacity of the saliva to act as a buffer, and 4) a genetic make-up making the host more susceptible to caries (2). These factors also represent various opportunities for the prevention of caries.

Dental plaque is a natural biofilm that consists of different bacterial species and extracellular matrix with soluble and insoluble glucans. It is affected by numerous external factors such as diet, saliva composition, and salivary flow rate (3). The resident oral microbiota has an intrinsic capability to protect the host against invading microbes and to contribute to the development of the host's defense mechanisms (3). More than 700 bacterial species have been identified within plaque samples, and around 40 species have been connected to caries alone (2, 4). The composition of bacterial species within plaque varies between individuals, sites within the oral cavity, diet, behavior, and other factors (4). In a biofilm different bacterial species exist in close proximity to each other. They live either in symbiosis or in competition with each other, and they communicate by quorum sensing (5). The structure of the biofilm is highly organized. There is a clear hierarchy, with different organisms occupying specific positions and having distinct roles within the biofilm (5). The behavior of the bacteria within a biofilm differs in comparison to bacteria under planktonic conditions; for example, metabolism is different and their susceptibility to the host's defenses and antimicrobials is diminished (3, 5). Nyvad et al. (6) have suggested that the biofilm can be considered a single unit rather than a collection of individual bacterial species. Further studies should address how the biofilm functions as a whole.

Caries models, in general, are commonly used to help us understand complex processes and the factors affecting them. They help us to accurately predict, in a controlled and simplified way, a clinical outcome which can lead us to preventive actions for a disease (1). When using a model, it is important to consider the research question in order to carefully evaluate which model type should be used so that results are interpreted correctly. The complexity of biofilm research requires different approaches to address various questions. First, the development of *in vitro* models should be based on prior knowledge of

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the *in vivo* situation. Then, as our understanding of the oral cavity progresses, model systems can be improved. There are many interactions and processes between bacteria in the biofilm which can vary depending on which bacteria are present and prevalent external conditions – factors which may complicate the interpretation of findings (4). Even though a model cannot capture all of the details involved with caries formation, it can give us a means of performing reproducible experiments under controlled conditions. Obviously there are ethical limitations with *in vivo* studies in relation to caries and periodontal diseases. Therefore, different *in vitro* techniques have been developed and are continuously improved to better address the study question, to help interpret the results and to obtain as much information as possible with other than clinical testing (4).

This short review presents an overview of the most common *in vitro* models used to study dental caries. It also highlights some of the results from their use.

Bacterial biofilm model systems for caries

Bacterial biofilm caries models can roughly be divided into two groups: closed batch culture and open continuous culture models (Table 1). Continuous methods can be further divided into artificial mouth models (AMM) and flow cells. Batch and continuous culture methods are used to grow a monoculture biofilm, a defined consortium biofilm (from two up to ten species) or a microcosm biofilm (using saliva or plaque sample as inocula). The different bacterial biofilm models are used to study the origins of caries, caries prevention, how cariogenicity changes with different bacteria and how diet or other compounds and materials affect cariogenicity (7). Biofilm models can be difficult to compare due to the differences in biofilm formation times, different growth media, and varying bacterial species used in different situations.

Batch biofilm models

With batch biofilm models, a biofilm is formed either on a plate wall, on the surface of discs, coupons or pegs or on human or bovine enamel within the well. A closed system is used so that the environment inside the well changes during the test as nutrients are consumed and metabolic products accumulate unless the growth media are replaced (8). The frequency of the growth media changes depends on the model set up. Unlike the oral cavity, there is no flow of fluids and nutrients with these models, although some models do create a liquid shear force by dipping the biofilms in saline or other liquid during biofilm formation (9). However, batch models do offer means of comparing multiple test compounds or conditions simultaneously; they only require small amounts of reagents and are convenient, reproducible, and economical to use (8).

One of the most commonly used batch biofilm models is the Zürich biofilm model which uses six microbial species (*Streptococcus oralis*, *Streptococcus sobrinus*, *Actinomyces naeslundii*, *Veillonella dispar*, *Fusobacterium nucleatum*, and *Candida albicans*) (9). Using fluorescently labeled antibodies and confocal laser scanning microscopy (CLSM), this model allows the interspecies associations to be studied with respect to biofilm formation and how macromolecules of different sizes can penetrate the biofilm *in vitro* (9). This model and its variants have been used extensively to evaluate the effect of different substances in the biofilm formation process [e.g. plant extracts (10), chlorhexidine (11), and xylitol (12)]. Furthermore, the model has been used to study the effect

Table 1. The main differences between batch biofilm model, artificial mouth model (AMM), and flow cell biofilm models

		Continuous	
	Batch	AMM	Flow cell
Advantages	Multiple compounds tested simultaneously	Flow conditions	Flow conditions
	Multiple conditions tested simultaneously	Conditions closely mimicking *in vivo* situation	Possibility to analyze biofilm formation real-time
	Small volumes of reagents	Product and nutrient concentration stable during biofilm formation	Intermediate complex equipment
	Easy to perform	Perfect mixing	
	Simple equipment		
Disadvantages	Closed system	Requires larger volumes of reagents	Conditions vary at different sites in the reactor
	No flow	Only one condition/run can be tested	Only one condition/run can be tested
		Complex equipment	Usually less replicates
		More expensive	
		Usually less replicates	

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of oral probiotics on a growing biofilm (13), as well as de- and remineralization (9, 14) in a biofilm with variable formation times. It has also been used for developing methods to analyze biofilm microbes (15). For example, Marttinen et al. (16) used a variation of this model incorporating three species of bacteria (*Streptococcus mutans*, *Streptococcus sanguinis*, and *A. naeslundii*) to study the effect of xylitol in a young biofilm. They observed that 5% (w/v) xylitol diminished the *S. mutans* counts in a young (8 h) biofilm, while total bacterial counts were unchanged, indicating a shift in the composition of the biofilm through a small change in the environment. Another modification of this model is the three-species version (*S. mutans*, *S. oralis*, and *A. naeslundii*) developed to mimic ecological changes with respect to cariogenic biofilm formation and to investigate the relationship between *S. mutans* and exopolysaccharides (17). The biofilm was grown in tryptone yeast broth in the presence of glucose within 24-well plates, and sucrose was added after 29 h to create a cariogenic challenge. In the multispecies biofilm, the addition of sucrose changed the proportion of the bacterial species favoring *S. mutans*. This also resulted in an increased biofilm mass due to augmented exopolysaccharide production (17). Klein et al. (18) further showed that *S. mutans* adapts to the multispecies environment by changing the expression of the genes associated with glucan synthesis, remodeling, and glucan-binding. In this way, *S. mutans* out-competes other bacteria by optimizing its metabolism to a sucrose environment, thus increasing its competitiveness and thereby its virulence. A fluorescent pH indicator dye was used to determine the pH changes within the biofilm, and the results indicated that the exopolysaccharide matrix helps to create low pH niches in the biofilm which favor the acid-tolerant *S. mutans* (19).

The Calgary Biofilm Device (a 96-well plate system using lids with 96 pegs for biofilm formation) was developed in 1999 (20). This model allows rapid testing for antibiotic susceptibility in a biofilm model, with or without agitation. As biofilm growth differs in comparison to planktonic growth, it was important to develop a means of testing susceptibility of the bacteria within biofilm to antimicrobials. In a Calgary device, inhibitory concentrations can be analyzed by comparing the positive control to the lowest concentration of the antimicrobial with minimum 10% of difference in OD_{650nm}. In addition, biofilms can be visualized using scanning electronic microscopy (SEM) or CLSM (5). The method has been used extensively to determine the Minimal Biofilm Inhibitory Concentration, Minimal Biofilm Eradication Concentration and Biofilm Bactericidal Concentration for various antibiotics and antimicrobials, but mainly in non-caries related biofilm studies (5, 20).

Many batch biofilm models have a constant exposure to sucrose during biofilm growth; however, this is usually not the case in the oral environment. To address this discrepancy, Ccahuana-Vasques and Cury (21) modified the *S. mutans* batch biofilm model. Originally designed to test short exposure of antiplaque agents on bovine enamel demineralization (22), it was modified to test the cariogenic challenge of sucrose exposure eight times a day. To validate the model, the effect of different concentrations of chlorhexidine and 0.05% NaF was tested on biofilm formation and demineralization two times a day. A dose–response effect of chlorhexidine on the *S. mutans* biofilm was demonstrated. This was also shown in clinical trials indicating the sensitivity of the model to detect changes in biofilm formation and enamel demineralization (21). The same model has more recently been used to evaluate anticariogenic properties of an apple concentrate, where a decrease in enamel demineralization and extracellular polysaccharide production was seen (23). As antibiofilm compounds are tested against a mature biofilm in this model, it cannot be used to evaluate the effect of bacterial adhesion properties; instead the model focuses on intermittent exposure to sucrose and a test substance. A similar approach was used by Steiner-Oliveira et al. (24) to study caries formation in human dentin. The *S. mutans* monospecies biofilm model was applied with artificial saliva as a growth medium with periodical exposures to sucrose. In accordance with *in vivo* studies, the model demonstrated that sucrose increased lesion development, but as the model had no saliva clearance it was not able to achieve a proper remineralization between sucrose exposures.

A microcosm batch biofilm was grown in a polystyrene based coverslip with different media, and it was evaluated using Checkerboard DNA–DNA hybridization analysis (25). This model was used to assess responses to environmental factors such as changes in growth media, growth volume, and sucrose addition. It showed a behavior similar to an *in vivo* biofilm, and the model was able to illustrate individual responses to environmental changes. van de Sande et al. (26) developed a microcosm batch biofilm model for estimating demineralization using bovine enamel discs, saliva analogue growth media, and periodical sucrose exposures. The model was used to compare mother–child pairs and their susceptibility to a regular sucrose exposure. It was seen that under sucrose exposure biofilms showed similar microbiological changes and mineral loss regardless of the individuals, thus suggesting that diet and behavioral factors can be more important causes of caries development than transmission of microbes (27). New and improved detection methods using the Quantitative Light-induced Fluorescence-Digital illuminator™ (QLF-D) can quantify biofilm bacteria and the red fluorescence observed by QLF-D was shown to correlate with cariogenicity of the biofilm in a microcosm model (28, 29). It was therefore effective in monitoring biofilm maturation, and the results from this *in vitro*

method suggest that QLF-D could be used to monitor cariogenic biofilm maturation also in clinical practice.

Continuous biofilm models

The term *artificial mouth model* (AMM) is usually used to describe dental biofilm systems with a continuous, open-surface fluid flow rather than flow cells with closed flow (30). The AMM provides intermittent or continuous flow of nutrients over the biofilm, mimicking the *in vivo* situation as closely as possible (31). An AMM simulates oral conditions in terms of temperature, humidity, sucrose supply, pH, and nutrient (i.e. saliva) flow rate, but still there are differences between different AMMs in biofilm formation time, nutrient media, and equipment used. As the equipment is more complex than in batch systems, AMMs usually have less replicates, but instead they offer a means to investigate the mechanism of action of microbes and the compounds being tested as well as the overall growth and structure of plaque. This is due to the controlled environment that more closely mimics the oral cavity *in vivo* (32). Tang et al. (31) provide a review of the history, development, and structure of the AMM.

A defined multispecies biofilm AMM allows for a more detailed and easier analysis of bacteria present in comparison to a microcosm AMM. An AMM with four-species (*S. mutans*, *S. sobrinus*, *A. naeslundii*, and *Lactobacillus rhamnosus*) has been used to study enamel and root caries and to compare single and multispecies models (30). Consortia biofilms were usually larger than monospecies biofilms, and they also tended to cause more enamel softening. The addition of sucrose to the consortia biofilm created a similar pH curve as that found *in vivo*. A defined multispecies AMM with a different set of bacterial species (*S. mutans*, *S. sobrinus*, *Lactobacillus acidophilus*, *L. rhamnosus*, and *A. naeslundii*) was used to study the mechanism of action of silver diamine fluoride on the biofilm. It was found that it inhibits biofilm formation, and it also reduces demineralization (32). The arrangement of bacteria within the biofilm was determined by CLSM. Lactobacilli mostly inhabit the upper parts of the biofilm, while mutans streptococci are found in the lower layers. Results suggest that high concentrations of silver and fluoride ions inhibit biofilm development. A slightly modified AMM using a three-species (*S. mutans*, *S. sobrinus*, and *Streptococcus gordonii*) system was developed to evaluate the formation of secondary caries around restorations and to assess the effectiveness of bonding material (33). For the formation of secondary caries, a biofilm was first grown in a continuous flow reactor for 20 h on a saliva-coated specimen and subsequently incubated in a batch system for 7–30 days. The model produced caries lesions around composite resin restorations and the protective effect of the bonding system was verified.

Forssten et al. (34) present a dental caries simulator consisting of a continuous flow system with standardized artificial saliva flow (35). The temperature is controlled, and the artificial saliva is continuously mixed. Hydroxy apatite (HA) discs are used as a model tooth and as an adhesive support for the bacteria (Fig. 1). The system can be inoculated with single or multiple bacterial species and test substances can be added either continuously or in pulses during simulation. The system has 16 replicate vessels which enable parallel testing of multiple conditions (34). With this model, it is possible to monitor the initial steps of bacterial adherence to the HA-discs and the subsequent biofilm formation. It can then be used to study the effects of various substances such as polyols on bacterial quantities and adherence.

Of all the *in vitro* models mentioned, microcosm AMM comes closest to replicating *in vivo* conditions in the oral cavity. However, as the complexity of bacteria increases also the interpretation of the results becomes more complicated. The advances in the methods used for analyzing the biofilm and its components have led to a deeper understanding of the biofilm formation process and the factors connected to it. The microcosm AMM is a valuable tool to for studying the function and structure of dental biofilm. The focus with microcosm AMM studies was initially on biofilm growth, metabolism (pH changes, the effect of sucrose, and growth media), and de- and remineralization processes (36–38). A further variation of the AMM is the microcosm constant depth film fermentor which has been used to study the effect of chlorhexidine and tetracycline on the microbiota composition in biofilm (39–41). The structure and viability of the biofilm were found to be similar *in vivo* as judged by CLSM (42, 43). Thirty-six bacterial species were also identified in the supragingival biofilm using a combination of culture and molecular methods (PCR) (44). The method used to identify different microorganisms in a microcosm biofilm developed; that is, denaturing gradient gel electrophoresis (DGGE) allowed the individual variations and changes of the bacterial populations to be captured during the growth of the biofilm (45). More recent methods of detecting bacteria present in biofilm, such as qPCR and Human Oral Microbial Identification Microarray (HOMIM), have enabled more accurate analysis of the bacterial population composition (46, 47). In addition, newly developed methods such as cross-polarization optical coherence tomography (CP-OCT) enable the evaluation of the early stages of caries formation (48). CP-OCT allows visualization of the biofilm without disturbing it. With this method, the sample is kept hydrated, and images are taken within minutes as the sample is removed from the biofilm reactor. Lately, microcosm biofilm models have increasingly been used for studying the possibilities of different restoration materials (e.g. dimethylaminododecyl methacrylate and

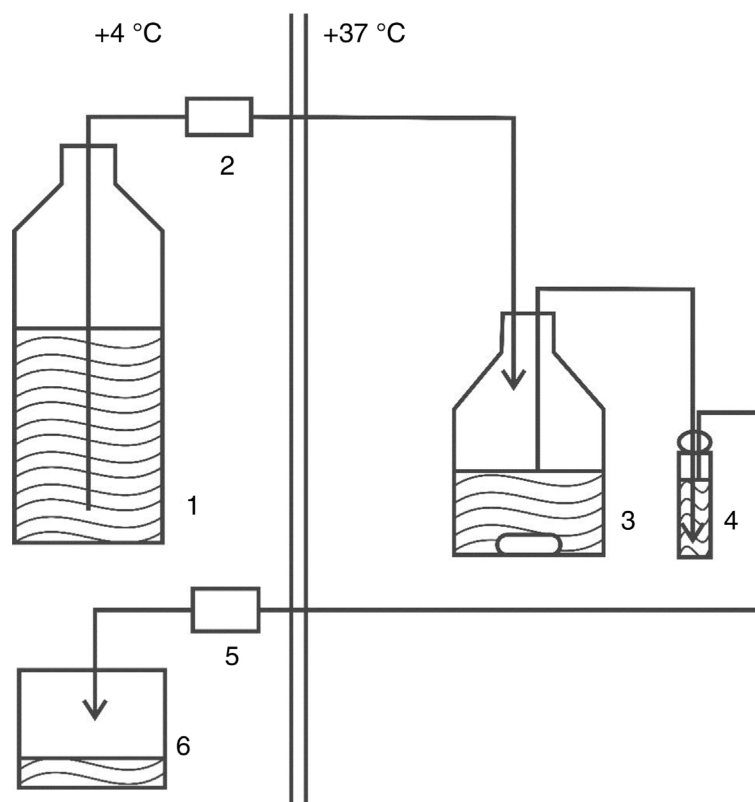

Fig. 1. Schematic diagram of the dental simulator (after Forssten et al., 2010). 1. Reservoir for artificial saliva/artificial saliva with test substances. 2. Inlet Pump. 3. Simulation vessel with constant stirring and added bacteria. 4. Sample collection during the simulation. 5. Outlet pump. 6. Waste.

nanoparticles of silver or calcium phosphate) to inhibit the formation of secondary caries (49–51). Publications describing the use of the microcosm AMM with next-generation sequencing have not been published yet, but this new technology will help to more accurately capture changes in the microbiota.

Flow cell biofilm models

In flow cells, the liquid phase moves only in one direction and mixing happens by diffusion; therefore, conditions vary at different sites within the reactor (8). Flow cells are especially useful for studying the development of biofilm formation and morphology. Sequential colonization can be observed in real-time using microscopic analyses of undisturbed biofilms (8). Hannig et al. (52) and Pamp et al. (53) provide reviews of various staining and visualization techniques that can be used with flow cell biofilms.

A four-species (*S. gordonii*, *A. naeslundii*, *Veillonella atypica*, and *F. nucleatum)* flow cell biofilm model was used to evaluate the mechanism of early biofilm formation. Biofilms were analyzed using fluorescent stains and fluorescent *in situ* hydridization (FISH) probes visualized by CLSM (54). It was found that species inoculated sequentially had more biomass than coaggregate-inoculated biofilms and *S. gordonii* was a major component of the formed biofilm.

Schlafer et al. (55) presented a variation of the flow cell biofilm model which focuses on changes in the early caries process when only mildly acidogenic bacteria are present. This five-species (*S. oralis*, *S. sanguinis*, *S. mitis*, *Streptococcus downei*, and *A. naeslundii*) flow cell biofilm model (26 h-old biofilm) is highly reproducible and shows structural similarity to *in vivo* biofilms. The structure and composition of the biofilms were analyzed using FISH with CLSM. In addition, the model also uses pH-sensitive ratiometric fluorescent dyes to evaluate pH-levels at the biofilm-substratum interface. The model can be useful for testing substances that affect early stages of caries development, and it has been used to evaluate the influence of osteopontin on biofilm formation (56). Osteopontin clearly decreased biofilm formation, but did not disrupt biofilms that had already formed. FISH analysis further indicated that osteopontin decreased *S. mitis* while the proportion of other bacteria increased.

Blanc et al. (4) developed a six-species (*S. oralis*, *A. naeslundii*, *Veillonella parvula*, *F. nucleatum*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*) biofilm model for evaluating biofilm development under flow and shear conditions that can be used to assess, for example, antimicrobial substances. Bacteria were first grown in a Lambda Minifor bioreactor, the bacterial suspension was transferred to a modified Robbins device

with HA-discs precoated with saliva and biofilm was formed in 3–9 days. SEM and CLSM were used to study the composition of the biofilm during formation of the biofilm, and the amount of bacteria was determined by culturing. The model indicated that chlorhexidine in combination with cetylpyridinium chloride is more effective in killing bacteria in the biofilm than chlorhexidine alone or in combination with NaF.

Conclusions

Dental caries is a common disease that affects almost all people at some stage of their life. Subsequent to caries being diagnosed, there are ways to minimize the damage caused. Different biofilm models display a practical and ethical way of exploring new opportunities to investigate and combat dental caries. The development of various biofilm models has increased the understanding of the biofilm formation process and the factors affecting formation and structure of a biofilm. The models are being used to develop new ways of influencing pH-levels in the oral cavity, to improve the remineralization of the enamel, to inhibit the growth of pathogenic bacteria by antimicrobials (e.g. chlorhexidine, sodium hypochloride) and to affect the metabolism of bacteria (e.g. by xylitol) so that they become less harmful (2). The research question should drive the choice of the model that is used. The main differences between the different types of models are presented in Table 1. Also, in the future biofilm models will be used to develop new restoration materials and to minimize possibilities for secondary caries to develop.

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The authors are employed by DuPont; DuPont manufactures and markets xylitol for oral health applications.

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