Protein Immobilization Hot Paper

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# Enzyme-Mediated, Site-Specific Protein Coupling Strategies for Surface-Based Binding Assays

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***Abstract:*** *Covalent surface immobilization of proteins for binding assays is typically performed non-specifically via lysine residues. However, receptors that either have lysines near their binding pockets, or whose presence at the sensor surface is electrostatically disfavoured, can be hard to probe. To overcome these limitations and to improve the homogeneity of surface functionalization, we adapted and optimized three different enzymatic coupling strategies (4′-phosphopantetheinyl transferase, sortase A, and asparaginyl endopeptidase) for biolayer interferometry surface modification. All of these enzymes can be used to site-specifically and covalently ligate proteins of interest via short recognition sequences. The enzymes function under mild conditions and thus immobilization does not affect the receptors' functionality. We successfully employed this enzymatic surface functionalization approach to study the binding kinetics of two different receptor–ligand pairs.*

The binding properties of receptor–ligand complexes have been studied in vitro with numerous assays developed during the last decades.[1–3] Mainly, covalent approaches for receptor immobilization have been established to precisely determine on-rate ($k_{on}$), off-rate ($k_{off}$), and equilibrium constant ($K_d$).[4] For these methods, the receptor is immobilized onto a surface and a change in signal upon ligand application is evaluated. While sometimes the terminology "ligand-analyte" is used, throughout this article the molecule immobilized to the sensor surface is called the receptor and its binding partner the ligand. In general, accessible side chains of corresponding amino acids (amine-, carboxyl-, or thiol-groups)[5] can be employed to covalently link the receptor to a surface. However, non-specific attachment requires an electrostatically driven surface pre-concentration step, where the pH and salt conditions of the buffer must be chosen such that the sensor surface and the receptors are predominantly oppositely charged. This pre-concentration step requires a buffer of low ionic strength in order to prevent screening of surface charges, which in turn may cause unfolding and aggregation issues.[6] Additionally, proteins with a low isoelectric point might not be sufficiently protonated, and thus remain negatively charged.

Another challenge with non-specific surface chemistry is that proteins often contain more than one reactive residue, which leads to inhomogeneous surface anchoring. Consequently, sensorgrams of a binding experiment represent the superimposed response of multiple populations of differently attached receptors. Varying attachment sites may strongly influence binding kinetics solely due to the molecules' orientation. The binding behaviour can be altered or binding may even be prevented, especially with receptors immobilized via reactive residues close to their binding interface (Figure 1 A).[7]

In this study, we expand the toolbox for surface functionalization by adapting advances in enzyme-based protein modification strategies to overcome the limitations of non-specific pull-down strategies in binding assays. The employed enzymes are a 4′-phosphopantetheinyl transferase from *Bacillus subtilis* (Sfp),[8] an evolved sortase A (SrtA) from *Staphylococcus aureus* (d59SrtA, P94R/D160N/D165A/K190E/K196T),[9] and an engineered asparaginyl endopeptidase from the plant *Oldenlandia affinis* (OaAEP1) (C247A).[10] All of these enzymes recognize specific amino acid sequences (tags) and covalently attach these tags to other

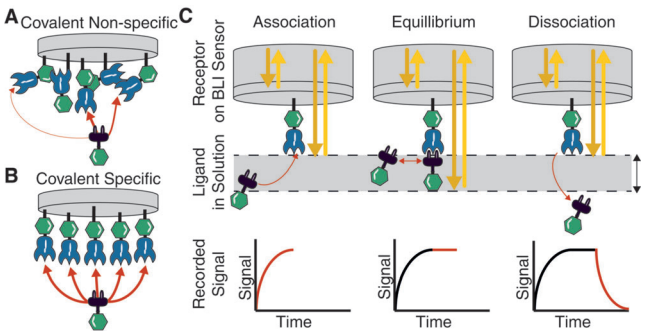


***Figure 1.*** Schematic of BLI Kinetics. A) Non-specific immobilization of the receptor on the sensor in different geometries as a result of several accessible amine-groups. B) Specific and site-directed immobilization of a receptor to a sensor. All receptors are homogeneously orientated. The red arrows in (A) represent different binding geometries with possibly different kinetics, whereas specific attachment (B) provides a uniform population of binders. C) The principle of a BLI kinetic experiment. A receptor-functionalized sensor is immersed into a ligand solution. The increasing signal shows binding of the ligand. When the sensor signal has reached a steady state, the rates of ligand association and dissociation are equal—the system has reached equilibrium. The sensor is then moved to a buffer solution, the receptor starts to dissociate and the detected signal decreases again.

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amino acid sequences (SrtA and OaAEP1) or to Coenzyme A (CoA; Sfp). In case of SrtA and OaAEP1, the tags have to be at the termini of the protein, whereas the ybbR-tag (11 amino acids) recognized by Sfp can also be internal (if accessible) since its ligation mechanism does not rely on peptidase activity. These tags can be fused to proteins and employed in surface pull-down strategies, hence allowing homogeneous loading of a surface (Figure 1B). In single-molecule approaches, such as single-molecule force spectroscopy, site-specific reactions[11–13] are already well established and ensure reliable mechano-probing of receptor–ligand systems without removing the proteins from the surface or cantilever. We adapted these enzyme-based techniques, which enabled us to link a receptor of interest to a sensor surface in very mild reaction conditions while using only low micromolar quantities of receptor.

We chose a biolayer interferometer (BLI) as a development platform because of its fast and flexible assay format. However, it should be noted that the approach presented here is applicable to other surface sensitive techniques, such as surface plasmon resonance (SPR) or quartz crystal microbalance (QCM), since the receptor immobilization relies on the same chemistry. The underlying principle of a BLI makes use of light reflection at interfaces between media of different optical densities to analyse the spectral shift of interference signals upon binding—which effectively modifies the optical path length—to the sensor.[14,15] The interference signal changes whenever binding/unbinding to the sensor fibre occurs (Figure 1C).

In order to establish our enzyme-based BLI binding assays, we selected two different systems (Table S1, Figure S1 in the Supporting Information). Firstly, we chose GFP-binding nanobodies (LaG9).[16] Nanobodies are small functional single-chain antibodies[17] and are popular tools in diagnostic as well as in therapeutic applications. As a second system, we chose the mechanically highly robust cohesin–dockerin type III complex (CohE–XDocIII) from *Ruminococcus flavefaciens*. As previous single-molecule force spectroscopy studies have shown, its unbinding behaviour under force depends on the anchoring geometry of the cohesin. When immobilized via its C-terminus, a most probable rupture force of around 700 pN (at 100 nN s$^{-1}$)[18] is observed, in contrast to only 100 pN (at 0.7 nN s$^{-1}$)[19] when anchored via its N-terminus. With the site-specific immobilization strategies presented here (Figure 2), we were able to probe the geometry dependence in the absence of force.

Experimental details, traces for Sfp-, SrtA- and OaAEP1-based sensor modifications (Figure S4–S19), and an overview of all possible immobilization geometries (Figure S20) can be found in the Supporting Information. Once the sensors were site-specifically loaded with the protein of interest, they were equilibrated in the same measuring buffer throughout all experiments.

In order to compare the different immobilization strategies, a kinetic binding series with each coupling approach was recorded. Figure 3A shows an example sensorgram of an SrtA-based experiment. Despite using another GFP variant which differs in the binding epitope (Figure S21), we obtained similar binding kinetics to the reported ones ($K_d = 3.5$ nM,

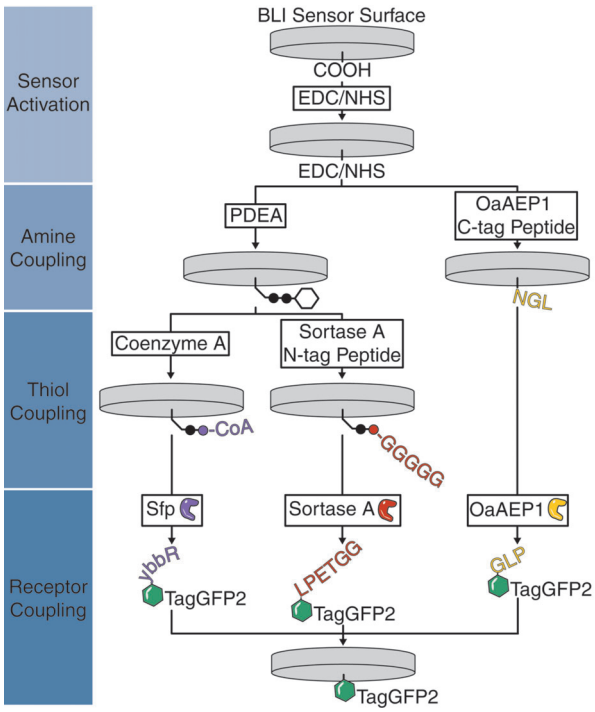
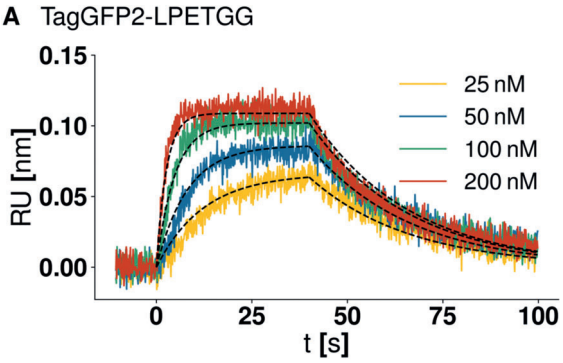



***Figure 2.*** Overview of the different covalent, site-specific immobilization techniques. Left: Sfp catalyses the reaction between ybbR-tag of the TagGFP2 and coenzyme a (CoA). First, the amine-group of PDEA reacts with the EDC/NHS-activated carboxyl groups of the sensor. PDEA can then undergo a thiol exchange reaction with CoA, which presents a free thiol. Middle: SrtA links C-terminal LPETGG with N-terminal GGG. In the case shown here, a C-GGGGG peptide was reacted with the EDC/NHS-PDEA-activated sensor. Right: OaAEP1 recognizes the C-terminal amino acids NGL and fuses it to TagGFP2 containing the N-terminal amino acids GLP. EDC/NHS-activated sensors were reacted with the amine-groups of a KK-GSGS-NGL peptide. All three immobilization methods yield a homogeneous TagGFP2-modified sensor ready for binding kinetic measurements.

$k_{on} = 2.3 \times 10^6$ M$^{-1}$ s$^{-1}$, $k_{off} = 8.0 \times 10^{-3}$ s$^{-1}$) determined with SPR[16] (compare Figure 3B).

The obtained kinetic rates were independent of the functionalization method (specific and non-specific). The site-specific approach anchors proteins at their termini and decreases the chance of binding site obstruction (spatial separation of surface coupling and ligand binding), which thus allows us to determine the unaltered (un)binding rates. This increased reliance is an intrinsic advantage of our site-specific surface functionalization. Based on this, we can compare the data with the non-specifically anchored proteins and conclude that the multiple lysine anchoring possibilities do not obstruct the binding behaviour in the case of TagGFP2–LaG9 interaction. TagGFP2 contains 17 lysines that may take part in the non-specific immobilization procedure. Hence, it is not surprising that enough primary amines non-adjacent to the binding epitope that do not disturb binding are available as anchoring sites. Other receptor–ligand systems might be more strongly affected by the non-specific anchoring (see cohesin–dockerin interaction below). Especially if the surface area at the ligand binding site is charged such that it is electrostatically favoured to make surface contact during the pre-concentration step, the binding site could be obstructed.




**B** Rates

| Ligand | $k_{on}$ [$M^{-1}$ $s^{-1}$] $\times 10^5$ | $k_{off}$ [$s^{-1}$] $\times 10^{-3}$ | $K_d$ [$M^{-1}$] $\times 10^{-9}$ |
|---|---|---|---|
| ybbR-TagGFP2 | 17.4 ± 1.2 | 36.3 ± 3.8 | 20.9 ± 0.7 |
| TagGFP2-LPETGG | 15.6 ± 1.2 | 35.2 ± 4.3 | 22.6 ± 2.7 |
| GGG-TagGFP2 | 17.3 ± 0.8 | 32.6 ± 0.4 | 18.8 ± 0.7 |
| GLP-TagGFP2 | 30.6 ± 1.3 | 70.6 ± 1.5 | 23.1 ± 0.7 |
| unspecific | 37.9 ± 5.0 | 48.8 ± 8.5 | 13.2 ± 3.3 |

***Figure 3.*** Binding kinetics of the TagGFP2-LPETGG receptor with the GLP-LaG9-HIS ligand. A) An example sensorgram of LaG9 ligand binding to the TagGFP2-LPETGG receptor at different concentrations (25, 50, 100 and 200 nM). B) The kinetic rates obtained from performing global fits to sensorgrams for each immobilization method. Values and the respective standard errors are obtained from triplicates.

TagGFP2-NGL and TagGFP2-ybbr could not be fused to a BLI sensor. However, both tags were functional to fuse protein domains in an in vitro bulk reaction. Thus, it appears that both tags are sterically hindered by the GFP domain when used for surface functionalization. A longer linker between GFP domain and the recognition sequence could possibly provide both enzymes (Sfp, OaAEP1) more space to ligate the protein to the sensor.

This enzyme-based and site-specific surface reaction also allowed us to probe the inverse geometry with the nanobody now immobilized to the sensor. Two kinetic titration series, one using the SrtA-based and the other using the non-specific immobilization approach, were recorded (Figure S10). Fits deviated notably from a 1:1 binding model, which might be explained by either ligand–surface interactions or by potential avidity effects should TagGFP2 present more than one binding interface. The ability to site-specifically anchor both binding partners allows us to exclude that the deviations from a simple 1:1 binding model stem from heterogeneous surface preparation due to non-specific protein anchoring. Based on the conducted experiments, we were able to show that all three enzymes can be used for sensor functionalization.

The advantages of defined surface immobilization emerge more clearly when investigating the CohE–XDocIII interaction of *R. flavefaciens*. This cohesin–dockerin pair has already been characterized in bulk studies[20] as well as single-molecule studies.[18,19] However, we were not previously able to non-specifically immobilize the cohesin in a functional state (Figure S3), possible due to a lysine in its binding pocket.[18] However, not only were we able to attach the cohesin site-specifically and in biologically active form with the enzymatic approach, we were also able to do so from either terminus. This was of particular interest since the unbinding behaviour of this complex under external force was shown to strongly depend on the anchoring geometry of the cohesin. The complete sensorgram of the sensor modification can be found in Figure S11. Because of the evidently very low off-rate of the cohesin–dockerin complex, and because no regeneration conditions could be found to force ligand dissociation, we chose to perform a kinetic titration experiment. Full dissociation of the complex would take too long and by far exceed the four hours of total experiment time suggested for BLI. Longer measurements would suffer from evaporation of liquids in the microwell plate, thus falsifying concentrations. Initial experiments showed that sensor-drift effects seemed to exceed the actual ligand dissociation due to the low off-rate (Figure S12). Thus, as recommended,[21] we modified our protocol such that both sensors are loaded with a receptor; in our case they were functionalized site-specifically with cohesin. For referencing, one sensor was only dipped into measurement buffer while the other was presented with ligand. Despite having minimized drift, it became clear that the off-rate of the complex is too low to be assessed through BLI. Too little dissociation occurred so that adequate fitting of the data was not possible. For an exact determination of the off-rate, alternative techniques such as SPR or QCM might be more promising, since they are not limited by evaporation effects and can thus measure for extended periods of time. The apparent low off-rate is common in cohesin–dockerin systems.[22,23] However, a qualitative statement about the (un)binding behaviour of the cohesin–dockerin complex is possible, namely that on- and off-rates appear to be independent of the anchoring geometry (Figure 4).

This stands in contrast to force spectroscopy experiments,[18,19] where the anchoring geometry strongly changes the force necessary to dissociate cohesin and dockerin. By comparing the findings obtained by force spectroscopy with those from site-directed BLI, we can conclude that the different rupture forces are indeed a consequence of force propagation within the receptor–ligand complex, rather than an artefact caused by surface effects or the employed anchoring chemistry.

In summary, the presented strategy provides an efficient means to covalently and site-specifically couple receptors under mild reaction conditions. The employed tags are all small and should not influence the overall functionality of a protein. This makes it viable to use the same constructs for characterization in a surface-based assay as well as for other bulk and single-molecule studies. Moreover, these small tags can be further used for post-translational protein modifications, that is, attachment of a fluorescent dye[24] or an additional protein domain,[13] or as a pull-down technique.[13] Hence, label-free and label-dependent techniques can be used with the same batch of proteins. While the enzymatic approaches presented here are shown for sensor modification in BLI, they can be easily adopted for other assays, such as SPR or QCM. Overall, the site-directed and covalent immobilization techniques present a viable, easily implementable alternative to the non-specific approach. Additionally, no buffer conditions suitable for pre-concentration need to be

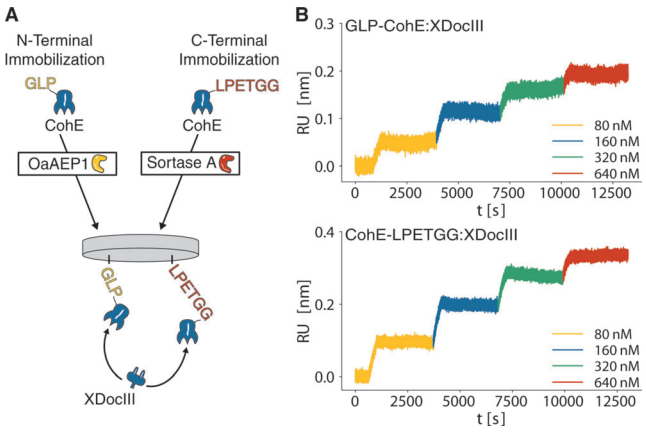



***Figure 4.*** Comparison of N- and C-terminal immobilization of CohE. A) Site-specific surface chemistry enables N- and C-terminal anchoring to be distinguished. B) Kinetic titration series of an OaAEP1-immobilized GLP-CohE (Top) and a SrtA-immobilized CohE-LPETGG (Bottom) sensor with XDocIII ligand binding. SrtA was employed to covalently couple CohE-LPETGG to a polyglycine-modified sensor. OaAEP1 immobilized the GLP-CohE to an NGL-modified sensor. A kinetic titration series was performed by subsequently dipping the sensor into different concentrations of XDocIII (80, 160, 320 and 640 nM) with dissociation steps in measurement buffer.

found, which provides a faster way of establishing assays on new receptor–ligand interactions. Also, due to the specific nature of the surface coupling, signal arising from ligands that bind to non-specifically adsorbed receptors can be subtracted since the reference sensor can be prepared by simply omitting the coupling enzyme. Most importantly, receptor–ligand interactions that were previously inaccessible due to reactive residues in their binding interface or due to electrostatic repulsion can now be site-specifically immobilized and characterized with the enzymatic approaches.

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## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** biolayer interferometry · protein immobilization · protein ligation · site-specific protein modification · sortase A, Sfp, OaAEP1

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# Communications

**Protein Immobilization**

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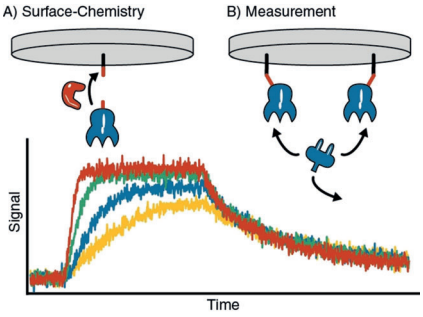
Enzyme-Mediated, Site-Specific Protein Coupling Strategies for Surface-Based Binding Assays




**Coupling strategies** for surface-based kinetic assays based on the enzymes Sfp, SrtA, and OaAEP were developed to site-specifically and covalently ligate proteins of interest via short recognition sequences. The enzymes function under mild conditions without affecting the receptor fold, thereby enabling study of the binding kinetics of receptor–ligand pairs that are inaccessible to non-specific surface chemistry.