CHAPTER 1

GENERAL INTRODUCTION

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Biofilms begin with bacterial adherence

The formation of bacterial biofilms starts with the binding of bacteria to a surface followed by growth and production of extracellular polymeric substances, maturation of the biofilm and subsequent dispersion of the cells (Costerton et al. 1999; Donlan and Costerton 2002; Fey 2010; Høiby et al. 2010).

In general, bacteria preferentially colonize on surfaces that are hydrophobic, have a certain surface roughness or have a conditioning layer. Conditioning layers alter the surface properties allowing microorganisms to adhere which would otherwise be unable to attach to the original surface. The salivary pellicle covering the hydroxyapatite surface of teeth is a well known example of such conditioning layer.

The adherence of bacteria to surfaces is governed by several factors such as the physicochemical properties of the surface, cell wall properties of the bacteria and the composition of the liquid medium. Physicochemical properties of the surface include short range forces like donor-acceptor, hydrogen, ionic, covalent and coordinate bonds, and long range forces such as electrostatic and van der Waals forces or stereochemical molecular recognition interactions (Katsikogianni and Missirlis 2004; Renner and Weibel 2011).

Biofilms: concern for health

Biofilms have been involved in a variety of microbial infections and diseases. For example, in health care sector, surfaces of indwelling medical devices are prone to biofilm formation such as the intravascular catheters, cerebrospinal fluid shunts, peritoneal dialysis catheters, intraocular lenses, cardiac pacemakers and prosthetic joints (Raad and Bodey 1992; Rupp and Archer 1994; Wang et al. 2009). In general, biofilms are difficult to control as they are resistant to some of the routinely used antibiotics (Sedlacek and Walker 2007; Høiby et al. 2010).

Dental biofilms and oral diseases

The accumulation of dental biofilms is often associated with the onset of dental diseases such as dental caries, gingivitis and periodontitis (Baehni and Takeuchi 2003). Despite the decline in The Netherlands and other developed countries since 1975, caries is still a major oral health problem in children. In 2010, 56% of the 5-year-old Dutch children suffered from caries, especially children with a relatively low socioeconomic status (Elfrink et al. 2010). In the USA,

47% of the individuals over the age of 30 suffer from periodontal disease, approximately 15% of them from severe periodontitis (Eke et al, 2012). As periodontitis is associated with an increased risk of cardiovascular disease, diabetes mellitus and adverse pregnancy outcomes (Shangase et al, 2013), prevention and treatment of periodontitis is a major public health problem (Watt and Petersen, 2012)

Dental caries, periodontitis and gingivitis are caused by multispecies dental biofilms (Becker et al. 2002; Socransky et al. 2002; Kumar et al. 2005; Brito et al 2007). During the course of a disease the bacterial composition often changes from a scanty biofilm dominated by Gram-positive bacteria, usually found in healthy individuals, to a biofilm with increased numbers of Gram-negative anaerobic rods, observed for example in periodontitis.

The acquired dental pellicle: a substratum for oral biofilms

The acquired dental pellicle is formed by the specific adsorption of salivary proteins and other macromolecules from the saliva (Lamkin et al. 1996) (Fig. 1). The acquired dental pellicle has several important roles in the maintenance of oral health: (i) lubrication of the tooth surface, making mastication and speech more comfortable (Tabak 1995). (ii) It acts as a semi-permeable barrier preventing demineralization and facilitating remineralization, thus maintaining the integrity of the enamel surface. (iii) The proteins of the pellicle inhibit precipitation of calcium salts on the enamel thus providing mineral homeostasis at the enamel-saliva interface. (iv) It exerts selectivity for bacterial adherence and is involved in the early stages of microbial biofilm formation.

The first stage of pellicle formation in the oral cavity is characterized by an almost instantaneous adsorption of salivary proteins on the enamel surface. The dental enamel is largely composed of mineral hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. Salivary Phosphoproteins with a high affinity to hydroxyapatite, such as statherin, histatin, and proline-rich proteins (PRPs), are among the first proteins which adsorb onto the hydroxyapatite surface (Lamkin et al. 1996). The phosphoproteins bind to the enamel surface by ionic interactions and inhibit precipitation of calcium phosphate salts from supersaturated solutions and inhibit crystal growth of precipitated calcium phosphate (Schwartz et al 1992).

Rapid protein adsorption is followed by a comparatively slower phase of the formation of salivary protein complexes onto the protein-coated enamel surface. Heterotypic complexes formed by noncovalent binding between salivary mucins MUC5B and other salivary proteins like amylase, PRPs, histatins, statherin, cystatins and lysozyme have been identified (Iontcheva

et al. 1997). Mucins are heavily glycosylated glycoproteins with strong water-binding, visco-elastic and gel-forming properties. Heterotypic complexing (Iontcheva et al. 1997), enzymatic cross-linking (Yao et al. 1999; Yao et al. 2000), or proteolytic processing can alter the properties of salivary proteins resulting in the formation of unique molecular species during pellicle formation. Salivary agglutinin (SAG) is another glycoprotein secreted by parotid glands and is naturally present in the dental pellicle (Carlén et al. 1998).

Hence, pellicle formation is a dynamic process that involves continuous adsorption-desorption processes, modification of adsorbed molecules by microbial or host enzymes and intermolecular complexing (Vacca et al. 2000).

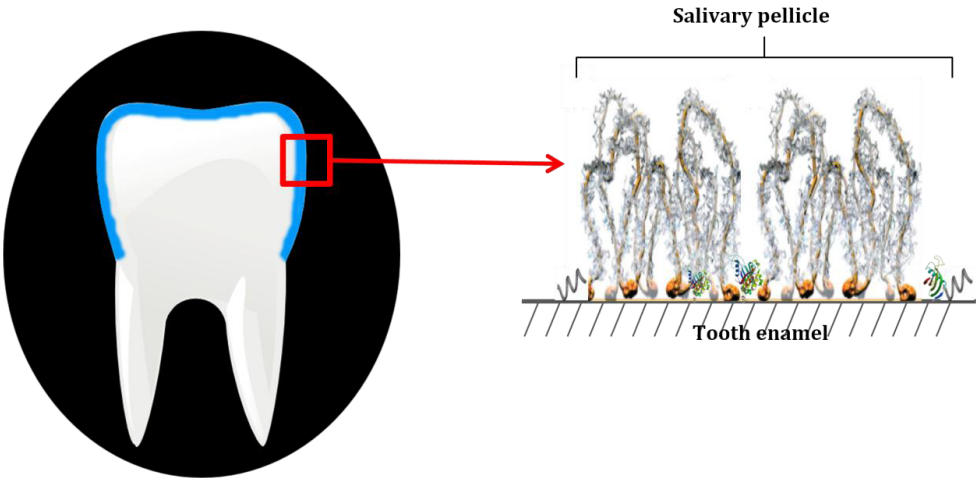

Figure 1. Schematic representation of the salivary pellicle on the tooth enamel

The salivary pellicle: substratum for bacterial adherence

The oral biofilm communities are complex and dynamic structures that accumulate through the sequential and ordered colonization by several oral bacteria (Kolenbrander et al. 2002). By providing multiple bacterial binding sites the salivary pellicle is involved in the early stages of microbial biofilm formation which begins when the early colonizers, mainly the oral streptococcal species; adhere to proteins in the acquired salivary pellicle (Li et al. 2004). *Streptococcus oralis*, *Streptococcus gordonii*, *Streptococcus sanguinis*, and *Streptococcus mitis* are examples of primary colonizers (Cassels et al. 1995). The oral streptococci bind to proteins such

as alpha-amylase, PRPs, and proline-rich glycoproteins (Kolenbrander et al. 2002). Previously it has been found that *S. gordonii* binds to acidic PRPs and salivary amylase while *S. sanguinis* binds to a complex enriched in secretory immunoglobulin A and alpha-amylase (Gibbons et al. 1991; Gong et al. 2000; Rogers et al. 2001). SAG present in the acquired pellicle facilitates adherence of bacteria such as *Streptococcus mutans* (Lamont et al. 1991; Loimaranta et al. 2005). Subsequently the planktonic bacterial cells that cannot colonize the tooth surface may bind to receptors on the cell surface of early colonizers. This is known as co-aggregation and is a specific cell-to-cell interaction that occurs between genetically distinct bacterial species. For example, *S. gordonii* co-aggregates with secondary colonizers such as the periopathogen *Porphyromonas gingivalis* (Haffajee and Socransky 1994) (Fig. 2). *S. gordonii* also promotes adherence of opportunistic yeast pathogens such as *Candida albicans* (O'Sullivan et al. 2000; Egland et al. 2001; Lamont et al. 2002; Kuboniwa et al. 2006). *Fusobacterium nucleatum* can co-aggregate with streptococci and obligate anaerobes and serves as a coordinator between late and early colonizers (Kolenbrander et al. 2002).

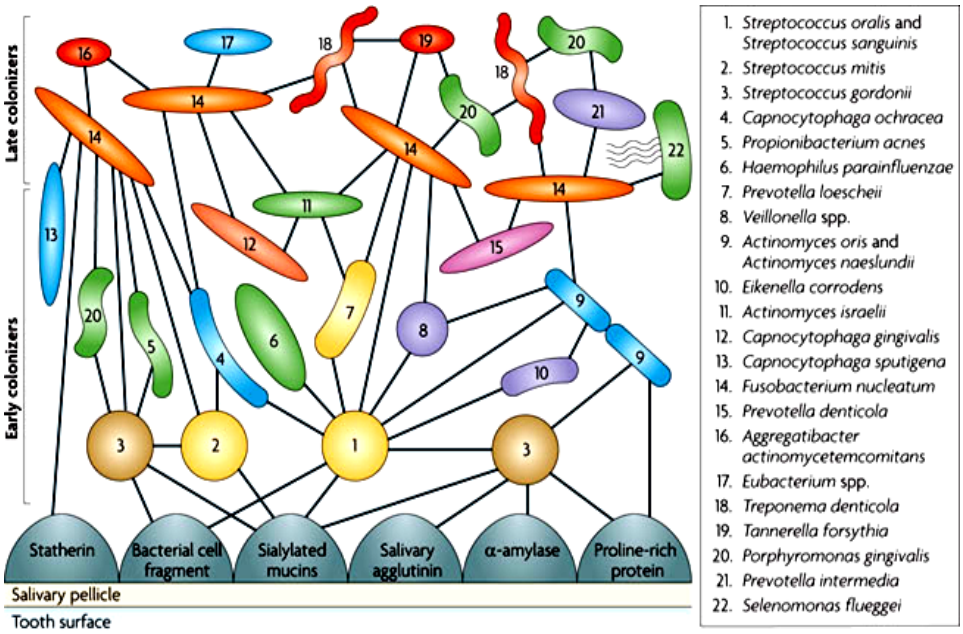

Figure 2. Schematic diagram of the interactions between salivary pellicle receptors, initial colonizing bacteria and late colonizers of the tooth surface. (Kolenbrander et al. 2010. *Nat Rev Microbiol*. 8:471-480).

Co-aggregation may lead to the development of a multi-species biofilm in which metabolic communication, genetic exchange, quorum-sensing and survival of obligate anaerobic bacteria might occur under aerobic conditions (Bradshaw et al. 1998, Chalmers et al. 2008; Sedgley et al. 2008). Extracellular polymeric substances, produced by bacteria during biofilm development strengthen adhesion between cells and can also act as receptors for co-aggregation.

Control of dental plaque: current strategies

Currently several methods exist by which dental plaque can be controlled. These methods include:

1. Mechanical removal of dental plaque using toothbrushes, dentifrices and interdental cleaning devices.

   Tooth brushing is the most commonly used method of daily oral hygiene and the removal of dental plaque is achieved through direct contact between the filaments of the toothbrush and the surfaces of the teeth and soft tissues. However, it is estimated that most individuals remove only 40-50% plaque by tooth brushing. Dentifrices are substances used in combination with a toothbrush to remove bacterial plaque and debris from the gingiva and teeth. Therapeutic dentifrices contain chemical agents with specific treatment or preventive activities such as fluoride, plaque-inhibiting agents (chlorhexidine, lactoperoxidase, triclosan), desensitizing agents (potassium nitrate and strontium chloride) and tartar control agents (pyrophosphate and zinc citrate).

2. Inhibition of biofilms and of the planktonic population of bacteria with antimicrobials.

   Periodontal disease, in some cases, is controlled by antibiotic therapy (Walker et al. 2004). The rapid development of antibiotic resistance in biofilms paved way for the development of compounds with novel mechanisms of action. Natural products, namely antimicrobial peptides (AMPs), prove to be promising candidates in laboratory studies but are not yet being routinely used. Most AMPs permeabilize microbial membranes, inducing either a large-scale failure or small defects that dissipate the transmembrane potential, which results in cell death (Sang and Blecha 2008; Wimley and Hristova 2011). Studies on AMPs such as, Lanticin 3147, decapeptide KSL, pleurocidin and chrysophsin-1 have shown bactericidal activity on planktonic cells and on biofilms of *S. mutans in vitro*(Dobson et al. 2011; Liu et al. 2011; Tao et al. 2011; Wang et al. 2012).

3. Prevention of adhesion of bacteria to the tooth surface

    a. Adhesion inhibitors targeting bacteria.

i) Synthetic peptide adhesion analogues. The strategy for the use of adhesion analogues is based on the assumption that isolated adhesion molecules or synthetic or recombinant fragments will bind to the receptor and thereby competitively block adhesion of the bacteria. *In vitro* studies have shown an inhibition in adherence of *S. mutans* to saliva coated hydroxyapatite beads and biofilm formation using analogues of the SspB peptides (Okuda et al. 2010). Similar synthetic peptides, mimicking a fragment of the fimbrillin adhesin of *P. gingivalis*, inhibited its adhesion to hydroxyapatite *in vitro* (Lee et al. 1992; Lee et al. 1995).

ii) Dietary adhesion inhibitors. Food products contain either a mixture of inhibitors or an inhibitor with a broad spectrum of activity. For example, constituents of milk and plant products inhibit bacterial adherence. Milk constituents, such as, casein proteins inhibit adherence of *S. mutans* to hydroxyapatite *in vitro* (Halpin et al. 2008). Plant constituents such as polyphenols have a bactericidal effect and inhibit adherence of *S. mutans in vitro*(Ferrazzano et al 2011; Yano et al. 2012).

iii) Anti-adhesin antibodies. This method involves the use of antibodies that block the adhesins of the pathogen thereby inhibiting its adhesion and infection. For example, monoclonal antibodies directed against the bacterial SA I/II adhesins of *S. mutans* inhibited adhesion of this bacteria to the tooth surface of human volunteers (Ma et al. 1998).

iv) Host derived anti-adhesins. Inhibition of adhesion of pathogens and subsequent reduction of colonization by constituents in body fluids are important components of the innate immunity. For example, the hydrophobic molecule sphinganine, a component of sphingolipids, decreased adhesion of *S. mitis* to buccal epithelial cells (Bibel et al. 1992b).

    b. Adhesion inhibitors targeting the tooth surface.

Bacteria need a surface to which they adhere and eventually grow and develop into a biofilm. Bacteria preferentially colonize surfaces that are hydrophobic, have a certain roughness on the nano- and micro scale, and are covered with a conditioning layer, rather than to smooth, hydrophilic surfaces. Hence a strategy would be to prevent the formation of a conditioning layer and to create coatings that are antimicrobial and/or antifouling. Bacterial repelling compounds such as phosphorylated polyethylene glycol

and pyrophosphate have been shown to inhibited biofilm formation *in vivo* and adherence of *S. mutans* to the salivary pellicle *in vitro* completely (Shimotoyodome et al. 2007).

Outline of the thesis

The studies in the present thesis were aimed at the development of novel anti-adherence and antifouling coatings for HA to control oral biofilms.

- In chapter 2, we identified and characterized the hydroxyapatite binding domain in SAG. SAG is composed of 13 highly homologous scavenger receptor cysteine-rich (SRCR) domains. Previously our group has mapped the bacterial binding domain of SAG to a peptide loop of 16 amino acids that is present in 10 out of 13 SRCR domains, designated SRCRP2 (P2, amino acids QGRVEVLYRGSWGTVC) (Bikker et al. 2002; Holmskov et al. 1997; Ligtenberg et al. 2001). We aimed to identify the hydroxyapatite binding domain of SAG using the peptides derived from the SRCR domain of SAG. A peptide designated as P3 bound maximum to hydroxyapatite. Since polyethylene glycol (PEG) has bacterial repellant activity, P3 was conjugated with PEG and the antifouling property of this conjugate was explored.
- In chapter 3, we explored the approach of functionalizing surfaces that are primed with an appropriate coating. This approach will allow a higher flexibility since different functional groups might be coupled to the preconditioned surface. As a proof of principle we aimed to functionalize a preformed peptide-coating on a polystyrene surface with an anti-adhesive PEG moiety using the sortase A enzyme. The resultant P3-PEG conjugate exhibited a significant reduction in bacterial adherence to the surface.
- In chapter 4, we utilized the phage display method to find a novel *in vitro* salivary pellicle binding peptide (SPBP) with antifouling properties. The use of a phage peptide library (displaying approximately 10^9 different peptide sequences) enabled the selection of a specific peptide with a high selectivity to the *in vitro* salivary pellicle. We successfully found a peptide designated SPBP 10 having antifouling activity against *S. gordonii* on hydroxyapatite treated surfaces.
- In chapter 5, we discovered antiadherence properties of sphingosine on hydroxyapatite surfaces against *S. gordonii* and *S. sanguinis*. Previous studies have demonstrated that sphingosines have bactericidal activity against Gram-positive and Gram-negative

bacteria as well as candidacidal activity against *C. albicans* (Bibel et al. 1992a; Bibel et al. 1993; Fischer et al. 2012). Sphinganine also inhibits the adherence of *S. mitis* to buccal epithelial cells (Bibel et al. 1992b). Sphingosines contain a positively charged amine by which they potentially bind to the phosphate of hydroxyapatite (Valentijn-Benz et al, submitted). Among the tested sphingosines, sphinganine inhibited the adherence of *S. gordonii* and *S. sanguinis* and also inhibited their growth.

- In chapter 6, we explored the antiadherence and bactericidal activity of sphinganine on *S. mutans.*
- In chapter 7, we summarize the results of the study, and discuss the potential application of these findings and future research options.

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