Oral Biofilm as a Reservoir for Antimicrobials

Otten, Marieke Petronella Theodora

Chapter 1

Mechanical removal and chemical control of oral biofilm

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The oral biofilm

The oral biofilm, or dental plaque, can be defined as a complex microbial community, embedded in a polymeric matrix and adhered to a surface. The development of an oral biofilm over time can be described in a few basic steps. The first step in biofilm formation in the oral cavity is the adsorption of a salivary conditioning film on the surfaces of teeth, restorations, prosthetic devices and soft tissue surfaces. In a second step, individual bacteria adhere to these surfaces and co-adhesion of other strains and species may take place. However, in this initial phase, adhesion is still reversible and bacteria may actually detach quite easily from the surface back to their planktonic state. When adhering bacteria start to produce extracellular polymeric substances (EPS), adhesion becomes irreversible. The next step comprises maturation of the biofilm, including the development of microcolonies and water channels to form a large, matrix enclosed structure[1]: the oral biofilm or also called “dental plaque”[2-4].

Figure 1 schematically presents the initial steps in the formation of a complex, multispecies biofilm, like the oral biofilm.

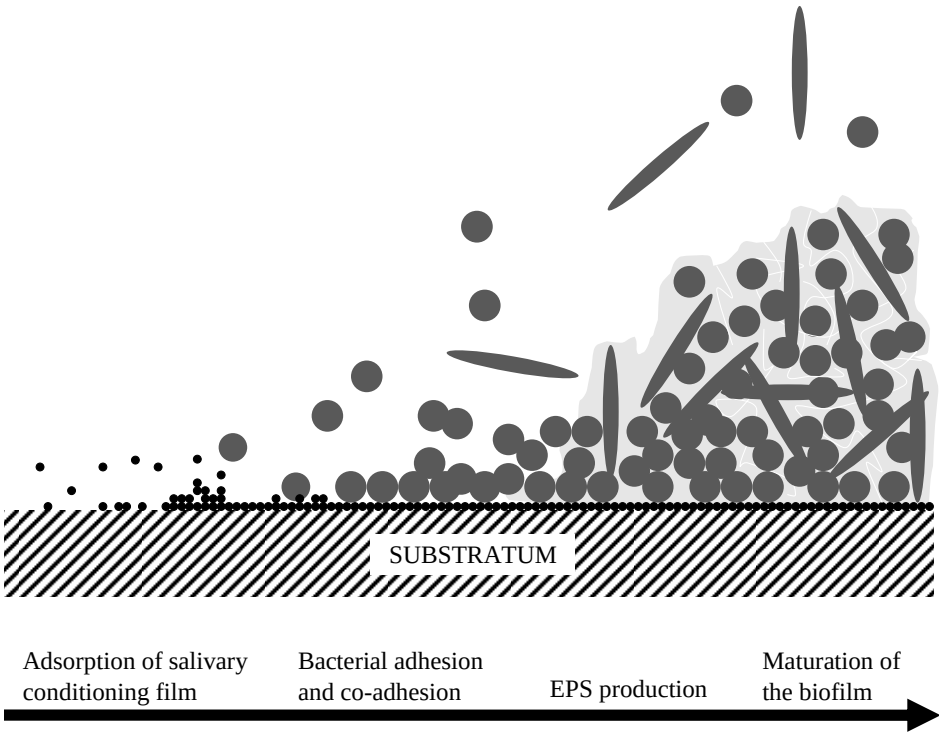

Figure 1. Schematic, sequential presentation of the initial steps in biofilm formation.

The resident oral microflora consist of microorganisms that live in harmony with the host, helps to maintain oral health and potentially harbors low numbers of pathogenic microorganisms[2]. Pathogenic bacteria can develop in high numbers under appropriate circumstances and cause oral diseases like caries, gingivitis and periodontitis[4;5]. Key environmental factors, like diet and the performance of proper oral hygiene can trigger shifts in the balance of the resident microflora in the biofilm, a process which is also called the "ecological plaque hypothesis"[6]. The goal of oral healthcare is to maintain the equilibrium between the resident flora and the host. This can be done either by reducing the total biofilm mass, targeted reduction of the prevalence of specific pathogens or by interfering with the

environmental factors responsible for the shift of the resident flora into a more pathogenic direction. Currently, the most wide spread and accepted way for the untargeted removal of oral biofilm is toothbrushing with a toothpaste, advisably supported by the use of mouthrinses and interdental cleaning aids. A large cohort study in adults by Axelsson *et al.*[7], studying the effects of a 30 years plaque control program on tooth mortality, caries and periodontal disease showed that self-performed biofilm control leads to an adequate maintenance of oral health. Nevertheless and although incidence was small, subjects included in this study suffered from caries and periodontal diseases and had an average plaque coverage of 20%, despite the extensive preventive program and supplementary use of interdental cleaning devices. In children however, adequate daily oral hygiene is much more difficult to achieve than in adults. Moreover, orthodontic appliances have become popular recently in both juvenile and adult patient populations, creating numerous retention sites in the oral cavity that are difficult to clean. Therewith the need for improved plaque control programs has greatly increased over the past decade.

Caries and periodontal diseases each have their own causative microorganisms out of thousands different bacterial strains and species that inhabit the human oral cavity[8-10]. Caries develops when specific types of acid producing bacteria, like mutans streptococci and *Lactobacilli* convert fermentable carbohydrates into acids, dissolving the enamel surface (demineralization) and finally resulting in cavities. For about half a century, caries was regarded as an “infectious and transmittable” disease, caused by a particular microorganism[11]. This would suggest that caries can be prevented by vaccination. Research focused on anti-caries vaccines with mutans streptococcal cells or antigens[12;13] showed that vaccination can prevent colonization by mutans streptococci, but clinical studies have failed hitherto to prove that vaccination is effective against caries. Currently, caries is much more considered as a “multi-factorial” disease, caused by a complex interplay between saliva, diet,

biofilm composition, and without a simple causative pathway[11]. Therewith, the idea that caries is an infectious, transmittable disease has been abandoned. Plaque induced gingivitis is due to the accumulation of bacteria in the biofilm around the gingiva, promoting an inflammatory response of the host, resulting in a red and swollen gingiva. In some patients and due to pathogenic bacteria (e.g. *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*, gingivitis progresses to periodontitis, with destruction of the supporting fibers and surrounding alveolar bone, finally resulting in tooth loss. Next to dental diseases like caries and gingivitis, associations between oral focal infections due to oral biofilm with the risk for developing e.g. cardiovascular diseases[14;15], and preterm low birth weight babies[16;17] have been reported.

Oral diseases remain a public health problem despite major achievements in oral health care[18]. Since there is a close relationship between oral biofilm and the occurrence of oral diseases like caries and periodontitis[19], mechanical removal[20;21] and chemical plaque control[22] are still widely applied.

Controlling oral biofilms: mechanical removal

The toothbrush is the most employed tool to remove oral biofilm, 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 are fulfilled. Biofilm removal from pits and fissures, interproximal spaces and around orthodontic appliances and imperfect restorations is never achieved by manual toothbrushing only, and a number of tools have been advocated to the market to assist biofilm removal in difficult to reach places, such as dental floss, toothpicks, mini-brushes and most importantly, interdental brushes. In terms of risk analysis for the development of caries and periodontitis, the interproximal area is most at risk. Therefore, it is of

utmost importance that interdental cleaning devices are used in combination with mechanical brushing. The choice for an interdental cleaning device can differ from patient to patient and should be based on the specific dental status of each patient[23;24]. In order to compensate for a poor brushing technique and to facilitate biofilm removal from hard to reach places, powered toothbrushes have been developed. Powered toothbrushes with a rotating, oscillating or sonic action remove biofilm and reduce gingivitis significantly better than manual brushes[25-29] and it is suggested that biofilm removal may even extend beyond the reach of the bristles[30;31]. Nevertheless, it remains difficult to completely remove oral biofilm by means of habitual brushing and the use of interdental cleaning[23;24;32]. A biofilm reduction of 50%-60% can be achieved by a single-time, self-performed brushing[23;33], meaning that biofilm is inevitably left behind. Two frequently used ways to score the amount of plaque on teeth is the planimetrical analysis (see Figure 2) and the plaque index[34]. In the plaque index plaque coverage is evaluated on a scale from 0 (no plaque) to 5 (plaque covering > two thirds of the surface).

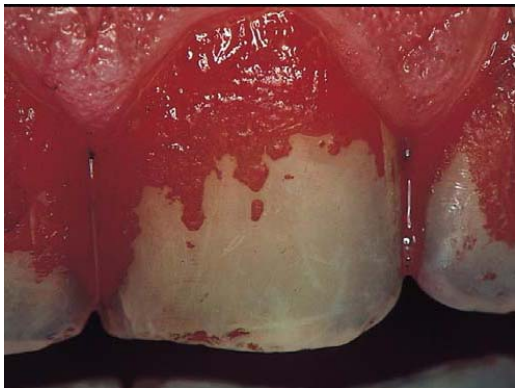

Figure 2. Picture of nine days undisturbed plaque development in the oral cavity. Plaque was stained by 0.5% aqueous neutral red solution. Subsequently planimetrical analysis can be used to express the percentage plaque area of the total buccal tooth surface[35].

The resident microflora contributes to oral health and is well tolerated by the oral cavity[2] and the human body in general. Therefore, the real challenge is to prevent a shift in the microbial composition of oral biofilm into a pathogenic direction. Yet, untargeted removal of oral biofilm every 48 h has been demonstrated to be sufficient to prevent the development of gingivitis and caries[24;36]. However, a brushing frequency of twice a day is usually recommended by dental professionals. This high brushing frequency is justified by the idea that a higher brushing frequency increases the efficacy of biofilm removal and enhances the beneficial effects of therapeutic toothpaste components, like e.g. fluoride[24] and stain-removing abrasives[37] and also meets patients demands for fresh breath. Note, that toothpastes with a moderate or high abrasivity do not necessarily yield more efficient removal of oral biofilm[33].

Controlling oral biofilms: chemo-therapeutic approaches

Therapeutic adjuncts, like fluoride and antibacterial agents, delivered by toothpastes or mouthrinses can help to prevent oral diseases by altering the pathogenicity of oral biofilms[38]. A wide range of chemo-therapeutic agents is available in oral health care products like toothpastes, gels and mouthrinses (see Table 1). In general, chemo-therapeutic agents can control biofilm formation by reducing accumulation of new biofilm, reducing or removing existing biofilm, suppressing growth and development of pathogenic bacteria and inhibiting production of virulence factors[39]. It is important that antibacterials in these processes should not disrupt the healthy oral microbiome, although most often their action is still untargeted[39].

Table 1. Chemo-therapeutic agents used in oral health care products

Class of inhibitor	Active ingredient	Modes of action
Amine alcohol	Octapinol Delmopinol	-Plaque inhibition[40-42], by interfering with plaque matrix formation and reducing bacterial adherence
Bisbiguanide	Chlorhexidine	-Antibacterial[40;42] -Cell wall damage[39-41] -Plaque inhibition by binding to cell membranes[40;41]
Enzymes	Lactoperoxidase Lysozyme Glucose oxidase Amyloglucosidase	-Antibacterial[42] -Enhances host defense mechanisms[39;41;42]
Essential Oils	Thymol Eucalyptol	-Antibacterial[40;42] -Antioxidative activity[40] -Inhibition of enzyme activity[39;43], reducing glycolysis[39], reducing bacterial adherence[39]
Fluorides	Sodium fluoride Stannous fluoride Amine fluoride Monofluorophosphate	-Prevents demineralization[44] -Enhances remineralization[44] -Antibacterial effects derived from non-fluoride portion[41-43]
Metal ions	Stannous Zinc Copper	-Antibacterial[39;41;42] -Plaque inhibition[39;40;42] -Inhibiting enzyme systems and glycolysis[39-41]
Oxygenating agents	Hydrogen peroxide Sodium peroxycarborate	-Antibacterial[42;43]

Plant extracts/ Natural products	Sanguinarine extracts	-Antibacterial[42] -Plaque inhibition[40;43] by suppression growth of bacterial strains and enzyme activity[41]
Phenols	Triclosan	-Antibacterial[39-42] -Plaque inhibition[39;40;42;43] -Interference with plaque metabolism[39] -Disruption of bacterial cell[41]
Quaternary ammonium compounds	Cetylpyridium Chloride	-Antibacterial[40-42] -Plaque inhibition[40;42;43] by interaction with microorganisms [41;43]
Surfactants	Sodium Lauryl Sulphate	-Antibacterial[41;42] -Inactivate bacterial enzymes[39;41]

Antibacterial agents can perform their action in different ways, i.e. causing leakage of cellular contents or affecting microbial metabolism[3;38]. Compared to planktonic or free-floating bacteria, organisms in a biofilm mode of growth are less sensitive to antimicrobial agents[45] and take advantage of the protective functions of the biofilm[2]. Biofilm bacteria differentiate themselves from planktonic ones by producing EPS. Bacterial EPS is comprised of biosynthetic polymers that can be highly diverse in chemical composition and may include polysaccharides, proteins, nucleic acids and phospholipids[1]. Apart from acting as a glue and providing structural support to the biofilm, EPS also acts as an extremely protective slime encasing. Antibacterials often bind to or are inactivated by the EPS matrix of the biofilm. As a result, the agents do not reach the deeper layers of a biofilm, as was first shown for vinegar by Antonie van Leeuwenhoek in the 17th century and later

confirmed for chlorhexidine treatments using confocal laser scanning microscopy (see Figure 3)[46].

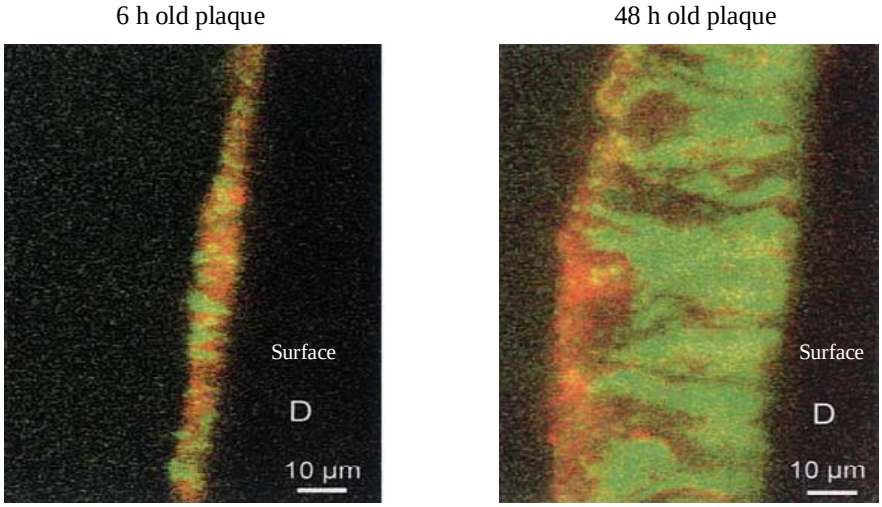

Figure 3. Confocal laser scanning micrographs[46] showing changes in plaque viability, grown in situ on a surface (on the right), after rinsing with chlorhexidine mouthrinse. Green and red areas represent live and dead bacteria. Note that chlorhexidine influenced the 6 h old plaque from top to bottom, and only influenced the outer layer of the 48 h old plaque, demonstrating a resistant nature of oral biofilm to a single chlorhexidine treatment.

Numerous studies have shown that in general up to 1000-fold higher concentrations of chemo-therapeutics are needed to kill bacteria in biofilms compared to free-floating or planktonic ones[9;47]. Chlorhexidine is the most employed antimicrobial in the oral cavity. It has a broad spectrum of activity against Gram-negative and Gram-positive bacteria and yeast, and proven anti-plaque activity. At high concentrations it is bactericidal by damaging the bacterial membrane[3]. At lower concentrations, it is bacteriostatic by inhibiting sugar transport and membrane functions[3;48]. Fluoride inhibits the metabolism of bacteria[49;50] by affecting cell glycolysis[51], and therewith inhibiting bacterial growth[52].

Inhibition of bacterial growth by fluoride ions is most effective under acidic conditions[52]. Fluoride can also reduce demineralization and enhance remineralization[44]. Essential oils, like thymol and eucalyptol, can reduce the level of Gram-negative anaerobic bacteria, resulting in a reduction in oral biofilm mass and gingivitis[48]. Triclosan has a broad spectrum of antimicrobial activity[3], and at sub-lethal levels it can inhibit acid production by oral streptococci[51]. Detergents like sodium lauryl sulphate can damage cell membranes, kill bacteria and inhibit enzymes. Metal salts, like stannous fluoride are very well retained in the oral cavity and possess bactericidal activity against both Gram-positive and Gram-negative bacteria[3;48].

Antibacterial photodynamic therapy can activate photosensitizers added to the biofilm to yield a reactive state, forming oxygen derived free radicals that lead to bacterial cell death[53;54]. However, the antibacterial effect of photodynamic therapy using methylene blue on biofilm bacteria was less than on bacteria in planktonic state[54]. Yet, the beneficial effects of photodynamic therapy in periodontal treatment may be plural and include not only antibacterial effects but also inactivation of proteases and inflammatory cytokines[53].

It is not always clear in what concentration oral chemo-therapeutics are actually applied in the oral cavity, although this can greatly affect their efficacy. Large individual variations exist for instance, in the amount of toothpaste used during brushing and water rinsing afterwards. Most people add water to the toothbrush with toothpaste on it, therewith diluting the active ingredients in the toothpaste. Further dilution takes place in the oral cavity due to salivation and swallowing. Also post-brushing, rinsing with water will reduce the concentration of active ingredients in saliva. A water rinse after the use of a 5,000 ppm F-containing toothpaste decreased the fluoride retention in oral biofilm to levels comparable to fluoride retention after using a 1,450 ppm toothpaste without water rinsing[55]. Rinsing with toothpaste-foam for 1 min after brushing resulted into a high fluoride

concentration in both saliva[56] and in oral biofilm[57], which is more effective than rinsing with a NaF-containing mouthrinse[57] after toothbrushing. A similar mechanism can be found for post-rinsing behavior: extrinsic factors like eating, drinking and chewing after using a chlorhexidine mouthrinse reduced the activity of the product[58]. Also other factors, like the concentrations used[59], the frequency of application[59] or the age of the product[60] influence the efficacy of antibacterial mouthrinses and toothpastes.

Retention of chemo-therapeutic agents in the oral cavity

An important property of effective oral antibacterial agents is their ability to retain in the oral cavity by interaction with oral surfaces and their subsequent slow release in active concentration at relevant places. This process is called substantivity and is defined by the Oxford Dictionary of Dentistry[61] as a characteristic of an antibacterial product whereby it remains active in the oral cavity for a longer period than the average brushing or rinsing time. Substantivity leads to the long-lasting effectiveness of oral hygiene products, especially of importance since the concentration of toothpaste and mouthrinse components in the oral cavity will decrease rapidly after use because of rinsing and swallowing in the absence of retention mechanisms.

Examples of substantive action are numerous. Antibacterial effects on bacterial viability in saliva e.g. of a 0.2% chlorhexidine rinse[59] could be perceived up to 7 h post-use, and include significant reductions in plaque re-growth and biofilm viability until 24 h post-use[62]. Effects of an amine fluoride/stannous fluoride containing mouthrinse and toothpaste on biofilm flora[60] on bacterial viability can be found up to 7 h after its application. A Triclosan containing toothpaste had a substantive effects on bacterial viability in plaque until 24 h after the last use[62] Substantivity therefore contributes to the biological action of toothpastes and mouthrinses containing antimicrobials.

Hypothesis on the reservoir function of plaque left behind after brushing

Recently, it was shown that biofilm viability and re-attachment on top of a biofilm that was exposed to antibacterial chemo-therapeutics tended to decrease as compared to an untreated biofilm[63]. These findings suggest that the oral biofilm may act as a reservoir for oral antimicrobials, in addition to the known contribution of their adsorption to intra-oral hard and soft surfaces. This yields the question, whether biofilm-left-behind after mechanical cleaning can be used as a reservoir for antibacterial chemo-therapeutic agents after brushing or mouthrinse use. It has already been shown in several studies that biofilms, after exposure to fluoride, which is a much smaller ion than most antibacterial agents, can become a reservoir for fluoride[55;57]. Increased fluoride retention in biofilms can be found until 12 h after the last brushing with a 1030 ppm fluoride toothpaste[64]. Cenci *et al.*[65] showed that both fluoride-containing toothpastes and fluoride releasing restorations maintain increased fluoride levels in a biofilm.

Conclusions and Aim of the Thesis

Maintenance of the equilibrium between a healthy and pathogenic biofilm is of utmost importance in oral health. The most efficient way to control the oral biofilm is mechanical removal, for which manual brushing and additional use of interdental cleaning devices are highly effective. Powered toothbrushes not only remove significantly more plaque than manual brushes, but their rotating, oscillating and sonic action may also extend beyond the reach of the bristles end. Nevertheless, a 100% plaque removal can never be achieved by brushing, not even when combined with the use of antibacterial chemo-therapeutic agents added to toothpastes and mouthrinses. In this chapter, a new possible mechanism is forwarded for the substantive action of antibacterial chemo-therapeutic agents in oral health care

products based on the hypothesis that biofilm-left-behind can act as a reservoir for oral chemo-therapeutics.

The overall aim of this thesis is to collect in vitro *and* in vivo *evidence in support of the hypothesis that oral biofilm can act as a reservoir for oral antibacterial agents and that biofilm-left-behind can therewith have a positive effect on oral health.*

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