

CHAPTER 2

**Identification of the hydroxyapatite binding domain of
salivary agglutinin**

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equal contribution

Abstract

The salivary agglutinin glycoprotein (SAG) is present in saliva but is also part of the salivary pellicle, playing a seemingly paradoxal role with regard to bacterial homeostasis. On the one hand, SAG aggregates bacteria in solution, thereby preventing bacterial colonization. On the other hand, when bound to the tooth surface, SAG facilitates bacterial colonization and microbial growth.

The protein part of SAG is predominantly composed of conserved Scavenger Receptor Cysteine-Rich (SRCR) domains. Previously it was found that bacteria binding and aggregation by SAG is mediated by a single peptide loop within the SRCR domains, designated SRCRP2 (P2). The current data suggest that the SRCR domains also harbour a hydroxyapatite (HA) binding moiety, SRCRP3 (P3). The observation that P2 and P3 individually play a unique role in SAGs function contributes to the understanding of SAGs dualistic role in bacterial binding.

Inspired by SAGs bacterial modulating capacity we created a P3-polyethylene glycol (PEG) conjugate. It was found that a P3 coating resulted in an increased antifouling activity of 20% compared to the uncoated surface *in vitro*. An additional PEG moiety resulted in an antifouling activity, up to 40% and 30% for *S. mutans* and *S. epidermidis*, respectively.

Introduction

The salivary agglutinin glycoprotein (SAG) is a high-molecular-weight glycoprotein in human saliva which mediates the adhesion and aggregation of bacteria in the oral cavity. Among other host defence factors and specific bacterial-binding proteins in saliva, such as mucins, statherin, histatins, proline rich proteins, SAG modulates microbial growth and colonization on the dental enamel, which is mainly composed of hydroxyapatite (HA) (Jensen et al. 1992; Johnsson et al. 1993; Carlen et al. 1998). In the oral cavity SAG is believed to play a dual role with regard to bacterial homeostasis. SAG facilitates bacterial clearance and bacterial colonization, depending on whether it is in solution or adsorbed on a surface (Loimaranta et al. 2005). In solution SAG binds and mediates aggregation of bacteria (Brady et al. 1992; Loimaranta et al. 2005), whereas bound to HA SAG provides a site for bacterial colonization and microbial growth (Kishimoto et al. 1989; Brady et al. 1992).

SAG is encoded by *Deleted in Malignant Brains Tumors 1 (DMBT1)*, and is identical to the lung protein gp-340 and DMBT1, which was found in the brain (Holmskov et al. 1997; Mollenhauer et al. 1997; Ligtenberg et al. 2001). SAG is composed of 13 highly homologous Scavenger Receptor Cysteine-Rich (SRCR) domains, separated by glycosylated SRCR-interspersed domains, two CUB (C1r/C1s Uegf Bmp1) domains, separated by a 14th SRCR domain, and a Zona Pellucida domain (Holmskov et al. 1997; Mollenhauer et al. 1997; Bikker et al. 2002) and belongs to the highly conserved, SRCR superfamily (Holmskov et al. 1997; Mollenhauer et al. 1997). This group of glycoproteins comprises cell surface molecules as well as secreted proteins that are characterized by the presence of multiple SRCR domains showing broad ligand binding spectra (Resnich et al. 1994; End et al. 2009; Dzik 2010; Liu et al. 2011). In line, SAG/ gp-340/ DMBT1 exhibits a broad binding spectrum to a variety of molecules playing a role in innate immunity, for example SP-A, SPD and IgA (Ligtenberg et al. 2004; Ligtenberg et al. 2007; Leito et al. 2008; End et al. 2009; Hansen et al. 2011). Moreover, SAG/ gp-340/ DMBT1 interacts and aggregates several Gram-negative and Gram-positive bacteria including *Streptococcus mutans*, a bacterium related to dental caries (Tino and Wright 1999; Bikker et al. 2002), via binding to the cell wall-associated adhesin P1 (Brady et al. 1992; Ligtenberg et al. 2007; Madsen et al. 2010). Other studies showed that SAG/ gp-340/ DMBT1 interacts with dextran sulfate sodium (DSS) and carrageenan, a structurally similar sulfated polysaccharide, which is used as a texturizer and thickener in human dietary products (End et al. 2009). Furthermore, SAG/ gp-340/ DMBT1 binds to negatively charged poly-sulfated and poly-phosphorylated structures including typical

Chapter 2

pathogen-associated molecular patterns such as lipopolysaccharide (LPS), and lipoteichoic acid (LTA) (End et al. 2009).

In earlier work we found that bacteria binding and aggregation by SAG is mediated by a single peptide loop within the 14 conserved, unglycosylated SRCR domains designated SRCRP2 (P2) (Bikker et al. 2002; Bikker et al. 2004). Here we report that the SRCR domains may also harbour another peptide stretch with HA binding capacity, represented by SRCRP3 (P3). Subsequently, having identified the surface-binding moiety of SAG, it was investigated *in vitro* whether the peptide could be upgraded towards an antifouling peptide conjugate by covalently link moieties of hydrogel-forming polyethylene glycol (PEG) to P3. This way, P3 would serve as an anchor peptide immobilizing the peptide conjugate on the surface exposing the PEG to the environment as a repellent moiety for bacteria.

Material and Methods

Peptide synthesis

Based on the published amino acid sequence of SAG, a consensus sequence of the 13 SRCR domain was determined using alignment software (Vector NTI, InforMax Inc., Oxford, United Kingdom), as performed previously (Bikker et al. 2002; Bikker et al. 2004). Seven peptides, together spanning the complete consensus sequence of the SRCR domains of SAG, as well as the PEG-conjugated P3 peptide were synthesized by solid phase peptide synthesis using Fmoc chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Bioscience, Bedford, MA, USA) (Table 1). α,ω -Bis-carboxy poly(ethylene glycol) (PEG) MW 20.000 Dalton, (Iris Biotech GmbH, Marktredwitz, Germany) was used as conjugate on the N-terminus of peptide P3. Purification by Reverse Phase-HPLC and confirmation of authenticity by mass spectrometry were conducted as described previously (Veerman et al. 2007). In order to prevent dimerization by the formation of disulphide bridges, the C-terminal cysteines (Bikker et al. 2002) were replaced by alanine residues.

Peptides were purified by reversed-phase HPLC on a JASCO HPLC System (Tokyo, Japan). Peptides were dissolved in 0.1% trifluoroacetic acid and applied on a VYDAC C18-column (218TP, 1.0 x 25 cm, 10- μ m particles, Vydac, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid. Elution was performed with a linear gradient from 30 to 45% acetonitrile containing 0.1% trifluoroacetic acid in 20 min at a flow rate of 4 ml/min. The absorbance of the column effluent was monitored at 214 nm, and peak fractions were pooled, lyophilized, and reanalyzed by RP-HPLC. The purity of the peptides was at least 90%.

Peptide binding to HA

At a concentration of 50 μ M peptides were incubated with 50 mg of HA beads (0.075-0.180 mm, Merck, Darmstadt, Germany) in a total volume of 500 μ l saliva buffer (2 mM K_2HPO_4 , 50 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.0). 0.1% Tween-20 was added to prevent non specific binding. The suspension of HA and peptide was continuously mixed for 90 min at room temperature (RT) and then centrifuged at 10,000 g for 3 min to collect HA. In parallel, control samples for each peptide, containing peptide but no HA, were treated at same conditions as the test samples. The amount of peptide in the supernatant of the control and test samples of each peptide was determined by Capillary Zone Electrophoresis (CZE, Bio-Rad, Hercules, CA, USA)

Chapter 2

essentially as described earlier (Den Hertog et al. 2004). The run time for peptides was 20 min. The amount of peptide bound was quantified by comparative analysis of peak areas of control and test samples using BioFocus Integrator software (Bio-Rad). The difference in the areas of peaks of control and test samples of each peptide corresponded to the amount bound to the HA and is represented as μM of peptide bound to 10 mg of HA. These experiments were performed in duplicates and were repeated at least three times.

Bacteria

Streptococcus mutans (Ingbritt) was cultured on Tryptic Soy Agar (TSA) plates under anaerobic conditions with 5% CO_2 for 24 h at 37 °C. *Staphylococcus epidermidis* (clinical isolate) BM185 was cultured on TSA plates under aerobic conditions for 24 h at 37 °C. For binding studies cells were harvested and washed twice in Tris-buffered saline (TBS, 50 mM Tris, pH 7.5, containing 150 mM sodium chloride). Bacteria were diluted in buffer to a final OD_{700} of 0.5, corresponding with approximately 5×10^8 cells/ml, respectively.

Antifouling activity of peptide P3 and P3-PEG

The antifouling property of the peptides and conjugates was examined using a microtiter plate method based on labelling of microorganisms with cell-permeable DNA-binding probes (Bikker et al. 2002; Bikker et al. 2004). For this, microtiterplates Fluotrac 600 (Greiner, Recklinghausen, Germany) were coated with or without 50 μM of P3 and P3-PEG conjugate. 50 μM P2, which was previously identified as the bacteria binding moiety of SAG, (Bikker et al. 2002) was used as control peptide (Table 1). The peptides and P3-PEG conjugate were dissolved in coating buffer (100 mM sodium carbonate, pH 9.6). After incubation at 4 °C for 16 h, plates were washed twice with TBS containing 0.1% Tween 20 and 1 mM Ca^{2+} . Subsequently, 100 μl of a *S. mutans* or *S. epidermidis* suspension, corresponding to $\sim 5 \times 10^8$ bacteria / ml were added to each well and incubated for 2 h at 37 °C. Plates were washed two times with TBS supplemented with 0.1% Tween 20 using a plate washer (Mikrotek EL 403, Winooski, VT). Bound bacteria were detected using 100 μl /well of 1 μM SYTO-9 solution (Molecular Probes, Leiden, The Netherlands), a cell-permeable fluorescent DNA-binding probe. Plates were incubated in the dark for 15 min at ambient temperature and washed two times with TBS supplemented with 0.1% Tween-20. Fluorescence was measured in a fluorescence microtiter plate reader (Fluostar Galaxy, BMG Laboratories, Offenburg, Germany) at 488 nm excitation and 509 nm emission wavelength. These experiments were performed in triplicates and were repeated at least three times.

Hydroxyapatite binding domain of salivary agglutinin

As reference, the number of bacteria that bound to an uncoated well was determined by culturing. For this, bacteria were removed from the surface by vigorously and repeatedly pipetting 200µl of TBS along the surface of each well. Subsequently the solution was plated on TSA plates and incubated for 24 h at 37 °C. Finally, the number of colony forming units were determined.

Crystallographic analysis in silico

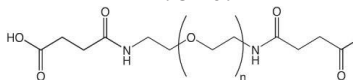
A model of the SRCR domain of SAG, represented by a crystal structure of the Mac-2 binding protein (Protein Data Bank accession code 1BY2), was studied using RasWin Molecular Graphics (www.rasmol.org) as described earlier (Bikker et al. 2002).

Results

Identification of the hydroxyapatite binding peptide of SAG

As SAG binds to HA (5) we hypothesized that, in parallel to the bacteria binding capacity of SAG, the SRCR domain may harbour a HA binding site. Therefore we synthesized peptides with confirmed identity, representing loops of the SRCR domains of SAG (Table 1). The peptides were incubated with HA granules, as a mimic of the mineral phase of dental enamel. The amount of bound peptide was analysed and calculated by CZE and integrated software. It was found that of all peptides tested, peptide SRCRP3-C, also designated as P3, showed high avidity for HA, compared to other SAG-derived peptides (Fig. 1). Virtually 10 μ M of P3 was bound by HA (Fig. 2).

Table 1. Peptides and the peptide-polyethylene glycol (PEG) conjugate used in this study

SAG peptide	Sequence	Residue numbers of peptides in the SRCR domain consensus sequence
SRCRP1-C/P1	GSESSLALRLVNGGDRA	1-17
SRCRP2-C/P2	QGRVEVLYRGSWGTVG	18-33
SRCRP3-C/P3	DDSWDTNDANVVCRQLGA	34-51
SRCRP4-C/P4	GWAMSAPGNARFGQGSPIVLDDVRA	52-77
SRCRP5-C/P5	SGHESYLWSA	78-87
SRCRP6-C/P6	PHNGWLSHNA	88-97
SRCRP7-C/P7	GHHEDAGVICSA	98-109
SRCRP3-C-PEG/ P3-PEG	DDSWDTNDANVVCRQLGA	

Sequences of salivary agglutinin glycoprotein (SAG)-derived scavenger receptor cysteine-rich (SRCR) consensus peptides were obtained from earlier studies (Bikker et al. 2002; Bikker et al. 2004).

Hydroxyapatite binding domain of salivary agglutinin

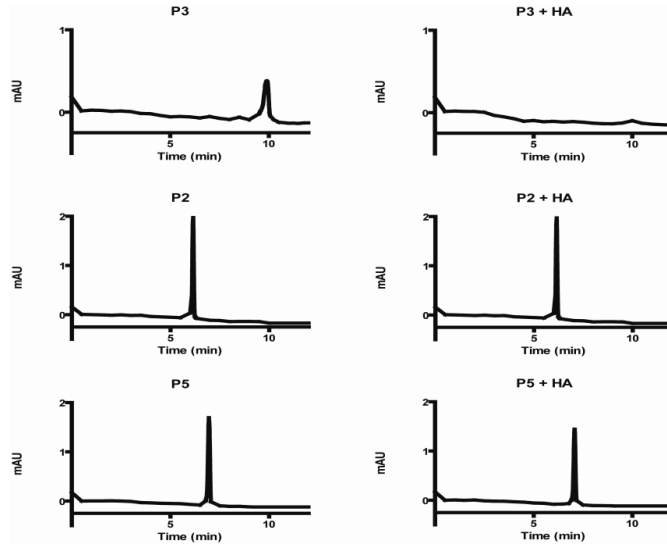


Figure 1. SAG derived SRCR consensus peptides were incubated with HA beads. After this, the amount of peptide in the supernatant was analysed with Capillary Zone Electrophoresis. (A) Only peptide P3 bound with high affinity to HA. Typical examples of non-binding peptides are (B) P2; (C) P5.

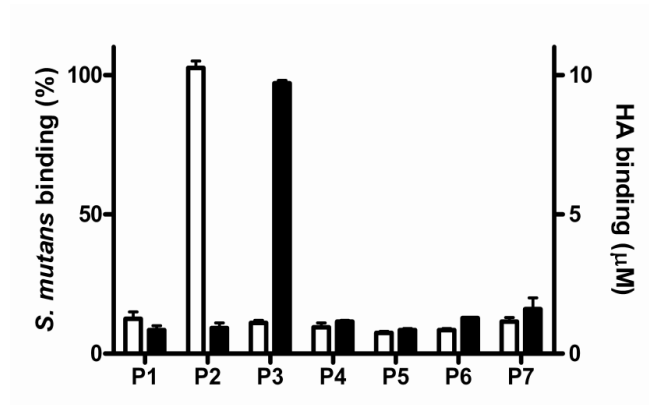


Figure 2. Adherence efficiency of SAG derived peptides to HA granules and *S. mutans*. The black bars depicts the amount of peptide bound to HA, represented as μM of peptide/10 mg in a total volume 1 ml. P3 clearly shows maximum binding to HA in comparison to the other peptides tested. Earlier it was discovered that only P2 bound to bacteria (white bars) (Bikker et al. 2002). The error bars represent the standard error.

Antifouling effect of a P3 and P3-PEG coating

P3 was selected as surface anchoring peptide for an antifouling conjugate SRCRP3-C-PEG (P3-PEG); P3 was conjugated to Polyethylene glycol (MW 20.000 Dalton) serving as a bacterial repellent moiety (Table 1). Both P3 and P3-PEG were investigated for anti-fouling properties *in vitro*. As proof of concept the bacteria repellent characteristics to *S. mutans* and *S. epidermidis* were observed in a solid-phase adherence assay, in which adhered bacteria were quantified using a fluorescent DNA stain, as described before (Bikker et al. 2002; Bikker et al. 2004). P2, which was previously identified as representative of the bacterial binding moiety of SAG (Bikker et al. 2002) was included as positive control. For both strains approximately 5×10^6 cells/well adhered to the surface of an uncoated microplate (not shown). Binding was enhanced by ~80% and ~50% for *S. mutans* and *S. epidermidis*, respectively by a coating of P2 (Fig. 3). In contrast, it was found that a coating with peptide P3 resulted in a decreased binding of bacterial cells to the surface of approximately 20% for both *S. mutans* and *S. epidermidis*. Finally, coating with the P3-PEG conjugate enhanced the antifouling activity of an uncoated surface, represented by a decreased amount of bound bacteria of 40% and 30% for *S. mutans* and *S. epidermidis*, respectively.

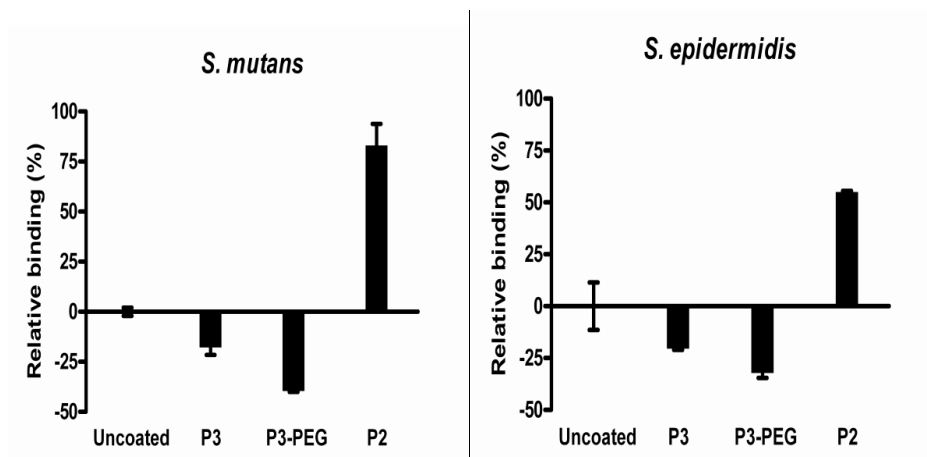


Figure 3. Relative bacterial binding to 50µM SAG-derived peptides, P2 (black), P3 (light gray), and pegylated P3 (P3-PEG, dark grey). A, binding to *S. mutans*; B, binding to *S. epidermidis*. The error bars represent the standard error.

Crystallographic analysis in silico

Using a crystallographic representation of a SRCR domain, the putative bacteria binding domain (P2) and putative HA binding domain (P3), were highlighted, showing an opposite orientation within the SRCR domain (Fig. 4A). Within the full SRCR domain all negatively charged amino acids were indicated (Fig. 4B): P3 contains 3 negatively charged amino acids, *i.e.* aspartate (D) residues D34, D35 and D38. Other negatively charged amino acids E101, D102 located at peptide P7, and D73 and D74 located at peptide P4. The orientation of the aspartate residues within P3, P4 and P7 putatively comprises the SRCR domains of SAG with a negatively charged region.

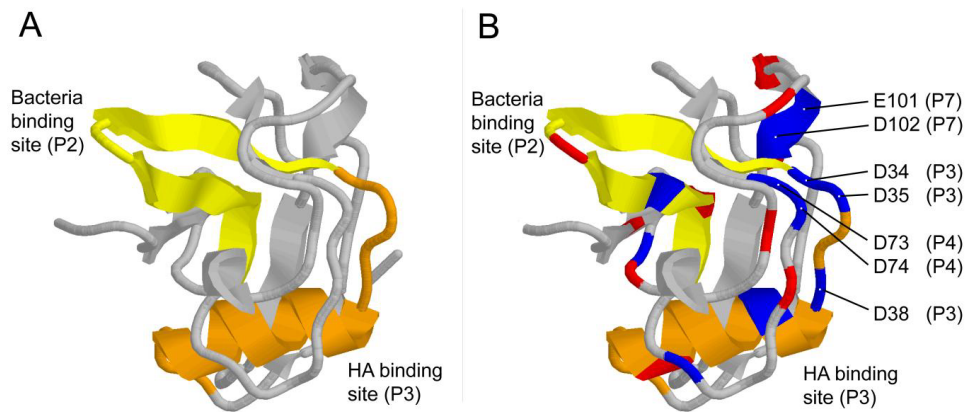


Figure 4. Schematic representation of the SRCR domain, highlighting the putative HA binding domain and bacteria-binding domain of SAG. (A) The bacteria binding stretch (P2, yellow) and hydroxyapatite binding stretch (P3, orange) are highlighted. (B); P3 contains 3 negatively charged amino acids, *i.e.* aspartate (D) residues D34, D35 and D38 which are putatively involved in HA binding. HA binding by P3 might be supported by other negatively charged amino acids E101, D102 located at P7, and D73 and D74 located at P4. The orientation of the aspartate residues within P3, but also P4 and P7 comprise the SRCR domains of SAG with a negatively charged region at, playing a role in HA binding. Positively charged residues are indicated in red.

Discussion

With regard to the microbial balance in the oral cavity SAG is believed to play a dual, even paradoxical role. On the one hand, in saliva SAG binds and agglutinates bacteria in solution. Thereby SAG promotes clearance from the oral cavity and prevents bacterial colonization *e.g.* on the tooth surface. On the other hand, adsorbed onto the tooth surface SAG promotes bacterial binding, the first step in colonization (Brady et al. 1992; Kishimoto et al. 1989; Loimaranta et al. 2005; Ligtenberg et al. 2007).

Previously we have been able to identify 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) (Fig. 2). The minimal bacteria-binding site on P2 was pinpointed to an 11-amino acid motif (GRVEVLYRGSW). Furthermore, an alanine substitution scan revealed that VEVL and Trp are critical residues in this motif (Bikker et al. 2004). Thus, the bacterial binding part of SAG had been mapped in great detail.

However, so far the putative HA binding part of SAG had not been elucidated. In order to achieve this goal, peptides were synthesized covering the complete SRCR consensus sequence of SAG, and their binding to HA was analyzed (Table 1). Of all peptides tested, only one 18-mer peptide, designated P3 (DDSWDTNDANVVCRLGA) was found to bind significantly to HA (Fig. 2). All other peptides hardly showed affinity to HA. Though, absence or weak binding by the other peptides does not necessarily mean that the corresponding domains are not involved in the binding of SAG to HA. It is possible that these peptides are derived from conformational domains of SAG and that their lack of conformational restraints leads to poor binding of the HA tested.

Based on crystallographic analysis *in silico* of a SRCR domain we could deduce that P2 and P3 are located at another site within the SRCR domain (Fig. 4A). Based on this observation it is tempting to hypothesize that the opposite orientation of P2 and P3 could contribute to the understanding of the dual role in bacterial homeostasis. We therefore postulate that both in saliva as well in the pellicle P2 plays a role in bacterial binding. And bound to HA, P3 putatively forms the surface anchoring part of a SRCR domain, exposing P2 to the microenvironment facilitating bacterial binding.

A detailed view on P3 reveals the presence of four Aspartic acid residues (D) within its sequence (Table 1), three of which *i.e.* D34, D35, D38, seem to be presented to its surface (Fig. 4B). Aspartic acid, which is negatively charged, has been described earlier as an amino acid which associates to HA via calcium ions. For example, both as single amino acid as well as part

of a Poly-aspartic acid peptide aspartic acid has been found to exhibit a remarkable affinity to HA (Guan et al. 2003; Murphy et al. 2007; Uddin et al. 2010). These observations underline the potential candidacy of P3 in HA binding by SAG *in situ*. Besides, *in silico* analysis revealed that four other negatively charged residues are exposed to the surface of an SRCR domain in the close vicinity of residues D34, D35, D38: aspartic acid D73, D74, situated on P4 and Glutamic acid E101 and Aspartic acid residue D102 on P7 (Fig. 4B). Based on these observations we postulate that these residues in concert contribute to HA binding of SAG.

In situ SAG plays a role in the microbial composition on the tooth surface (Carlen et al. 1998; Loimaranta et al. 2005). Now, having identified the surface-binding moiety of SAG, we were inspired to mimic SAGs bacterial modulating capacity *in vitro* by creating a P3-conjugate to influence microbial surface binding. In order to demonstrate this concept it was chosen to covalently link P3 to a moiety of PEG, a hydrophilic, bacterial repellent moiety macromolecule (Table 1) and study the antifouling activity of this peptide conjugate. Next to the cariogenic *S. mutans*, *S. epidermidis* was chosen as model organism. *S. epidermidis* is known to cause infections related to indwelling medical devices such as intravascular catheters, cerebrospinal fluid shunts, peritoneal dialysis catheters, intraocular lenses, cardiac pacemakers and prosthetic joints (Inman et al. 1984; Raad and Bodey 1992; Rupp and Archer 1994; Wang et al. 2009). *S. epidermidis* is an important member of the human skin microflora. When devices are implanted or manipulated *S. epidermidis* can easily be transmitted to the surfaces of these devices.

It was found that both P3 as well as P3-PEG, coated on a polystyrene surface repelled both *S. mutans* and *S. epidermidis* (Fig. 3). In line with our expectations, the P3-PEG showed a decrease in bacterial adherence, compared to the control. But, it was observed that P3 itself showed bacterial repelling characteristics as well, albeit to a lesser extent compared to P3-PEG. An explanation for this phenomenon could be that in contrast to the other SAG-peptides P3 exhibits a net negative charge (-3), potentially repelling the negatively charged bacterial membrane and membrane anchored structures such as LPS and LTA. Though, this needs to be addressed in future experiments. Apparently, the presence of an additional conjugated hydrophilic PEG moiety increased the intrinsic repellent capacity of the P3 peptide even more.

Chapter 2

Acknowledgements

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Chapter 2

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