CHAPTER 3

Sortase A as a tool to functionalize surfaces

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Abstract

A widely accepted approach to combat surface fouling is based on the prevention of biofoulants to attach to a surface by the functionalization with polyethyleneglycol (PEG). The goal of this study was to generate a proof of concept for the enzymatic coupling of PEG to a peptide pre-coated surface by using the enzyme Sortase A (SrtA). A hydrophobic polystyrene surface was primed with anchoring peptide P3 equipped with a pentaglycine acceptor motif for SrtA, to enable subsequent transpeptidation with either biotin, or a PEG-tail containing the sortase recognition motif LPETG. High levels of surface-bound biotin were detected only in cases with biotin-LPETG and SrtA. Little if any reactivity was detected in wells treated with the SrtA scrambled motif EGLTP, or in the absence of SrtA. Conjugation of PEG resulted in a significant decrease of bacterial adherence to the surface.

Introduction

The Sortase family of transpeptidase enzymes catalyzes sequence-specific ligation of proteins to the cell wall of Gram-positive bacteria. *In vivo* Sortase A (SrtA) covalently attaches proteins to the bacterial cell wall by cleaving the LPXTG motif between the threonine (T) and the glycine (G) and then links the carboxy terminus of the cleaved protein to the terminal amino group of penta-glycine cross-bridges in the peptidoglycan (Schneewind et al. 1992; Mazmanian et al. 1999; Ton-That et al. 1999; Bierne et al. 2002).

The enzyme activity of *Staphylococcus aureus* SrtA has been used *in vitro* to link various compounds displaying the C-terminal LPETG motif to compounds exposing either an N-terminal glycine motif or 6-aminohexose moiety (Samantaray et al. 2008; Levary et al. 2011). Examples include peptide nucleic acids, oligosaccharides, polyethylene glycol (PEG), lipids, fluorescent labels and Green Fluorescent Protein (GFP), streptavidin and alkaline phosphatase and has also been used for peptide cyclization (Parthasarathy et al. 2007; Pritz et al. 2007; Samantaray et al. 2008; Tanaka et al. 2008; Matsumoto et al. 2009; Sakamoto et al. 2010; Wu and Proft 2010; Bolscher et al. 2011; Popp et al. 2011).

Inspired by this straight forward methodology of enzyme coupling on the one hand and the broad applicability on the other hand it was investigated whether it was possible to generate a proof of concept to enzymatically functionalize a peptide pre-coated surface towards an antifouling surface. Earlier, Parthasantary and co workers, used SrtA to couple GFP to polystyrene beads which were chemically modified with either alkylamine or the *in vivo* SrtA ligand, Gly-Gly-Gly, on their surfaces (Parthasarathy et al. 2007). Besides, Chan and co-workers, modified various solid supports including cross linked polymer beads, beaded agarose, and planar glass surfaces with an oligoglycine motif by standard Fmoc chemistry and using the SrtA as coupling agent (Chan et al. 2007). Instead of direct modification of the surface of interest, in this study the surface was first coated with a surface binding peptide contained with a peptapeptide glycine motif, $(Gly)_5$P3. A part of this peptide, P3, was recently identified as the surface, *i.e.* hydroxyapatite, anchoring peptide of the salivary agglutinin glycoprotein (SAG) (Bikker et al. 2013). Polystyrene, which has a hydrophobic character, was chosen as model surface. After peptide coating, a hydrophilic, bacteria-repellent moiety of PEG equipped with a C-terminal LPETG motif was enzymatically coupled to the surface-bound peptide using SrtA. To analyze the potential altered bioadhesion characteristics, the effect on adhesion of *Yersinia pseudotuberculosis*, which readily adsorbs onto this type of surfaces, was studied (Paerregaard et al. 1991; El Tahir and Skurnik 2001).

Materials and Methods

Bacteria

Y. pseudotuberculosis (DSM 8992) was cultured on Tryptic Soy Agar (TSA) plates under anaerobic conditions maintained in tryptic soy broth (TSB) under aerobic conditions at 30°C. For binding studies cells were harvested by centrifugation for 10 min at 10,000x*g* at and washed twice in TRIS-buffered saline (TBS, 50 mM TRIS, pH 7.5, containing 150 mM NaCl) supplemented with 1 mM $CaCl_2$. Bacteria were diluted in buffer to a final OD_{600} of 0.5, corresponding with approximately 10^8 cells/ml.

Solid-phase peptide synthesis

The peptides and peptide conjugates, including the biotinylated peptides as well as the PEG-conjugated peptide were synthesized by solid phase peptide synthesis using Fmoc chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Biosearch, Bedford, MA, USA). Biotin (Biotin p-nitrophenyl ester,5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid p-nitrophenyl ester) (Novabiochem, Darmstadt, Germany) was used as conjugate. alpha,omega-Bis-carboxy poly(ethylene glycol) (PEG) MW 20.000 Dalton, (Iris Biotech GMBH, Marktredwitz, Germany) was used as conjugate. All peptides and peptide-conjugates used in this study are summarized in table 1.

Table 1. Peptides used[a].

Peptide	Sequence
$(Gly)_5$P3	HOOC-**GGGGG**DDSWDTNDANVVCRQLGA-NH_2
Biotin-LPETG	Biotin-**LPETG**-NH_2
Biotin-EGLTP	Biotin-**EGTLP**-NH_2
PEG-LPETG	PEG-**LPETG**-NH_2

[a]Sortase recognition motifs are indicated in bold. PEG; Polyethylene glycol (MW 20.000 Da).

Peptides and conjugates were purified by semi preparative RP-HPLC (Jasco Corp., Tokyo, Japan) on a Vydac C18-column (218MS510; Vydac, Hesperia, CA, USA). Peptides were dissolved in H_2O containing 5% acetonitrile (AcN; Biosolve) and 0.1% TFA. Elution was performed with a linear

gradient from 15 to 45% AcN containing 0.1% TFA 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 and lyophilized. Reanalysis by RP-HPLC on an analytic Vydac C18-column (218MS54) developed with a similar gradient at a flow rate of 1 ml/min revealed a purity of ≥ 95%. The authenticity was routinely confirmed by mass spectrometry (MS). Mass spectra were recorded with a Thermo LTQ ion-trap mass spectrometer in nanospray configuration (Thermo Fisher Scientific, Hampton, NH, USA) or a Microflex LRF matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer equipped with an additional gridless reflectron (Bruker Daltonik, Bremen, Germany). The purity of the peptides and conjugates was at least 90%.

Expression and purification of SrtA

A soluble version of SrtA was created as reported earlier comprising the catalytic domain of the *S. aureus* SrtA (aa 26 to 206) and a hexa-histidine tag at the N terminus (cloned in to pQE30; Qiagen, Valenica, CA, USA)(Bolscher et al. 2011). Briefly, the SrtA-expression plasmid containing *Escherichia coli* BL-21 (DE3) was cultured in the presence of 10 µg/ml ampicillin until OD600 ~ 0.7. SrtA production was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After an additional 3 h of culturing, bacteria were harvested by centrifugation at 3500x*g* at 4°C for 30 min and resuspended in ice-cold lysis buffer (50 mM TRIS-HCl, pH 7.5, containing 150 mM NaCl, 20 mM imidazole, and 10% glycerol). Bacteria were lysed by passing through a prechilled cell disruption press (One Shot Model; Constant Systems Ltd., Daventry Northants, UK) operating at 1250 kpsi (8618 MPa). The lysate was cleared by centrifugation at 14,000x*g* at 4°C for 30 min. The supernatants were subjected to affinity chromatography on a recharged Ni^{2+}-HisTrap HP column (GE Healthcare, Uppsala, Sweden). The column was washed extensively with lysis buffer containing 50 mM imidazole and eluted with lysis buffer containing 500 mM imidazole. The imidazole was removed by a buffer exchange step on a PD-10 desalting column (GE Healthcare). Purity was analyzed by SDS-PAGE; if necessary, affinity purification was repeated. The affinity-purified SrtA was stored in 10% glycerol, 50 mM TRIS-HCl (pH 8.0), and 150 mM NaCl at −80°C until further use. Alternatively, SrtA was purified directly from the HisTrap elution fractions, without buffer changes or imidazole removal, by semipreparative RP-HPLC, using a linear gradient from 25 to 45% AcN containing 0.1% TFA in 20 min at a flow rate of 4 ml/min. HPLC-purified SrtA was lyophilized and stored at −20°C. The latter method produced SrtA of >95% purity.

Determination of SrtA activity

SrtA activity was monitored using a FRET bacterial sortase substrate I, being LPETG equipped with the fluorophore EDANS at the C terminus and the quencher DABCYL at the N terminus (AnaSpec, Fremont, CA, USA), according to manufacturer's procedure (Bolscher et al. 2011). Fluorescence was monitored with a Fluostar Galaxy microplate fluorimeter (BMFG Labtechnologies, Offenburg, Germany).

Surface Sortase mediated conjugation of peptide $(Gly)_5P3$

Microtiterplates Fluotrac 600 (Greiner, Recklinghausen, Germany) were coated with 50µM peptide $(Gly)_5P3$, which was recently identified as a surface binding peptide with affinity for hydroxyapatite in 200 µl coating buffer (100 mM sodium carbonate, pH 9.6) (Bikker at al. 2013). After incubation at 4 °C for 16 h, plates were washed twice with 300 µl TBST to prevent non-specific binding and washed twice with sortase reaction buffer (50 mM TRIS, pH 7.5, containing 150 mM NaCl and 5 mM $CaCl_2$). Then the conjugates biotin-LPETG and biotin-EGLTP and PEG-LPETG dissolved in sortase reaction buffer were added. For conjugation 1/5 (w/w) SrtA was added and incubated at 37 °C for 16 hrs.

Detection of SrtA conjugated biotinylated peptides

Microplates with conjugated biotinylated peptides were washed three times with TBST. Then, 1:10.000 diluted HRP-Conjugated Streptavidin (Sanquin, Amsterdam, The Netherlands) was added and incubated for 1 h at RT. After thee washes with TBST peroxidase activity was detected after incubation with a solution containing 3,3′,5,5′-tetramethylbenzidine (10 mg/ml) and 0.5 µL of hydrogen peroxide (30%) in 0.1 M NaOAc and 0.1 M citric acid at pH 4. The color reaction was stopped by adding 25 µL of 4 M H_2SO_4, and the absorbance was read at 450 nm with a microplate reader. The assay was performed twice in triplicate.

Antifouling assessment of the SrtA conjugated PEG-LPETG coating

Antifouling activity of the SrtA functionalized coating was examined essentially as described earlier using a microtiter plate method based on labeling of microorganisms with cell-permeable DNA-binding probes (Bikker et al. 2002; Bikker et al. 2013). For this 200µl ~10^8 cells/ml *Y. pseudotuberculosis* supplemented with 1 µM SYTO-13 solution (Molecular Probes, Leiden, The Netherlands), a cell-permeable fluorescent DNA-binding probe, was added to each well and incubated for 3 h at 37°C at 150 rpm. In order to prevent evaporation, the microplate

was sealed. Subsequently plates were washed twice with TBS supplemented 1mM $CaCl_2$ using a plate washer (Mikrotek EL 403, Winooski, VT). Bound bacteria were detected using by measuring fluorescence 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 duplicates and were repeated at least three times.

Results

Pre-coated surface conjugation by Sortase

Peptide $(Gly)_5P3$ was coated on a microplate, as represented by Figure 1A. Then, after washing, the biotinylated peptides were added, possessing either the normal sortase motif (LPETG) or the scrambled sortase motif (EGLTP), which was used as control substrate. At the same time SrtA was added to enable the formation of a covalent amide bond between the α-carboxyl group of the N-terminal glycine of peptide $(Gly)_5P3$ (HOOC-GGGGGDDSWDTNDANVVCRQLGA-NH2) and the α-amino group of the C-terminus of threonine of peptide Biotin–LPETG (Biotin-LPETG-NH_2), removing the N-terminal glycine (Figure 1B). After washing, to remove the uncoupled peptides, the presence of coupled biotin was detected using streptavidin-HRP (Figure 1C). High levels of surface-bound biotin were only found in the wells that were treated with biotin-LPETG and SrtA (Figure 2). Little if any reactivity was detected in wells treated with the scrambled motif, or in the absence of SrtA. These data clearly indicate that SrtA conjugation using the normal SrtA sequence was successful.

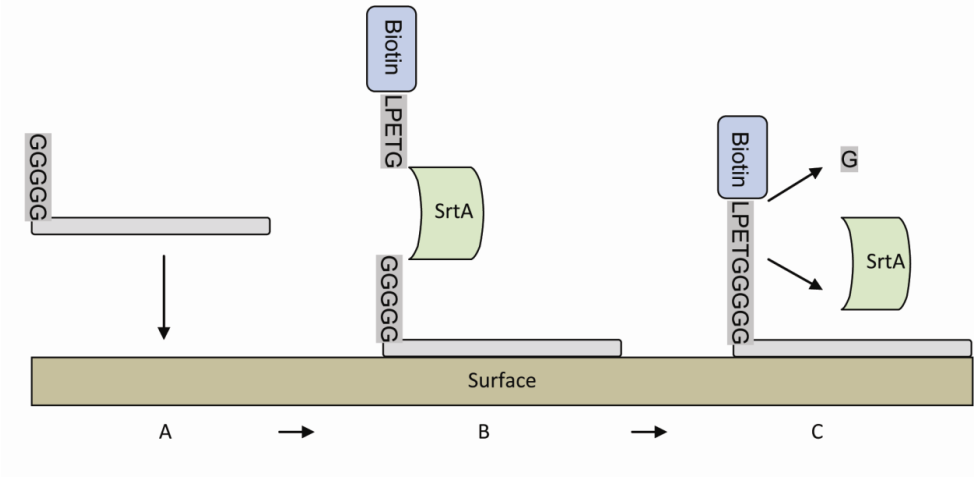

Figure 1. Schematic representation of surface functionalization of a peptide coating by SrtA. A: at first peptide $(Gly)_5P3$ adheres to the surface. B: Secondly, the peptide containing a N-terminal LPETG motif and SrtA are added to facilitate conjugation. C: Upon conjugation, the N-terminal glycine is removed and both peptides are covalently coupled

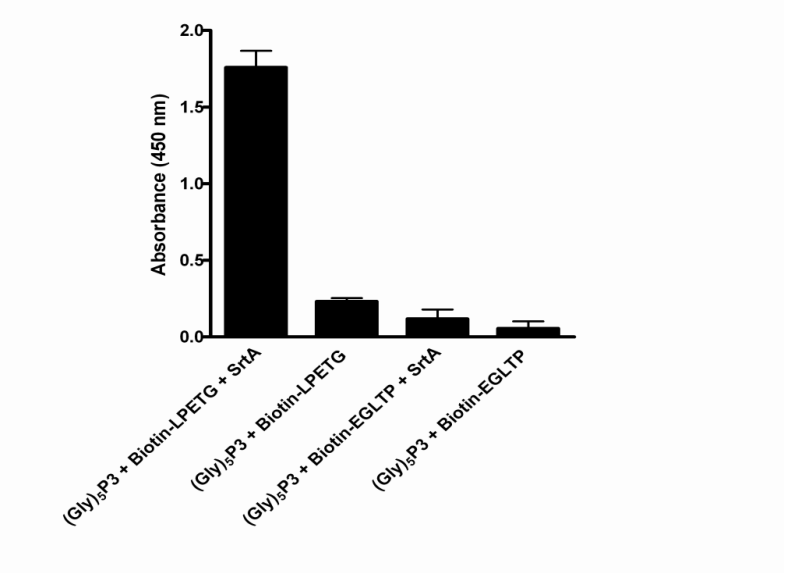

Figure 2. Enzymatic conjugation of surface binding peptide $(Gly)_5$P3 to biotinylated-sortase peptides: Biotin-LPETG (normal) and Biotin-EGLTP (scrambled) in the presence and absence of Sortase A. The presence of biotin was detected using a HRP-streptavidin conjugate. The error bars represent the standard error.

Antifouling analysis of SrtA mediated PEGylated surface

To examine if this strategy is feasible to change the surface characteristics, we aimed to PEGylate the surface, using SrtA in combination with a PEG-LPETG conjugate. Again, $(Gly)_5$P3 was coated on a microplate. Then, after washing, PEG–LPETG was added in the presence of SrtA to enable the formation of a covalent amide bond between the N-terminal glycine of peptide $(Gly)_5$P3 and the α-amino group of the C-terminus of threonine of PEG–LPETG (PEG-LPETG-NH2), removing the N-terminal glycine. After removal of the non-bound material, the bacteria repellent characteristics of the surface to *Y. pseudotuberculosis* in a solid-phase adherence assay was evaluated. Peptide P2, which was previously identified as representative of the bacterial binding moiety of SAG was included as positive control (Bikker et al. 2002; Bikker et al. 2013). Bacteria adhered to the surface of an uncoated microplate. Binding was enhanced by ~45% by a coating of 50 μM P2 (Figure 3). In contrast, it was found that the presence of the $(Gly)_5$P3-PEG conjugate resulted in a decreased binding of bacterial cells to the surface of approximately 30% compared to the uncoated surface (Figure 3). In the absence of SrtA, no significant antifouling activity was found.

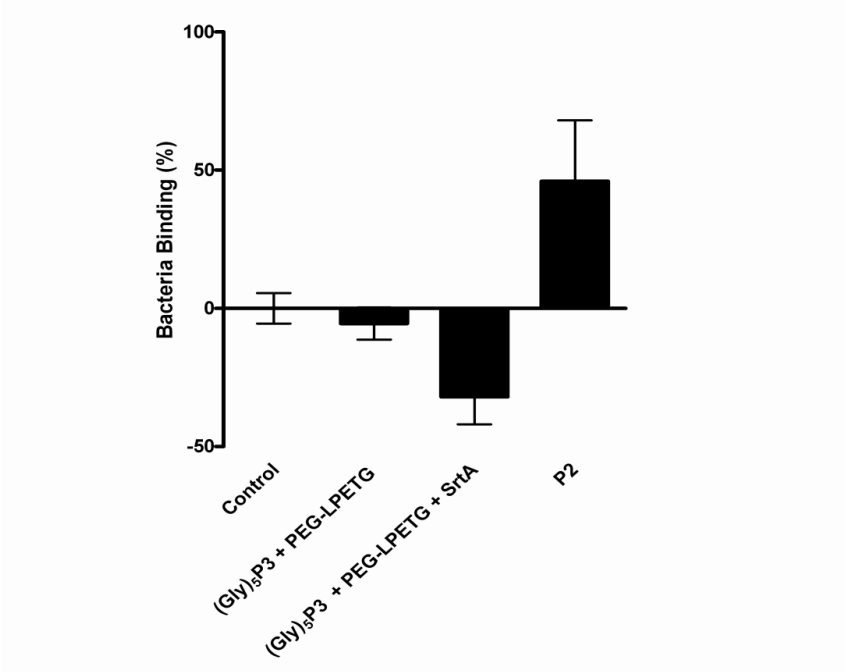

Figure 3. Antifouling activity of PEG-LPETG conjugated to surface coated $(Gly)_5P3$ by Sortase. P2 was used as positive control (Bikker et al. 2002). The error bars represent the standard error.

Discussion

The goal of this study was to generate a proof of concept for the enzymatic coupling of an antifouling moiety to a peptide pre-coated surface by using SrtA. A widely accepted approach to combat surface fouling is based on the prevention of biofoulants such as bacteria to attach to a surface by the functionalization with PEG. So far, physical adsorption, chemical adsorption, direct covalent attachment and block or graft copolymerization are some of the techniques that have been used to attach PEG to surfaces covalently (Llanos and Sefton 1993; Gabriel et al. 2006; Banerjee et al. 2011). Alternatively, also non-covalently bound biomimetic antifouling polymers have been developed. For example, Dalsin and co-workers used 3,4-dihydroxyphenylalanine as surface anchoring moiety to tether PEG to titanium dioxide (Dalsin et al. 2003).

Polystyrene surfaces have a hydrophobic character and bind cells and biomolecules through hydrophobic interactions. Because of its hydrophilic character, PEG has no affinity for hydrophobic surfaces. So, the surface was primed with a peptide equipped with the pentaglycine acceptor motif of sortase, to enable subsequent transpeptidation with a PEG-tail containing the sortase recognition motif LPETG. The polystyrene plates which were used in this study are high binding polystyrene surfaces showing affinity for bacteria, including *Y. pseudotuberculosis* (Paerregaard et al. 1991; El Tahir and Skurnik 2001). By SrtA mediated conjugation of anchoring peptide $(Gly)_5$P3, *i.e.* a variant of surface binding peptide P3, to PEG-LPETG it was hypothesized that *Y. pseudotuberculosis* binding to the polystyrene would be influenced since PEG has, in contrast to polystyrene, an hydrophilic character. Indeed, it was found that the SrtA mediated conjugation of PEG resulted in a significant decrease of bacterial adherence (Figure 3) (Bikker et al. 2013). These data strongly suggest that, PEG was successfully conjugated to the precoated $(Gly)_5$P3 peptide.

In summary, so far SrtA has been used widely for linkage and functionalization of various biomolecules compounds (Parthasarathy et al. 2007; Samantaray et al. 2008; Matsumoto et al. 2009; Wu and Proft et al. 2010; Bolscher et al. 2011; Levary et al. 2011;). Though, to our knowledge we are the first who have used SrtA to generate an antifouling conjugate *in vitro*, under mild conditions. This strategy offers promise for directed functionalization of biomedical materials which are not amenable for direct covalent linkage.

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