Oral biofilms
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CHAPTER 1

GENERAL INTRODUCTION

AND

AIMS OF THIS THESIS
History of Oral Health

Evidence for the existence of dental caries and chronic periodontal disease has been found already in Paleolithic material, dating these diseases back to as early as prehistoric times. Although scientific evidence for a relationship between oral hygiene and oral health was still thousands of years away, personal oral hygiene has already been practiced since ancient history. Early tooth cleaning devices were, for instance, toothpicks - used by Greeks and Romans, and probably before them by Babylonians and Chinese - and chew sticks, already mentioned in India around AD 120 – 162 and promoted in Islam by Muhammed (Fischman, 1997; Carranza and Shklar, 2003).

The development of the chewing stick into the toothbrush dates back to AD 1000 in China. After the introduction in China, toothbrushes were reinvented in the western part of the world in the late 18th and early 19th centuries and nowadays life without a toothbrush is beyond imagination. Yet, the toothbrush is inadequate for maintaining oral health and also in ancient times additional means of chemical oral health care support were introduced next to mechanical cleansing. Genesis, the first book of the Bible, already mentions the use of labdanum (mastic), a resin that has been used in Mediterranean countries for breath freshening for thousands of years. The word mastic derives either from the Greek verb mastichein, which means "to gnash the teeth" or massein, which means "to chew", thus one could say that mastic could be seen as the first and original chewing gum. The use of mouth rinses is mentioned for the first time in Chinese medicine around 2700 BC, as a treatment of diseases of the gums. The recommendation was to rinse with the urine of a child. The Roman natural philosopher and army commander of the early Roman Empire, Pliny, simply recommended salt water rinsing. The founding father of medicine, Hippocrates, advocated a mixture of salt, alum and vinegar (Fischman, 1997).
Although oral hygiene has been practiced and advocated for centuries and mechanical as well as chemical cleaning was common, the mechanisms of their effectiveness were not known. Actually the reason of cleaning the teeth was not always to preserve oral health, but often merely for cosmetic or religious purposes (Fischman, 1997). It was only since 1676 that Antonie van Leeuwenhoek discovered the existence of “animalcules” in dental plaque, later to be known as bacteria, that research started to recognize bacteria as the cause of oral health problems (Porter, 1976). Still, it took until 1965 before it was proven that the bacteria in dental plaque or biofilm were the cause of gingivitis. In 1965 Löe and coworkers demonstrated in their landmark experimental gingivitis study, that persons with a clinically healthy gingiva developed clinical symptoms of gingivitis within 2 to 3 weeks when dental plaque was allowed to accumulate undisturbed. Once adequate oral hygiene was resumed, the gingival inflammation subsided within a week (Löe et al., 1965).

Concerning adequate oral hygiene, there is strong evidence that tooth brushing reduces gingivitis (Lang et al., 1973).

Although the consequences of dental plaque formation are well known, adequate daily oral hygiene is still difficult to obtain for many if not most people. Nearly no-one grows old without suffering at least once from caries or periodontal disease. Caries and periodontal diseases each have their own cause related microorganisms out of the thousands of different bacterial species that inhabit the human oral cavity (Kolenbrander et al., 2006; Ten Cate, 2006; Keijser et al., 2008).

Maintaining proper oral hygiene can be further hampered in patients with fixed orthodontic appliances. Orthodontic treatment is becoming increasingly popular, while only two decades ago it was exclusively for juveniles, adult orthodontic treatment is now common practice. There are more than four million juvenile and one
million adult patients in North-America alone reported by the American Association of Orthodontists. The downside of orthodontic treatment is that the region of the tooth surface around the brackets is prone to adhesion of oral bacteria and subsequent biofilm formation, which is difficult to control in retention sites, such as around orthodontic brackets. Adequate oral health care in orthodontic patients is one of the new challenges in the field. Also in patients with low dexterity or physical handicaps, proper oral hygiene can be difficult to maintain.

**Oral Biofilm - Plaque Formation**

The development of a biofilm can be sketched in a few basic phases as depicted schematically in Figure 1. The first phase in biofilm formation in the oral cavity is the forming of a salivary conditioning film. In the second phase, individual bacteria adhere to a surface and co-adhesion may take place. However, this is still a reversible adhesion and many bacteria may actually leave the surface back to their planktonic state. When the bacteria start to produce extracellular polymeric substances (EPS), adhesion becomes irreversible. The next phases comprise the maturation of the biofilm from the development of microcolonies and waterchannels to large matrix enclosed structures. The mature biofilm is a supplier of microcolonies or revertant planktonic bacteria (Bos et al., 1999; Stoodley et al., 2002b). The hallmark of bacterial biofilms that differentiate them from bacteria that are simply attached to a substratum is that biofilms contain EPS surrounding the resident bacteria. Microbial EPS are biosynthetic polymers that can be highly diverse in chemical composition and may include polysaccharides, proteins, nucleic acids and phospholipids (Stoodley et al., 2002b). Apart from acting as glue and giving structural support for the biofilm, EPS also acts as an extremely protective slime encasing. Antimicrobials often bind to
Figure 1. Artistic impression of the development of a biofilm. The first phase in biofilm formation in the oral cavity is the formation of a salivary conditioning film by adhering salivary proteins. In the second phase, individual bacteria adhere to a surface and co-adhesion may take place. This is still a reversible adhesion and many bacteria may actually leave the surface back to their planktonic state. When the bacteria start to produce extracellular polymeric substances (EPS), adhesion becomes irreversible. The next phases comprise the maturation of the biofilm, with the hallmark property of bacterial biofilms, the production of a slime layer containing EPS, surrounding the resident bacteria.
or are inactivated by the EPS matrix of the biofilms. As a result, the agents do not reach the deeper layers of the plaque, as was shown for chlorhexidine treatments using confocal laser scanning microscopy (Zaura-Arite et al., 2001) and earlier in the 17th century by Antonie van Leeuwenhoek for vinegar (Carranza and Shklar, 2003).

Oral microorganisms find shelter against swallowing and subsequent death in the gastro-intestinal tract, the host immune system and oral antimicrobials through adhesion to oral hard and soft tissues. Although bacteria never adhere directly to such surfaces, but always to an adsorbed film of salivary proteins. Due to their smaller size, proteins can migrate much faster than microorganisms and cover a surface within seconds after exposure to the oral cavity, after which bacteria start to adhere. Typically, initial colonizers of the protein coated tooth surface are Actinomyces and Streptococcus (Li et al., 2004). In supragingival plaque, Actinomyces species are the dominant part of the microbiota, other prominent species are Streptococcus, Veillonella, Eikenella and Neisseria among others (Ramberg et al., 2003; Li et al., 2004; Kolenbrander et al., 2006).

In a mild form, oral biofilms cause discoloration, or so-called white spot lesions on teeth or around orthodontic brackets, indicative of sub-surface decalcification. White spot lesions occur in approximately 50% of all orthodontic patients (Gorelick et al., 1982) and are reversible provided the use of proper oral health care products and improved brushing techniques (O'Reilly and Featherstone, 1987). Caries and plaque induced gingivitis are more severe effects of oral biofilm and constitute very common diseases. Approximately the complete Dutch population suffers from a form of gingivitis or periodontitis (Kalsbeek et al., 1996). Moreover, the role of dental plaque in the onset of gingivitis, which in turn is regarded as the precursor of periodontitis, has been well studied (Löe et al., 1965). Periodontal
diseases are prevalent in populations around the world (Albandar and Tinoco, 2002). In western Europe, 36% of those aged 35-44 years have moderate periodontitis and approximately 10% have severe periodontitis (Sheiham and Netuveli, 2002). Considering the prevalence of periodontal diseases, Axelsson & Lindhe promoted a strict plaque control regime as a prerequisite for stable and healthy periodontal conditions (Axelsson and Lindhe, 1981). Note that biofilms in the oral cavity are often referred to as “dental plaque”. In this thesis the words “oral biofilm” and “dental plaque” will be used interchangeably.

Mechanical and Chemical Plaque Control

Maintaining oral health can only be reached by effective daily oral hygiene measures. The use of a toothbrush in combination with toothpaste and interdental cleaning devices are the most common way to achieve this. The use of mouthrinses is considered as a beneficial addition, although it may not be regarded as a replacement for mechanical plaque removal (Moran, 2008). Considering the extensive literature available, it is beyond the purpose of this introduction to give a complete overview on the multitude of all available plaque control measures. Therefore, only the main aspects of mechanical and chemical plaque control are pointed out.

The toothbrush is the most used tool to remove dental plaque, although its proper use is not trivial and requires quite some skill. When performed with an adequate technique and duration of time, manual brushing is highly effective. However, for most patients, neither of these criteria is fulfilled. Removal from pits and fissures, interproximal spaces and around orthodontic appliances is seldom or never achieved by manual tooth brushing only, and a number of tools have entered the market to assist plaque removal in places difficult to reach. These tools include dental
floss, toothpicks, mini brushes and most importantly, interdental brushes. In order to compensate for a poor brushing technique and removal at hard to reach places, powered toothbrushes were developed. Powered toothbrushes with a rotating, oscillating or sonic action remove plaque and reduce gingivitis significantly better than manual brushes (Tritten and Armitage, 1996; Ho and Niederman, 1997; Moritis et al., 2002; Biesbrock et al., 2008; Rosema et al., 2008).

Chemotherapeutic agents have been developed in order to assist in the control of gingivitis and plaque. In 1985 the Council on Scientific Affairs of the American Dental Association (ADA) established guidelines for the acceptance of anti-gingivitis and/or plaque agents. These have been revised in 1997 and now state that:

“Examples of products evaluated under these guidelines include mouth rinses and toothpastes containing agents that would:

1. destroy, inhibit or modify plaque, including its pathogenicity for gingivitis, and microbiologic growth in general,

2. those that modify the attachment of plaque microorganisms to their natural sites, and

3. those that act by other antimicrobial mechanisms to reduce or prevent gingivitis”.

Toothpastes or dentifrices assist in maintaining good oral health in many ways. Therapeutic components in toothpaste formulations generally involve fluorides to decrease enamel demineralization (Lynch et al., 2004) and enhance remineralization (Feng et al., 2007; Altenburger et al., 2007), abrasives and detergents to enhance plaque removal and antimicrobials to kill remaining plaque organisms on the tooth surface. Nowadays, toothpastes also carry cosmetic functions, such as to whiten the dentition.
Mouth rinses are used for a variety of reasons: to freshen breath, to help prevent or control tooth decay, to reduce plaque, to prevent or reduce gingivitis, to reduce the speed at which calculus (calcified plaque) forms on the teeth, or to produce a combination of these effects. Mouth rinses are available as cosmetic and therapeutic products. Cosmetically, they merely give a temporarily control of bad breath and leave a pleasant taste. Therapeutic mouth rinses however, are meant to help control or reduce plaque, gingivitis, caries and bad breath.

Chemotherapeutic agents such as in toothpastes and mouth rinses should possess some key properties in order to be appropriate for use in oral hygiene procedures. Some of these properties are: good oral substantivity, biologically active conform its specific mode of antiplaque action, low toxicity and low permeability into the oral mucosa. When an antiplaque agent enters the oral cavity it can adhere to the hard and soft surfaces in the mouth: the dentition, dental pellicle, supragingival plaque and oral epithelia. Binding to these receptor sites determines whether the antiplaque agent will have a good oral substantivity, being sites of biological action or reservoirs. Oral substantivity is the effect of active substances on oral surfaces over an extended period of time (Cummins and Creeth, 1992). For toothpastes and mouth rinses substantivity is important as their efficacy should last after brushing and prevent re-deposition or regrowth of plaque as long as possible.

**Biofilm Models in Oral Health Care**

To study the features and dynamics of oral biofilms, a variety of models can be used. The purpose of a model is to simplify a complex system, enabling the study of specific aspects under controlled experimental conditions. The major advantage of this lies in the ability to reveal the effects of specific parameters. Hence, the merits of
in vitro models lie in their explanatory power and ability to predict. Consequently, it is essential that the advantages and disadvantages of the model are well-known, especially when results are to be extrapolated to the in vivo situation. Because of the huge complexity of in vivo oral biofilms, there is a need for in vitro oral biofilm models. For instance, due to the many different species identifiable in the oral cavity and their numerous interactions, in vitro models provide with an insight into in vivo processes (Sissons, 1997; Wimpenny, 1997).

Over time, a range of in vitro biofilm models have been developed, including amongst others single and multiple strain models and a variety of different devices in which biofilms are grown, each with their specific properties. In Table 1, the advantages and disadvantages of different in vitro models are presented, giving a short overview of the models used in oral research. When performing experiments using in vitro models, the selected biofilm model needs to meet the requirements dictated by the goal of the research. For instance, in vivo oral biofilms are composed of numerous different bacterial species. When there is a need for a strong in vivo relevance, a multi-species or whole saliva biofilm is the better model to use. However, the downside of such a model is its microbiological complexity or at least its difficulty to control bacterial composition and therewith reproducibility. Moreover, the understanding of all possible bacterial communications is rather difficult as well. Single-strain biofilm models are on the other hand easy to control in bacterial composition and thus reproducible, while dual-strain biofilm models also provide a possibility to explore bacterial interactions. Of course, these models only give a fundamental understanding and are not easily extrapolatable to in vivo reality. Next to the choice of a biofilm model, one has the choice of different oral devices, each with their specific properties. For example, if in real life the biofilm grows under shear,
like in the oral cavity, shear should also be present in the *in vitro* model. Both the modified Robbins device as the parallel plate flow chamber have this property. Although the first can take multiples samples at the same time, while the latter cannot, making it a time consuming device. Moreover, the parallel plate flow chamber has the ability of real-time observation, making confocal laser scanning microscopy easily applicable, allowing distinction after live-dead staining when appropriate. Also a very important property is the non-disrupting air-liquid interface, which is an important factor in removal of biofilm. Both devices can however not control the biofilm thickness or growth as in the constant depth film fermentor.

Summarizing, single strain biofilms are the most straightforward, easy to control and reproduce. In order to mimic the oral biofilm, dual-species models are a logical first step towards multi-species biofilm models. These provide with the interaction factor, which comprises synergism and competition, influencing adhesion strength to the surface and between bacteria, but also morphology of the biofilm. Multi-species biofilms grown from human whole saliva would be the ultimate model, as they highly relate to the *in vivo* situation. Oral biofilm devices can be chosen from the type of biofilm needed. Parallel plate flow chamber provide with real-time observations, the modified Robbins device can analyze multiple samples, while both have the ability of controlled shear. When the former properties are less important compared to biofilm growth and thickness, the constant depth film fermentor is the better choice.

**Potential Synergy of Mechanical and Chemical Plaque Control**

Since complete removal of dental plaque is impossible, it is interesting to dwell on the potential synergy between mechanical and chemical plaque control. Effective
chemical plaque control is always said to be due to adsorption of antimicrobials to the abundantly available soft tissues in the oral cavity and their subsequent desorption, but it can be hypothesized that also plaque left behind after brushing, can act as a reservoir for oral antimicrobials that can slowly release over time to kill newly adhering bacteria in the plaque. In this respect, it is important to note, that bacteria do not only adhere to tooth surfaces and soft tissue, but also to dead biofilm as such. Plaque is already known to act as a reservoir for fluorides (Ekstrand and Oliveby, 1999), while plaque left behind after being fluffed-up by sonic brushing has been described to have an even greater ability to adsorb fluorides than undisturbed plaque (Sjögren et al., 2004).

Aims of this Thesis

The aims of this thesis are:

1. to compare different oral biofilm models with respect to their virtues for the evaluation of mechanical and chemical plaque control.

2. to test the hypothesis that plaque left behind can act as a reservoir for oral antimicrobials to provide additional substantivity.

- In Chapter 2 a comparison is made of the efficacies of three different modes of contact-brushing on bacterial removal and re-deposition in single strain biofilm models on a saliva-coated surface.

- Chapter 3 deals with the comparison between dual-species biofilms and multi-species biofilms (human whole saliva) after adhesion or growth, with respect to their ease of removal by different modes of brushing. Additionally, re-deposition of bacteria to the brushed surface from a streptococcal suspension and fresh human whole saliva were studied.
Chapter 4 compares different biofilm modes of mechanical plaque control (contact- and non-contact brushing) using different biofilm models (single- and dual-species biofilms as well as multi-species biofilms grown from human whole saliva).

Chapter 5 aims at chemical plaque control by testing whether the *in vitro* antibacterial efficacies of a herbal- and chitosan-based toothpaste formulation are equally high as of chlorhexidine in terms of immediate and delayed bacterial killing in oral biofilms of different composition and maturational status.
Table 1. Advantages and disadvantages of different oral biofilm- and device models to study

<table>
<thead>
<tr>
<th>Model</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Reference</th>
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<tr>
<td>Single strain biofilms</td>
<td>Good control of biofilm composition, reproducible.</td>
<td>No interspecies interaction, not representative for in vivo biofilm formation.</td>
<td>(Sissons, 1997)</td>
</tr>
<tr>
<td>Dual strain biofilms</td>
<td>Good control of biofilm composition, reproducible, synergism, competition.</td>
<td>Difficult to extrapolate to in vivo conditions.</td>
<td>(Bradshaw and Marsh, 1999)</td>
</tr>
<tr>
<td>Whole saliva biofilms</td>
<td>Strong in vivo relevance.</td>
<td>No control of biofilm composition and reproducibility, microbiologically complex.</td>
<td>(Kolenbrander et al., 2006)</td>
</tr>
<tr>
<td>Parallel plate flow chamber device</td>
<td>Controlled shear and mass transport, real time observation, no disrupting air-liquid interface, confocal laser scanning microscopy compatible.</td>
<td>Time consuming, no multiple sample analysis at the same time.</td>
<td>(Zimm et al., 1999)</td>
</tr>
<tr>
<td>Modified robbins device</td>
<td>Multiple sample analysis at the same time, controlled shear.</td>
<td>No real time observation, disruptive air-liquid interface.</td>
<td>(Stoodley et al., 2002a)</td>
</tr>
<tr>
<td>Constant depth film fermentor device</td>
<td>Controlled biofilm growth and thickness, reproducible.</td>
<td>No controlled shear and real time observation, disruptive air-liquid interface when sampling, compaction of the biofilm during growth.</td>
<td>(Linton et al., 1999)</td>
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<td></td>
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<td>(Kharazmi et al., 1999)</td>
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