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ORIGINAL RESEARCH PAPER

Immobilization of proteins to biacore sensor chips using *Staphylococcus aureus* sortase A

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Abstract The immobilization of proteins to surfaces is an active area of research due to strong interest in protein-based sensors. Here, we describe a novel method for immobilizing ligand proteins onto Biacore sensor chips using the transpeptidase activity of *Staphylococcus aureus* sortase A (SrtA). This method provides a robust and gentle approach for the site-directed, covalent coupling of proteins to biosensor chips. Notably, the high specificity of the sortase allows immobilization of proteins from less than pure protein samples allowing short cuts in protein purification protocols.

Keywords Biacore · Biosensor · Fibronectin-binding protein · Human factor H · Plasmon surface resonance · Protein immobilization · Sortase

Introduction

The Biacore system is a real-time bio-sensor based on surface plasmon resonance allowing accurate analysis of protein binding specificity, kinetics and affinity (http://www.biacore.com/lifesciences/index.html). Biacore sensor chips usually consist of a carboxymethylated dextran matrix covalently attached to a gold surface.

Ligand proteins must be firmly attached to the solid surface allowing the conservation of a reproducible chip surface after extensive washing. Furthermore, the immobilization must allow interacting molecules (analyte) to access and bind to the ligand protein efficiently. Chemical approaches where reactive groups, such as carboxyl-, amino- or sulfide groups are covalently linked to complementary groups on the solid surface (Tomizaki et al. 2005), occasionally lack specificity resulting in randomly orientated molecules on the chip surface. As a result, the binding site for the analyte might not be accessible for a number of immobilized molecules. In addition, the proteins have to be of great purity to allow selective immobilization and avoid coupling of contaminating proteins. A more uniform alignment to the chip surface can be achieved by site-specific immobilization via an affinity tag, e.g. protein fusions with histidine (His) tags (Schmid et al. 1997) or the immobilization of antibodies by the use of protein A or G (Wang and Jin 2003). However, these methods also have drawbacks. Disadvantages of the His-tag system are metal-dependent nonspecific protein adsorption and low affinity of the His-tag to the Ni^{2+}-NTA complex ($K_d = 10$ μM) whereas protein A-mediated immobilization lacks the control on the orientation of protein A itself (Rusmini et al. 2007).

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Recent studies have shown that *Staphylococcus aureus* sortase A (SrtA) provides a robust and gentle approach for the selective and covalent immobilization of proteins to solid surfaces (Chan et al. 2007; Parthasarathy et al. 2007). SrtA is a transpeptidase that cleaves precurser proteins between a threonine and a glycine residue within a conserved LPXTG motif near the *C*-terminus and covalently links it to the terminal amino group of the penta-glycine cross-bridges in peptidoglycan (Marraffini et al. 2006). This reaction can be performed in-vitro using oligo-glycine in solution (Ton-That et al. 2000) or attached to a solid surface via the terminal carboxyl group (Chan et al. 2007; Parthasarathy et al. 2007).

Here, we report the successful use of sortase A in the covalent immobilization of a protein onto a CM5 biosensor sensor chip followed by analysis of the ligand-analyte interaction using a Biacore 2000. For this analysis, we have chosen a recombinant form of a streptococcal cell wall-attached protein to which we fused a *C*-terminal LPETG SrtA recognition motif. A fibronectin-binding protein (Fba) from group A streptococcus (GAS) was described by Terao et al. (2001), who demonstrated its binding to human fibronectin in an in vitro assay. It was later found that Fba also mediated binding of human factor H (FH) and FH-like protein 1 to the GAS cell surface (Pandiripally et al. 2003).

Materials and methods

Cloning, expression and purification of recombinant SrtA and rFba-LPETG

The *srtA* gene, without the region encoding for the signal peptide, was amplified from *Staphylococcus aureus* RN4220 genomic DNA by PCR using SrtA.fw and SrtA.rev primers (Table 1) and Taq polymerase (Promega, Corp.). The *fba* gene without the regions encoding the signal peptide and the sortase sorting domain was amplified from *S. pyogenes* SF370 (serotype M1, ATCC 700294) genomic DNA using Fba.fw and Fba-LPETG.rev primers (Table 1). The PCR fragments were cloned into the BamHI/EcoRI restriction sites of pGEX-3c (a modified version of pGEX-2T, Pharmacia) (Proft et al. 2001). Recombinant SrtA and rFba-LPETG were expressed in *E. coli* DH5α as glutathione-*S*-transferase (GST) fusion proteins and were purified on glutathione (GSH) agarose, as previously described (Proft et al. 2001). The mature proteins were cleaved off from GST with picornavirus protease 3c. Recombinant SrtA was finally purified by cation exchange chromatography using carboxymethyl Sepharose and dialyzed against 25 mM Tris/HCl pH 7.4, 50 mM NaCl.

Coupling of tri-glycine linker peptide to the CM5 biosensor chip

The tri-glycine linker peptide (HOOC-Cys-Ser-Ser-Gly-Gly-Gly-NH_2, EZBiolab, Westfield, USA) was attached to the carboxymethylated dextran surface of a CM5 biosensor chip by thiol-coupling. In brief, the flow cell surface was modified using thiol coupling reagent, 2-(2-pyridinyldithio) ethaneamine (PDEA, 22.5 mg ml^{-1} in 0.1 M borate buffer pH 8.5), following activation with N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS), as described in the Biacore Application Note 9. The Gly-linker peptide was coupled at 10 μg ml^{-1} (10 mM sodium-formate pH 4.3) by multiple injections over 30 min at 5 μl min^{-1}. Excess reactive groups were deactivated using 50 mM L-Cysteine, 1 M NaCl, 100 mM sodium-formate pH 4.3. Gly-linker peptide was also coupled to a second flow cell to equivalent response levels and used as a negative control surface.

Table 1 List of primers used for PCR amplification

Primer	Sequence	Site
SrtA.fw	**GGATCC**AAACCACATATCGATAATTATC	*Bam*HI
SrtA.rev	**GAATTC**TTATTTGACTTCTGTAGCTAC	*Eco*RI
Fba-LPETG.fw	**GGATCC**GTAGATGGCATCCCTCC	*Bam*HI
Fba-LPETG.rev	**GAATTC**GAACCAGTTTCCGGGAGAGATCTACCTGACTCATGGGCC	*Eco*RI

Immobilization of rFba-LPETG to the CM5 biosensor chip

For immobilization of the ligand, an equimolar mixture of rFba-LPETG and GST was mixed with rSrtA in sortase reaction buffer (25mM Tris/HCl pH7.4, 50mM NaCl, 2mM $CaCl_2$). (Note that the GST part was not removed after digestion of the GST-Fba fusion protein with 3c protease.) The final concentrations were 4 mg rFba/GST ml^{-1} and 1.3 mg SrtA ml^{-1}. The mix was run over the CM5 sensor chip at 5 µl min^{-1} for 2h at 37°C or 50 µl of the reaction mix was dropped onto the chip surface and incubated in a sealed Petri dish overnight at 37°C. After the immobilization step, the chip was washed with 1 M NaCl at 20 µl min^{-1} until the baseline steadied.

Purification of human factor H (FH) from human plasma

FH was purified from human plasma according to the method of Licht et al. (2006). In brief, 20 ml human plasma was precipitated with 13% (v/v) PEG 6000 (polyethylenglycol, MW 6000 Da). The pellet was dissolved in 10 ml buffer A (10 mM sodium phosphate pH 7.3, 0.1 M glycine) and applied to heparin affinity chromatography using a column packed with 5 ml heparin-agarose (Sigma). After extensive washing with buffer A, FH was eluted with a gradient of NaCl from 30 to 500 mM. Fractions containing FH were pooled, dialyzed against buffer A and applied to anion exchange chromatography using a Poros 10HQ column on an AKTA explora FPLC system (Amersham Biosystem). Bound proteins were eluted with a linear salt gradient ranging from 30 to 500 mM NaCl. Purified FH was dialyzed against running buffer (25 mM Tris/HCl pH 8.0, 50 mM NaCl) and diluted with running buffer as required.

Analysis of FH binding to immobilized rFba on the CM5 biosensor chip

The binding of human FH to immobilized Fba was analyzed using a Biacore 2000 biosensor. Various dilutions of the analyte with or without heparin were injected for 2.5 min at 20 µg ml^{-1}.

The CM5 chip surface was regenerated by passing through 7 mg heparin ml^{-1}. Removal of FH resulted in a chip surface that was fully functional after storage at 4°C for 2 months.

Results and discussion

Recombinant mature SrtA (without the *N*-terminal signal peptide) was produced as GST fusion in *E. coli*. After affinity purification of the fusion protein, the GST part was cleaved off using a highly specific picornavirus 3c protease, and rSrtA was finally purified by cation exchange chromatography to a final yield of about 15 mg l^{-1} of *E. coli* culture. Recombinant Fba, without the *N*-terminal signal peptide and the *C*-terminal cell wall anchor domain, but with a *C*-terminal LPETG SrtA recognition motif, was produced using the same protocol. In contrast to rSrtA, the GST part was not removed after protease treatment, as the SrtA reaction is highly specific and therefore does not require very pure protein preparations.

We have successfully used the sortase-mediated reaction to site-specifically immobilize rFba-LPETG to the surface of a CM5 sensor chip (Fig. 1). First, a tri-glycine linker peptide was attached to the carboxymethylated dextran surface of a CM5 biosensor chip by thiol-coupling. This provided a suitable surface with *N*-terminal Gly residues as acceptor molecules for the SrtA mediated transpeptidation with rFba-LPETG. Coupling of the Gly-linker peptide resulted in an increase of 370 response units (RU). In a second step, a mixture of rFba-LPETG, GST and rSrtA was ran over the chip for 2h at 37°C. Note that the removal of GST part after digestion of the GST-Fba fusion protein was not necessary, as the SrtA reaction is highly specific (Ton-That et al. 1999). However, due to the slow kinetics of the sortase reaction, the coupling reaction was unsuccessful, even at the lowest flow rate of 5 µl per min. To overcome this problem, 50 µl enzyme/substrate mix were applied onto the chip surface and incubated over night at 37°C in a sealed Petri dish. This resulted in an increase of 34,600 RU compared to the untreated CM5 chip surface. No increase in RUs was observed when rSrtA was excluded from the coupling mix.

To confirm the site directed immobilization of rFba, human factor H (FH) was used as an analyte. FH was purified from human plasma and was

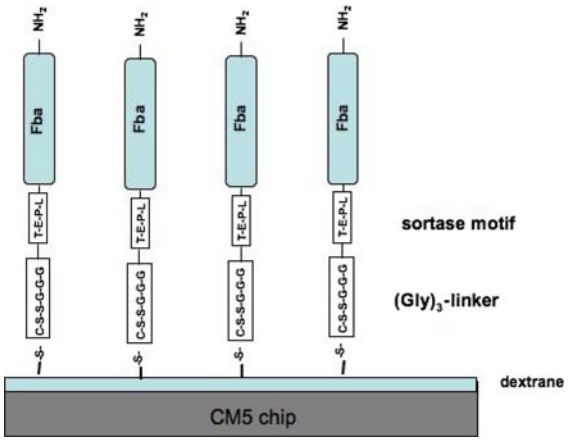

Fig. 1 Schematic presentation of a Biacore CM5 biosensor chip coupled with rFba-LPETG using the enzymatic activity of rSrtA

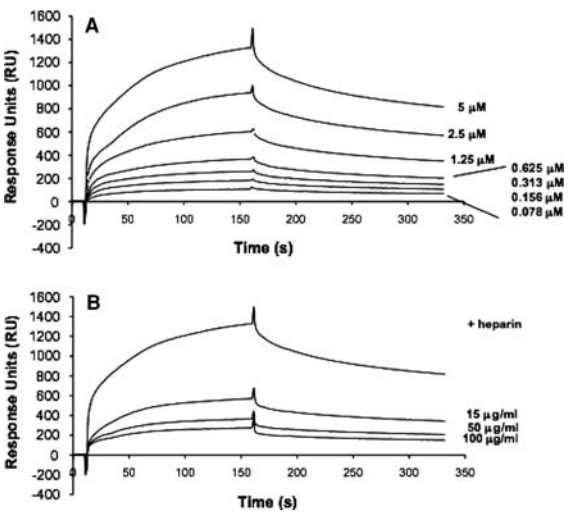

Fig. 2 Biacore analysis of immobilized rFba-LPETG on a CM5 biosensor chip and factor H (FH) purified from human plasma. Recombinant Fba-LPETG was linked to the CM5 chip via a pre-immobilized $(Gly)_3$-linker peptide in a sortase-mediated reaction. FH (5 µM, 2.5 µM, 1.25 µM, 0.625 µM, 0.3125 µM, 0.156 µM, and 0.078 µM) binds to rFba in a concentration-dependent manner (**a**) and competes for binding with heparin (**b**)

dialyzed against running buffer. Purified FH bound to the rFba-coated sensor chip in a concentration-dependent manner (Fig. 2a). FH dissociated slowly from the immobilized rFba over 15 min and could be completely removed from the chip with 7 mg heparin ml^{-1} (data not shown). The specific binding of FH to the immobilized rFba could also be demonstrated by adding heparin to the analyte (Fig. 2b). The addition of 15 µg heparin ml^{-1} to a 5 µM FH solution resulted in significantly reduced binding, whereas the addition of 100 µg heparin ml^{-1} almost completely abolished FH binding. Following removal of FH, the chip surface remained fully functional after storage at 4°C for 2 months.

In conclusion, we present a method for a site-directed immobilization of recombinant proteins via their *C*-terminus to a biosensor chip. This method has some advantages over other immobilization techniques. The site-specific coupling of the ligand allows the protein to be orientated uniformly on the chip surface in such a way that the *N*-termini are exposed to the analyte solution. Therefore, this method is useful for the analysis of proteins with specific binding sites in their *N*-terminal domains, e.g. proteins bound to the cell surface via their *C*-terminus. The covalent coupling allows for more stringent washing steps compared to, e.g. His-tagged proteins. Most of all, the high specificity of the sortase allows immobilization of proteins from less than pure protein samples allowing short cuts in protein purification protocols. Furthermore, the enzyme, SrtA, is easy to produce in large amounts, which makes this method very cost efficient. A current drawback of the method is that the amount of immobilized protein cannot be controlled, as the chip is coupled outside the machine. This could be circumvented by technical improvements of the Biacore to allow for smaller flow rates or stop the flow altogether. Alternatively, coupling could be controlled by restricting the amount of the $(Gly)_3$ linker coupled to the chip.

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