CHAPTER 7

SUMMARIZING DISCUSSION

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Dental biofilm i.e. dental plaques have been implicated in oral diseases such as dental caries, gingivitis and periodontitis (Baehni et al. 2003). The formation of dental biofilms is initiated by the adherence of early colonizers, predominantly streptococcal species, to the dental pellicle. This is subsequently followed by growth and production of extracellular polymeric matrices, maturation of the biofilm and subsequent dispersion of the cells (Costerton et al. 1999; Donlan and Costerton 2002; Fey 2010; Høiby et al. 2010). Proteins in the acquired dental pellicle such as alpha-amylase, proline-rich proteins and proline-rich glycoproteins provide bacterial binding sites to the early colonizers (Kolenbrander et al. 2002). Early colonizers such as *Streptococcus gordonii* bind to proline-rich proteins and salivary amylase while *Streptococcus 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).

Currently a variety of strategies are used for the removal of biofilms. These strategies include mechanical removal such as tooth brushing and non-mechanical removal with agents such as ethanol, sodium lauryl sulfate, triclosan, cetylpyridinium chloride, chlorhexidine digluconate, and nisin. However, *in vitro* studies with these antimicrobial agents revealed that these treatments hardly result in full eradication of the biofilm (Corbin et al. 2011).

The lack of efficient biofilm control measures has sparked interest in alternative strategies aimed at the inhibition of the development of biofilms on surfaces, for instance by inhibiting the interaction between bacteria and the surface (Ofek et al. 2003). Bacteria preferentially colonize on surfaces that are hydrophobic, have a certain surface roughness, and are covered with a conditioning layer. To prevent colonization of these surfaces, a strategy could include the development of coatings that are antimicrobial and/or bacterial repellent. Bacterial repelling compounds such as polyethylene glycol (PEG) and pyrophosphate have been shown to inhibit bacterial adherence *in vitro* to hydroxyapatite (HA) and biofilm formation *in vivo* (Shimotoyodome et al. 2007).

The present study was aimed at the development of novel coatings for HA, the dental mineral, with anti-adhesive properties. Using a protein naturally occurring in the pellicle, salivary agglutinin (SAG), as lead compound, we developed a HA-binding peptide, P3. Besides using phage display, we discovered a novel peptide, denoted SPBP 10, which binds to the salivary pellicle on HA. Both P3 and SPBP 10 exhibited anti-adhesive properties *in vitro* against bacteria.

Furthermore, it was found that sphingosines and their derivatives exhibited pronounced anti-adherence activity against oral streptococcal species *in vitro*.

Discovery of hydroxyapatite binding domain in salivary agglutinin

In **chapter 2** we describe the identification of the HA-binding domain of SAG. SAG is a high-molecular weight glycoprotein secreted by salivary glands and is naturally present in the dental pellicle (Carlén et al. 1998). In saliva, SAG mediates aggregation and oral clearance of bacteria, while in the acquired pellicle it facilitates adherence of bacteria such as *Streptococcus mutans* to early colonizing bacteria (Lamont et al. 1991; Loimaranta et al. 2005). SAG is composed of 13 highly homologous scavenger receptor cysteine-rich (SRCR) domains. Previously we have identified the part of SAG that mediated bacterial binding; a peptide loop of 16 AA present in 10 out of 13 SRCR domains, designated SRCRP2 (P2, amino acids QGRVEVLYRGSWGTVC) (Holmskov et al. 1997; Bikker et al. 2002; Ligtenberg et al. 2001).

In parallel, peptide sequences covering the complete SRCR consensus sequence of SAG were synthesized and their binding to HA was analyzed. Among all peptides tested, only one 18-mer peptide, (DDSWDTNDANVVCRQLGA, designated P3) bound significantly to HA. Detailed amino acid analysis of the P3 sequence revealed the presence of four negatively charged aspartic acid residues by which it may potentially bind to the calcium ions of HA (Guan et al. 2003; Murphy et al. 2007; Uddin et al. 2010; Bikker et al 2013). Interestingly, crystallographic analysis *in silico* of a SRCR domain revealed an opposite orientation of P2, the bacteria binding domain of SAG (Bikker et al. 2002), and P3 which could explain the dual role of SAG in bacterial homeostasis. When bound to HA, P3 putatively forms the surface anchoring part of a SRCR domain, exposing P2 to the microenvironment facilitating bacterial binding.

Functionalization of the peptides with polyethylene glycol

Previous studies have demonstrated bacterial repellant properties of PEG (Shimotoyodome et al. 2007; Müller et al. 2009). So, in the studies described in **chapter 2** PEG was selected to enhance the bacterial repellant property of P3. Covalent coupling of this peptide with PEG was performed chemically and enzymatically using sortase A (SrtA).

A P3-PEG conjugate was chemically synthesized by the covalent attachment of PEG to the primary amine of the amino acid at the N-terminal of the peptide. The bacterial repellent property of the P3-PEG conjugate was investigated for *S. mutans* and *Staphylococcus epidermidis*, the latter being a normal skin flora microorganism. *S. epidermidis* can get

transmitted to the surfaces of indwelling medical devices such as intravascular catheters, cerebrospinal fluid shunts, peritoneal dialysis catheters, intraocular lenses, cardiac pacemakers and prosthetic joints, and cause infections (Raad and Bodey 1992; Rupp and Archer 1994; Wang et al. 2009). We observed that P3 by itself exhibited bacterial repelling characteristics which could be due to the net negative charge of P3 peptide which may potentially repel the negatively charged bacterial membrane and membrane anchored structures such as LPS and LTA. As expected, the presence of a conjugated hydrophilic PEG moiety increased the intrinsic repellent capacity of the P3 peptide even more (**Chapter 2**).

In **chapter 3** PEG was enzymatically coupled to surface bound P3 using Sortase A (SrtA). SrtA is a transpeptidase which catalyzes sequence-specific ligation of proteins to the cell wall of Gram-positive bacteria. *In vivo*, SrtA conjugates proteins to the bacterial cell wall by recognizing the LPXTG motif and then cleaving the peptide bond between threonine (T) and glycine (gly, G) followed by the formation of a covalent bond between the carboxy terminus of the cleaved protein and the amino group terminal of penta-glycine cross-bridges in the peptidoglycan (Mazmanian et al. 1999; Ton-That et al. 1999). Previously, SrtA has been used by our group to cyclize histatin peptide (Bolscher et al. 2011). The potential of SrtA was further utilized to couple PEG-LPETG to $(Gly)_5$P3 peptide which was pre-coated on the polystyrene surfaces of a microtiter plate. The resulting P3-PEG conjugate was evaluated for its anti-adherence activity against *Yersinia pseudotuberculosis*. The conjugate decreased the binding of *Y. pseudotuberculosis* indicating successful conjugation of P3 peptide with PEG by SrtA enzyme (**Chapter 3**). Hence this strategy of generating anti-adherence compounds offers a promise for directed functionalization of biomedical materials which are not amenable for direct covalent linkage.

So, PEGylation of P3 peptide, either by chemical or enzymatic methods exhibited enhanced inhibition in bacterial adherence compared to the non PEGylated P3 peptide.

Discovery of peptide binding to the in vitro salivary pellicle

Phage display is a method for identifying novel peptides with selectivity for specific targets such as enzymes, cell-surface receptors and biomaterials such as HA and titanium (Rousch et al. 1998; Roy et al. 2008; Liu et al. 2010). We describe, **in chapter 4**, the identification of 10 salivary-pellicle-binding phages that were selected using this technique, displaying novel peptide sequences. The peptides were synthesized and evaluated for their binding to the uncoated HA and saliva-coated HA. Among the 10 salivary-pellicle-binding peptides (SPBPs),

SPBP 10 exhibited the highest binding to the saliva-coated HA. Sequence analysis of SPBP 10 revealed the presence of 5 hydrophobic amino acids that occur consecutively (NSAAVRAYSPPS), forming a hydrophobic region, which might assist in the interaction with the *in vitro* salivary pellicle. Furthermore, *in silico* analysis of the secondary structure of this peptide showed a tendency towards an alpha helical structure (spanning the residues AAVR). Thus, this conformational characteristic may play an important role in pellicle binding. Furthermore, the affinity of SPBP 10 to HA could probably be due to the positively charged arginine AA (R) that binds to negatively charged phosphates.

Binding of SPBP 10 to HA and saliva-coated HA was not affected by calcium ions, suggesting that its binding is not mediated by the calcium ions in the HA surface. This is in line with the fact that SPBP 10 does not contain negatively charged residues which potentially interact with positively charged calcium ions, as *e.g.* occurs in acidic proline rich proteins and statherin (Johnsson et al. 1993). The presence of the non ionic detergent Tween-20 did not abolish the affinity of SPBP 10 to the salivary pellicle indicating that the binding is probably not due to hydrophobic interactions. These assays confirm the selective binding of SPBP 10 to the *in vitro* salivary pellicle.

The limitation of using an *in vitro* salivary pellicle is that the pellicle composition can be different from the *in situ* formed pellicle. For instance, comparative analysis of protein composition of *in vitro* and *in vivo* salivary pellicle has revealed a difference in the amount of acidic proline-rich proteins (Yao et al. 2001). Hence, further studies will be required to explore the binding behavior of SPBP 10 to the *in situ* formed pellicle. This peptide also showed significant antifouling activity against the early colonizing bacteria *S. gordonii*. This study suggests that this approach shows potential for design of compounds that can be applied for controlling the early stage of bacterial adherence on biomaterials.

Anti-adherence and bactericidal activity of sphingosines and its derivatives

Sphingosines are amphoteric compounds which are composed of a positively charged amino alcohol head group and hydrophobic hydrocarbon chain which may be saturated or unsaturated. Previously it has been shown that sphingosines in solution exert killing effects on both Gram-positive and Gram-negative bacteria (Bibel et al. 1992; Bibel et al. 1993; Fischer et al. 2012). In **Chapter 5** and **6** we described that a sphingosine-coating on HA surfaces can effectively inhibit bacterial adhesion. The anti-adherence effects were most likely caused by the

altered physicochemical properties of the surface, such as change in net charge and hydrophobicity, which play an important role in the primary adhesion of bacteria to HA.

A coating of HA with sphingosines affected adherence of both *S. gordonii* and *Streptococcus sanguinis*. Remarkable differences were observed between structurally similar sphingosines. For instance, sphinganine which shows only minor structural differences with other sphingosines, exhibited maximum anti-adherence properties. Sphinganine structurally differs from sphingosine in the degree of saturation of the acyl side chain and from phytosphingosine (PHS) in the presence of a hydroxyl group. PHS differs from phytosphingosine-1-phosphate (PHS-1-PO_4) by a phosphate group. Strikingly, sphinganine inhibited adherence of *S. gordonii* and *S. sanguinis* by 80 and 100-fold respectively, whereas PHS did not exhibit any anti-adherence activity (**Chapter 5**). Sphinganine also inhibited the adherence of *S. mutans* to HA discs by 1000-fold (**Chapter 6**). Quantitative analysis of the sphingosines showed that even though sphinganine adsorbed 2 fold less to HA than PHS, it exhibited maximum inhibition of *S. gordonii* and *S. sanguinis*. Adherence of *S. gordonii* was inhibited by sphinganine, PHS-1-PO_4 and stearoyl PHS, whereas that of *S. sanguinis* was inhibited in addition by sphingosine and sphingomyelin. These differences may be attributed to the higher hydrophobicity of *S. sanguinis* compared to *S. gordonii*. It is hypothesized that the hydrophobic *S. sanguinis* will be more repelled by the charged head groups of sphingosines than the more hydrophilic *S. gordonii*. On the other hand PHS-1-PO_4, which is even more hydrophilic, inhibited *S. gordonii* to the same extent as sphinganine. This may be due to the repulsive forces between the phosphate group of sphingosine and the negatively charged groups on the bacterial surface.

On theoretical grounds it may be assumed that adsorbed on the HA surface, sphingosines will form aggregates (bilayers or micelle like structures) with the positively charged head groups exposed to the bulk fluid, as well as adhered onto the HA surface. Sphinganines having a saturated acyl chain will likely form a more rigid film than its unsaturated analogues. Therefore we envisage that sphinganine films are less vulnerable to disruption e.g. by bacterial adhesins than the more fluid films composed of sphingosine or PHS.

Interestingly, the evaluation of bactericidal activity of structurally similar sphingosines - PHS, sphingosine and sphinganine - against planktonic cells, intact biofilms and disrupted biofilms of *S. mutans* showed different activities when compared to their respective anti-adherence properties. Against biofilms, sphingosine exhibited maximum bactericidal activity (100-fold reduction) compared to PHS and sphingosine (5- and 10-fold respectively). This could be due to the ability of sphingosine molecules to penetrate the hydrophilic extracellular polysaccharide

layer of the biofilm and also the availability of more free sphingosines compared to PHS and sphinganine. PHS and sphingosine showed maximum bactericidal activity against planktonic and disrupted biofilms, while sphinganine was less effective for both planktonic and disrupted biofilms (1000-fold reduction). This supports the finding that inhibition of the adherence of *S. mutans* to sphinganine-treated HA discs is probably not due to its bactericidal activity.
Considering the anti-adherence and bactericidal activity of sphingosines, it can be suggested that they may have potential as an anti-biofilm agent to control and eliminate oral biofilms.

Peptides vs sphingosines
In this thesis the potential applications of peptides and sphingosines as anti-biofilm coating materials for HA have been explored. Potential advantages and disadvantages of these molecules are summarized in Table 1.

Table 1. Advantages and disadvantages of the application of peptides and sphingosines as anti-biofilm coating materials.

	Advantages	Disadvantages
Peptides	-Peptides can be synthesized chemically. -Optimization of peptides is possible by organic synthesis. By the substitution of single amino acids, optimization can be very accurate.	-Peptides are prone to proteolytic degradation. -Production of peptides is more expensive than that of sphingosines. -The function of peptides is relatively specific which narrows down its application. e.g. P3 peptide exhibited anti-adherence activity but no bactericidal activity. -Peptides may potentially exert a toxic activity or an allergic reaction.
Sphingosines	-Sphingosines exert versatile functions with wide applications. For e.g. they exhibit anti-adherence and bactericidal activity. -In general sphingosines are considered safe.	-Since sphingosines are isolated from natural sources its modification is difficult and cumbersome.

Future recommendations

Using different strategies we have discovered anti-adherence coatings for HA surfaces. These coatings which were composed of PEGylated peptides and sphingosines, exerted anti-biofilm activity and hence may have practical application to control biofilms on HA surfaces like the dental enamel or prosthesis that have HA coatings such as dental implants.

The studies described in the thesis were performed *in vitro* using monocultures of bacteria. Therefore, subsequent studies should explore the effects of the discovered anti-adherence coatings on the growth and composition of mixed biofilms. When the compounds also have *in vitro* effects on mixed biofilms, subsequent *in vivo* studies should explore the effects in laboratory animals and human subjects.

When the compounds have clinical effects against oral biofilms, they might be added to oral health care products such as mouth washes, dentifrices or mouth sprays. The method of enzymatic PEGylation of peptides can be used to regenerate protective coatings on medical or industrial surfaces that are prone to biofilm formation and biofouling, including prosthesis, catheters and tubings of dental units.

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