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An integrated, peptide-based approach to site-specific protein immobilization for detection of biomolecular interactions†

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We have developed an integrated solution for the site-specific immobilization of proteins on a biosensor surface, which may be widely applicable for high throughput analytical purposes. The gold surface of a biosensor was coated with an anti-fouling layer of zwitterionic peptide molecules from which leucine zipper peptides protrude. Proteins of interest, the autoantigenic proteins La and U1A, were immobilized via a simple incubation procedure by using the complementary leucine zipper sequence as a genetically fused binding tag. This tag forms a strong coiled-coil interaction that is stable during multiple consecutive measurements and under common regeneration conditions. Visualization of the immobilized proteins of interest via antibody binding with multiplex surface plasmon resonance imaging demonstrated 2.5 times higher binding responses than when these proteins were randomly attached to the surface via the commonly applied activated ester-mediated coupling. The proteins could also be immobilized in a leucine zipper-dependent manner directly from complex mixtures like bacterial lysates, eliminating the need for laborious purification steps. This method allows the production of uniform functional protein arrays by control over immobilized protein orientation and geometry and is compatible with high-throughput procedures.

Introduction

Interactions between biomolecules play a crucial and complex role in biological systems. This makes reliable and sensitive methods to study these interactions an important tool in (bio)chemical and medical research. A sensitive and versatile technique for label-free, real-time detection of such interactions is Surface Plasmon Resonance (SPR).[1] Like many of the methods for detection of biomolecular interactions, it relies on immobilization of biologically active molecules on a solid surface. The most commonly applied immobilization chemistry uses an activated ester functionalized surface to couple primary amines, present on the N-terminus or lysine side chains of proteins and peptides.[2–4] The widely applied amine-based coupling chemistry leads to surface heterogeneity due to variations in orientation, steric hindrance and multivalent binding of the ligand.[5] This is suboptimal for sensitivity and creates variability between measurements.

To improve ligand immobilization, several strategies for site-specific protein immobilization have been developed.[6,7] Some of these utilize bio-orthogonal reactions like copper-catalyzed and strain-promoted 'click' chemistry,[8–11] oxime ligation[10,12] Staudinger ligation[13] or enzymatic coupling reactions as those with sortase[14] or phosphopantetheinyl transferase.[15] Others make use of non-covalent binding, such as facilitated by the use of affinity tags frequently used for protein purification[16–20] or peptide tags that bind to the surface matrix, like pMMA.[21] More alternatives are DNA-directed immobilization[22,23] or enzymatic modification of the proteins,[24–26] like localized biotinylation of a specific peptide tag using the BirA enzyme.[27] However, these methods either rely on separate modification and coupling steps of the proteins involved, or have relatively weak binding properties.

Advances in SPR technology also put different demands on the immobilization chemistry. Surface Plasmon Resonance Imaging (iSPR) is a multiplex variant that is primarily used for the visualization of biomolecular interactions, generally on microarrays.[1] With this approach, instead of a single biomolecule, multiple different biomolecules need to be immobilized simultaneously. As a consequence, coupling or modification steps that need individual optimization for distinct biomolecules, are undesirable. An immobilization method that is specific, strong and well controlled, yet versatile and simple enough to be used for multiple different biomolecules is not yet available.

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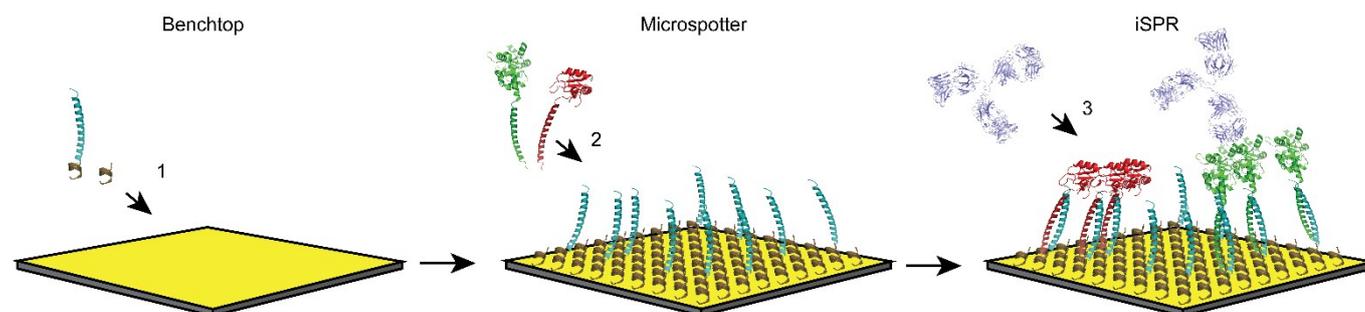
Figure 1: Schematic representation of leucine-zipper mediated immobilization. Initially the gold surface of an iSPR biosensor is coated with anti-fouling peptides mixed with anti-fouling peptides extended with leucine zippers (1). One or more fusion proteins containing a single complementary leucine zipper peptide are then immobilized on the surface by incubation in a microspotter (2). Following immobilization antibody binding to the immobilized proteins is visualized using iSPR (3).

Versatile, specific and strong binding can be found in so-called leucine zipper polypeptides. These are coiled-coil binding motifs, originating from the bZIP class of transcriptional regulator proteins.[28] Their structure consists of a heptad repeat with a hydrophobic leucine on the binding interface, which gives them their name. Next to these leucines, the binding interface contains pairs of oppositely charged residues. By variation of these charged residues, affinity and specificity of leucine zippers can be engineered, and sets of heterospecific leucine zipper peptides have been developed.[29,30] By optimizing binding strength, binding affinity up to a dissociation constant of 10^{-15} M has been obtained, comparable to the well-established biotin-streptavidin interaction.[31] Zhang et al. successfully applied leucine zipper polypeptides for immobilization of proteins, although in their approach an additional crosslinking reaction was still needed to functionalize the surface.[32] Immobilization with coiled-coil domains was also shown by Ferrari et al., using the larger tetra-helical SNARE protein complex as connector.[33]

The aim of our study was to provide a generic, integrated solution for well-controlled immobilization of proteins on gold surfaces. Our approach (Figure 1) utilizes a monolayer of short, zwitterionic peptides as anti-fouling layer on the gold surface.[34] A fraction of the anti-fouling peptides is extended with a leucine zipper. This functionalizes the surface to allow binding of a complementary leucine zipper sequence, which is genetically fused to the proteins that are to be immobilized. This approach allows a strong, well-controlled immobilization of the fusion proteins using simple incubation with the functionalized surface. The autoantigenic human La and U1A proteins were used to demonstrate the applicability of this method[35].

Materials & Methods

Cloning

An XhoI restriction site was introduced between the oligohistidine tag sequence and the attL1 site of a pDEST17 vector by site-directed mutagenesis. Partially overlapping oligonucleotides coding for the leucine zipper peptides ER (LEIEAAFLERENTALETRVAELRQRVQRLRNRVSQYRTRYGPLGG-GGK) and RE (LEIRAAFLRQRNTALRTEVAELEQEVQRLENEVSQY-ETRYGPLGGGGK) flanked by XhoI sites (Biolegio, Nijmegen, The Netherlands) were converted to double-stranded molecules by

PCR, digested by XhoI (New England Biolabs, Ipswitch, MA, USA) and the inserts isolated. The inserts were then ligated into the XhoI-linearized pDEST17 variant described above. *E. coli* ccdB survival 2 T1^R competent cells were transformed with these constructs and grown on media with 100 µg/mL carbenicillin and 34 µg/mL chloramphenicol. This resulted in the pDNz-VinER and pDNz-VinRE plasmids (see Supplementary Figures S1 and S2). La and U1A cDNAs were generated by PCR using cDNA constructs described previously[36,37] and introduced into pENTR/TEV/D-TOPO vectors. Subsequently these were recombined in an LR-recombination reaction with pDEST17 and pDNz vectors using Gateway LR Clonase II mix and the resulting pDEST17-La, pDEST17-U1A, pDNz-VinER-La, pDNz-VinRE-La, pDNz-VinER-U1A and pDNz-VinRE-U1A vectors were used to transform *E. coli* TOP10 competent cells. Following plasmid isolation, the integrity of all constructs was confirmed by DNA sequencing. Cloning supplies were acquired from Invitrogen (Carlsbad, CA, USA) and chemicals from Sigma-Aldrich (St. Louis, MO, USA).

Protein Expression

The constructs for the expression of unmodified and N-terminally leucine zipper-tagged U1A and La proteins were used to transform *E. coli* BL21 AI. An initial overnight culture in 5 mL LB was used to inoculate 1.5 L 2TY medium containing 100 µg/mL ampicillin. The bacteria were cultured at 37 °C and expression was induced (at OD 0.7) using 0.1% of L-arabinose and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (MP Biomedicals, Santa Ana, CA, USA), after which the bacteria were cultured overnight at 25 °C. Cells were harvested and resuspended in 20 mM Tris-HCl, pH 8.0, 500 mM KCl for Ni-NTA affinity purification or PBS for direct immobilization, lysed by sonication and cleared by centrifugation for 20 min at 29,000 g. For direct immobilization the crude lysates were cleared by gradual addition of poly(iminoethylene) to 0.02% and centrifugation for 20 min at 29,000 g. Lysates were then frozen by liquid nitrogen and stored in single-use aliquots at -80 °C. For purification the lysates were incubated with Ni-NTA sepharose (IBA Life Sciences, Goettingen, Germany) and the suspension was cast in a column. The column was washed with wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM KCl, 20 mM imidazole, 10% glycerol), wash buffer containing 1 M KCl and wash buffer containing 50 mM imidazole. Bound proteins were eluted (20

mM Tris-HCl, pH 8.0, 100 mM KCl, 500 mM imidazole, 10% glycerol) and analysed by SDS-PAGE. The buffer was then exchanged to PBS and samples were concentrated using 10 kDa MWCO centrifugation filters (EMD Millipore, Billerica, MA, USA).

Peptide Synthesis

Peptides were synthesized using standard Fmoc chemistry on Barlos resin. The resin was swollen in dimethylformamide (DMF). Fmoc groups were removed by washing with 20% piperidine in DMF while shaking for 20 min. The desired amino acids were coupled using 3 eq Fmoc-protected amino acid, 3.3 eq diisopropylcarbodiimide and 3.6 eq N-hydroxy benzotriazole for 40 min up to overnight as indicated by a negative Kaiser test.[38] After final Fmoc removal, the resin was washed with DMF, dichloromethane and methanol and dried. Cleavage from the resin was performed using 90% trifluoroacetic acid, 5% water, 2.5% triisopropylsilane and 2.5% thioanisole for 5 hours. After filtration from the resin the free peptide was precipitated in diethyl ether, dried in air, redissolved in water and lyophilized. LCMS was performed on a Thermo Finnigan LCQ-Fleet ESI-ion trap (Thermo Fischer, Breda, The Netherlands) equipped with Alltima C18 column, 2.1 x 150 mm, particle size 3 µm (Alltech Applied Sciences, Breda, Netherlands) using an acetonitrile/water gradient with 0.1% formic acid.

Chip preparation

SensEye Au iSPR sensors (Ssens, Enschede, The Netherlands) were cleaned by incubation for 10 min with a 3 : 1 solution of sulphuric acid (analysis grade, 95-97%) and 30% hydrogen peroxide. Following incubation the chip was rinsed with Milli-Q water, ethanol and dried under nitrogen. The surface was subsequently coated with a peptide layer by overnight incubation with a mixture of 4.75 mM anti-fouling peptide (MPA-LHDLHD) and 0.25 mM leucine zipper-anti-fouling fusion peptide (RE: MPA-LHDLHDLEIRAAFLRQRNTALRTEVAELEQEVQ-RLENEVSQYETRYGPLGGGGK or ER: MPA-LHDLHDLEIEAAFL-ERENTALETRVAELRQRVQRLRNRVSQYRTRYGPLGGGGK) in Milli-Q water. After incubation the chip was rinsed thoroughly with Milli-Q.

Protein immobilization

Immobilization of purified, zipper-tagged La to the peptide surface was achieved by incubation with 150 ng/mL of the protein in PBS containing 0.075% Tween-80 during 1h in a Continuous Flow Microspotter (Wasatch Microfluidics, Salt Lake City, UT, USA), equipped with a printhead for up to 48 spots of approximately 0.1 mm^2 each. This incubation was followed by a 2 min washing step.

For immobilization of zipper-tagged La using crude bacterial lysates, Tween-20 was added to a final concentration of 0.1% vol. before similar incubation in the microspotter.

Covalent immobilization to the peptide-surface was performed by activation for 30 min with 50 mM 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide and 250 mM sulfo-(N-hydroxy-succinimide) (NHS) in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) followed by 1h incubation with 150 ng/mL non-tagged La protein in sodium acetate pH 4.5.

iSPR analysis

Performance of the peptide anti-fouling layer was tested by preparing a chip as described under chip preparation. For comparison a SensEye Easy2Spot-P (Ssens, Enschede, The Netherlands) chip with planar NHS ester functionality was used and blocked by incubation for 10 min with a 1 M 2-aminoethanol solution in 10 mM MES pH 5.5. For analysis of the non-specific binding to these surfaces, zipper-tagged La and serum proteins were diluted in system buffer (PBS containing 0.075% Tween-80) to final concentrations of 100 µg/mL. Binding of these proteins was visualized in an IBIS MX96 iSPR over a period of 30 min and analysed using SPRintX software (IBIS Technologies, Enschede, The Netherlands) by equalization of baselines and determination of the response at the end of the binding phase.

Immobilization of the leucine zipper-tagged protein was visualized by preparation of the anti-fouling layer as described under chip preparation, followed by 30 min incubation in the IBIS MX96 iSPR with 60, 12 and 6 µg/mL zipper-tagged La in system buffer, 8 min buffer wash and 1 min regeneration with 6 M guadinine-HCl. Analysis was performed with SPRintX software and baselines equalized.

Visualization of the immobilized leucine zipper-tagged proteins using antibodies was performed by preparation of a leucine-zipper functionalized chip as described under chip preparation. After immobilization of either the purified proteins or the proteins from crude lysate, the first measurement and regeneration cycle was performed with system buffer (in case of purified proteins) or with 1 M NaCl (in case of the crude lysate). Binding of monoclonal antibody (a 1:20 dilution of culture supernatant containing 5% serum in system buffer, resulting in approximately 1 µg/mL antibody according to SDS-PAGE analysis) was visualised in the IBIS MX96 iSPR for 40 min, followed by 8 min dissociation and 1 min regeneration with 10 mM glycine-HCl, pH 2.0, unless stated otherwise. Analysis was performed with SPRintX software; the chip surface was calibrated according to the manufacturer's instructions, baselines were equalized and the response was determined at the end of the binding phase, when binding was close to equilibrium.

Statistical analyses

Significance of the differences in protein-binding to the antifouling layers and of the antibody-binding to the La protein immobilized using a leucine zipper tag or using covalent coupling was assessed using an unpaired, one-way ANOVA with Tukey-test. Significance between the responses from antibody-binding to leucine zipper-tagged and untagged protein pairs immobilized from crude lysate was assessed using a t-test. All statistical analyses were performed using Graphpad Prism 5 (Graphpad Software, La Jolla, CA, USA).

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Results and discussion

Peptide-based self-assembled monolayer for antifouling

The gold surface of an iSPR chip is typically covered with either a dextran hydrogel or a monolayer of carboxyl-terminated alkane thiols to avoid non-specific interactions with the gold and to provide functional groups for immobilization. Good results have also been reported with zwitterionic polymers.[39,40] More recently, Masson and co-workers developed monolayers of various zwitterionic peptides with an N-terminal 3-mercaptopropionic acid (MPA) for surface attachment,[34,41] which provide a similar function. For our purpose, we selected the anti-fouling peptide consisting of MPA – Leu – His – Asp – Leu – His – Asp, which showed good anti-fouling properties with serum as analyte solution.[19] This peptide was synthesized using Fmoc solid-phase peptide synthesis and its integrity and purity were confirmed with LC-MS (Supplementary Figure S3).

In order to assess anti-fouling performance, non-specific binding to the gold surface of an iSPR sensor coated with the anti-fouling peptide was investigated by exposing the coated surface to high concentrations of either a purified recombinant protein or a crude biological sample (serum). The coated surface was incubated with a high concentration, 100 µg/mL, of leucine zipper-tagged La protein, a putative RNA chaperone which is a target for autoantibodies in patients with systemic lupus erythematosus and Sjögren's syndrome,[35] or with a similar concentration of bovine serum proteins in PBS containing 0.075% Tween-80. Serum proteins were chosen because they were also present during subsequent antibody binding experiments (see below). The non-specific binding to the peptide layer was compared with binding to a comparable, commercially available alternative with a planar, NHS ester-functionalised surface chemistry blocked with 2-aminoethanol. After incubation with the purified protein solution for 30 minutes, non-specific binding to the iSPR chip was approximately equal for both tested layers, while after incubation with the serum proteins a considerably lower non-specific binding was observed with the peptide layer (Figure 2). These results showed that the peptide-based layer has indeed
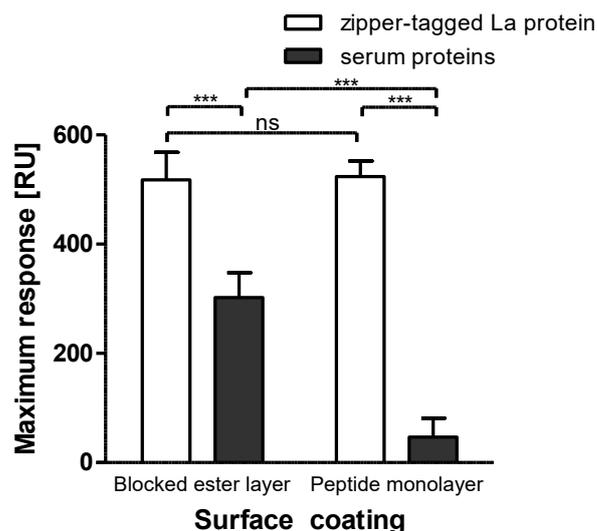

Figure 2: Anti-fouling properties of zwitterionic peptide monolayer. The response due to non-specific binding to the peptide monolayer is compared with a commercially available chip with planar, NHS ester-functionalised surface chemistry that was blocked using 2-aminoethanol. Analytes were 100 µg/mL zipper-tagged La protein and approximately 100 µg/mL serum proteins in PBS containing 0.075% Tween-80. Bars represent the mean with standard deviation of triplicate analyses. Significance of differences (***: P ≤ 0.001; ns: non-significant) was assessed using a one-way ANOVA with Tukey test. RU: response units.

good anti-fouling properties which are particularly useful for measuring antibody interactions.

Leucine zipper based immobilisation for iSPR

To provide stable immobilization, a leucine zipper pair with high affinity, yet low homodimerization properties is desired. Such a pair was previously described by Moll and coworkers (peptide RE: LEIRAAFLRQRNTALRTEVAELEQEVQRLENEVSQYET-RYGPLGGGGK and peptide ER: LEIEAAFLERENTALETRVAELR-QRVQRLRNRVSQYRTRYGPLGGGGK).[31] Both leucine zipper peptides were synthesized with the anti-fouling sequence and 3-mercaptopropionic acid at the N-terminus (Supplementary Figure S4 and S5). This allowed the attachment of either peptide to the gold surface of an iSPR sensor, with the leucine zipper peptide protruding from the monolayer of anti-fouling peptides
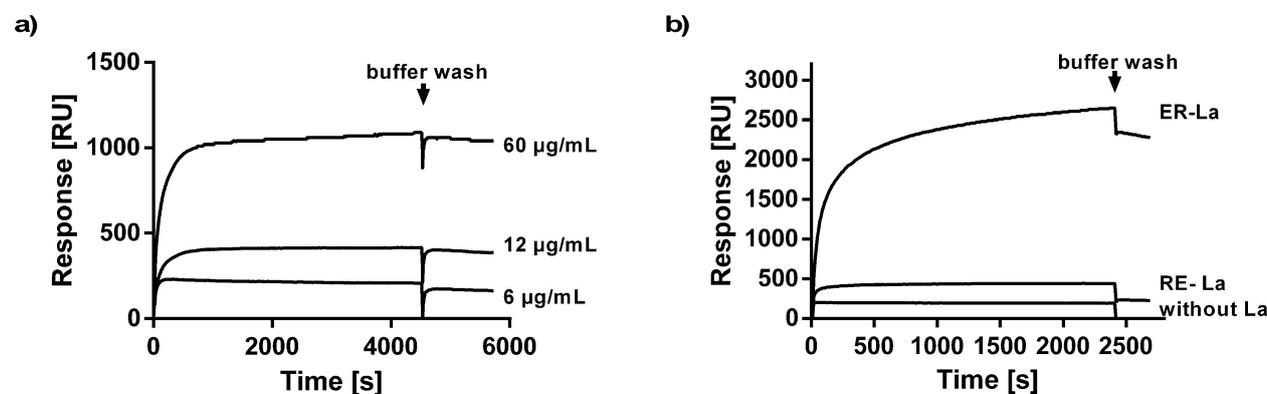

Figure 3: Leucine zipper-based immobilization of the La protein on an iSPR surface. a) Sensorgram visualizing binding of 60, 12 and 6 µg/mL of zipper-tagged La protein to the complementary zipper peptide on the iSPR surface. Binding was performed in PBS containing 0.075% Tween-80 for 4500 s, after which dissociation conditions (buffer wash) were applied for 1500 s. b) Sensorgram visualizing binding of 1 µg/mL anti-La monoclonal antibody SW5 to the La protein, immobilized using standard conditions (150 ng/ml) in a microspotter and tagged with a leucine zipper complementary (ER) or non-complementary (RE) to the sensor surface. Binding was performed in PBS containing 0.075% Tween-80 for 2400 s, after which dissociation conditions (buffer wash) were applied for 480 s. RU: response units.
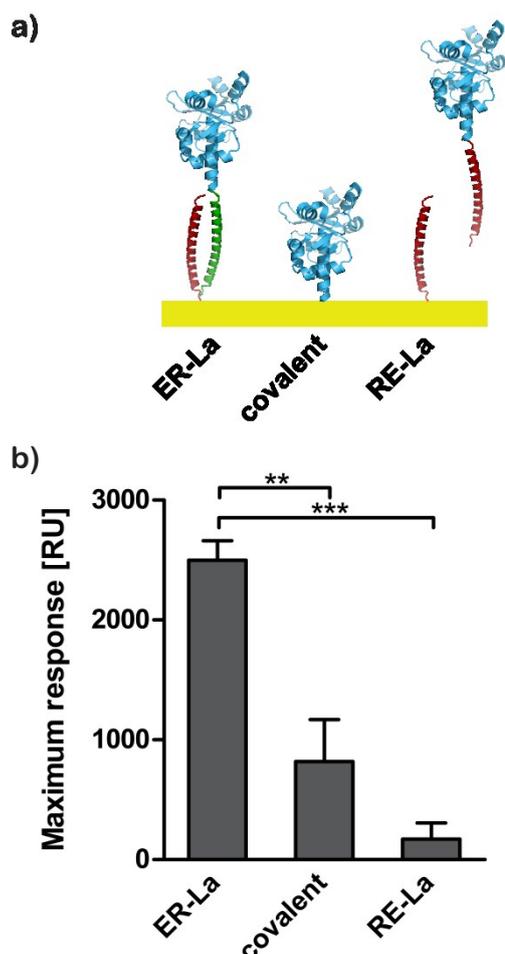
a)

b)

Figure 4: Specificity of leucine zipper-based immobilization detected by antibody binding. a) Zipper-tagged La protein was immobilized on a RE leucine zipper-functionalized sensor surface using, from left to right, a complementary (ER) leucine zipper-tag, activated-ester mediated covalent coupling and a non-complementary (RE) leucine zipper tag. b) Maximum iSPR responses for anti-La antibody binding to the immobilized La protein. Bars represent the mean of triplicate analyses with standard deviation for 1 μg/mL anti-La monoclonal antibody SW5 binding in PBS containing 0.075% Tween-80. Significance of differences (**: P ≤ 0.01, ***: P ≤ 0.001) was assessed using a one-way ANOVA with Tukey test. RU: response units.

(Figure 1). A 20-fold molar excess of anti-fouling peptide compared to leucine zipper peptide was used to create an anti-fouling layer which will yield a high density of immobilized leucine zipper molecules.

The cloning of sequences coding for a target protein fused to a leucine zipper was performed with the Gateway system[42], which allows efficient transfer of DNA-fragments between plasmids by recombination cloning. To facilitate high-throughput cloning of zipper-fusion constructs, the pDEST17 vector of the Gateway system was modified by the incorporation of either the ER or the RE sequence. This resulted in the pDNz-VinER and pDNz-VinRE plasmids (see Supplementary Figures S1, S2 and Table S1), which after incubation with the recombination enzyme mix and the pENTR vector containing the cDNA of the target La protein, led to the desired leucine zipper fusion expression construct. The constructs for the ER- and RE-tagged La protein obtained this way, were expressed in *E. coli* using standard techniques
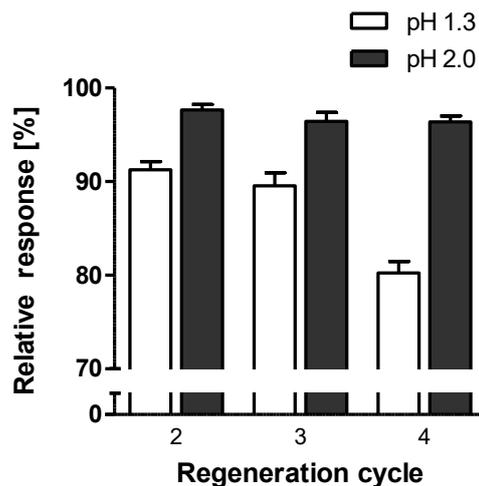

Figure 5: Relative response of antibody-binding after consecutive regeneration cycles. Each cycle consisted of incubation with antibodies, followed by regeneration with 10 mM glycine-HCl of pH 1.3 or pH 2.0. The mean is shown with standard deviation of triplicate analyses with 1 μg/mL anti-La monoclonal antibody SW5 in PBS containing 0.075% Tween-80.

(Supplementary Figure S6). After purification, the recovered yield of soluble fusion protein was somewhat lower compared to the unmodified La protein (data not shown), which might be due to an increased tendency of the tagged protein to aggregate.

To immobilize the fusion protein, the leucine zipper-functionalised surface was incubated with the purified fusion protein containing a complementary zipper sequence and the immobilization was monitored by iSPR (Supplementary Figure S7). The resulting sensorgram (Figure 3a) shows swift association of the fusion protein and very slow dissociation during a buffer wash, reflecting the very stable non-covalent interaction. Consequently, dissociation was negligible during the timeframe of SPR measurements.

Using a continuous flow microspotter with an array of microfluidics flow cells, individual 'spots' on a sensor chip were incubated with either fusion proteins with complementary and non-complementary zipper sequences to assess the specificity of immobilization. A traditional activated ester coupling was performed in parallel to compare leucine zipper-based with covalent immobilization. The entire sensor chip was incubated with antibodies against the La protein, to visualize the immobilized proteins (Figure 3b, Figure 4, supplementary Figure S8). The complementary zipper-tagged La protein indeed was efficiently immobilized on the surface, whereas the antibody-binding signals for the non-complementary zipper-tagged protein were hardly detectable, indicating that the latter was not, or only very inefficiently, immobilized. This substantiates the specificity of zipper-based immobilization. Antibody-binding to zipper-immobilized protein was higher than when the protein was covalently bound to the surface. These observations reflect those of previous reports[6,43] that site-specific protein immobilization improves the binding response. With site-specific immobilization the protein structure is better preserved, resulting in less non-binding protein molecules on

the surface. Also the accessibility of binding sites for the antibodies might have been improved due to a more favourable orientation of the immobilized protein. In addition, it should be noted that we can not exclude the possibility that covalent immobilization led to a lower amount of protein immobilized on the surface compared to the zipper-mediated immobilization.

For multiple binding experiments on a single chip it is important that the immobilized proteins are resistant to regeneration procedures. In typical SPR measurements the ligand is immobilized on the surface once and after a binding experiment, the bound analyte is removed from the surface by a regeneration step, allowing the next binding experiment. For bound antibodies this regeneration step is commonly performed by a short incubation with a low pH solution.[44] For optimal regeneration the maximum response should remain constant over multiple measurement cycles. With a relatively harsh treatment at pH 1.3 the maximum response for the binding of anti-La antibody to the zipper-La protein showed a clear reduction, with a total decrease after 4 cycles of approximately 20%, most likely due to detachment or denaturation of the immobilized protein (Figure 5). However, under somewhat milder regeneration conditions (pH 2.0) the response remained stable and the total decrease after 4 cycles was less than 5%. These data indicate that it is possible to selectively remove the bound antibody from the surface, while the immobilized proteins remain intact and available for binding during several consecutive measurements and surface regenerations.

Although the immobilization of proteins is simplified by using the leucine zipper-mediated approach, the laborious fusion protein expression and purification steps still remain. To reduce the amount of work involved, the specificity of leucine zipper-binding can also be employed to immobilize fusion proteins directly from crude bacterial lysates. Besides the previously used La protein, a second autoantigenic protein was used, U1A, a protein component of the U1 small nuclear ribonucleoprotein particle[35]. The lysates of both fusion proteins were first cleared from insoluble material and bacterial DNA by two centrifugation steps and precipitation using poly(iminoethylene). The cleared lysates were then applied to the zipper-functionalized surface and after incubation the surface was rinsed thoroughly to remove non-bound material. Immobilized proteins were visualized by measuring antibody binding using iSPR. The results in Figure 6 show that the leucine zipper-tagged U1A and La proteins were indeed immobilized by binding to the complementary zipper peptides, while untagged proteins were largely washed away, demonstrating that crude cell lysates can be employed for leucine zipper-based ligand immobilization.

While leucine zipper-based immobilization is potentially widely applicable, the leucine zipper sequence, although shorter than the previously mentioned SNARE complex[33], is relatively long compared to the moieties used for the execution of covalent coupling strategies. This might be a disadvantage in experiments where the length of this element is of importance. Additionally, the incorporation of a sequence of this length increases the risk that it interferes with proper protein folding or that steric effects influence the accessibility of important

regions of the protein. Like other techniques which rely on fused peptide tags, such as a sortase- or ybbR-tags[14,15], this potentially limits the available orientations in which a protein can be immobilized and may restrict applications particularly to proteins with free N- or C- termini. In contrast to covalent immobilization strategies, the non-covalent nature of the leucine zipper-tags provides an opportunity to regenerate a biosensor surface for the analysis of unrelated biomolecular interactions by denaturing the coiled-coil structure.

Conclusions

We have demonstrated that leucine zippers as fusion tags can successfully be used as an integrated system for well-controlled, site-specific immobilization of proteins on surfaces, e.g. for SPR analysis. A peptide-based anti-fouling layer on the bare gold surface of an iSPR chip allows functionalization of the surface with leucine zippers, without additional coupling or capturing steps and with comparable anti-fouling performance to commercially available alternatives. Two model proteins were genetically fused to a leucine zipper sequence using a high-throughput cloning procedure, and effectively immobilized on such a surface by leucine zipper heterodimerization. Availability of the immobilized proteins was subsequently assessed by incubation with antibodies, where the antibody binding response for protein immobilized using leucine zippers was higher than that observed for a classical, random, activated ester-based coupling strategy. Although non-covalent and reversible, leucine zipper-based immobilization was stable in the time-frame of SPR measurements and not affected by
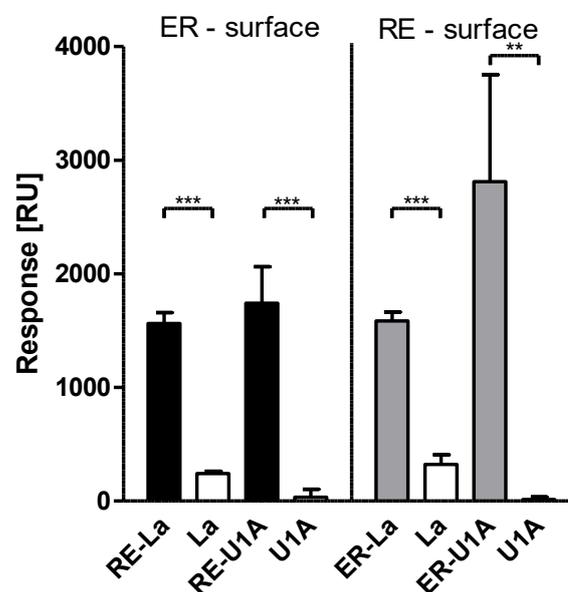

Figure 6: Immobilization of leucine zipper-tagged proteins from crude bacterial lysates. Antibody-binding response to spots incubated with crude cell lysates containing fusion proteins with complementary (RE-tagged protein on ER surface and vice-versa) zipper sequences and untagged proteins, on a surface functionalized with the ER peptide (black/white) and the RE peptide (grey/white). The bars represent the mean of triplicate analyses with standard deviations for 1 µg/mL anti-La monoclonal antibody SW5 and anti-U1A monoclonal antibody 9A9 in PBS containing 0.075% Tween-80. Significance of differences (**: P ≤ 0.01, ***: P ≤ 0.001) between tagged and untagged protein pairs was assessed using a t-test.

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regeneration conditions commonly used in antibody-based assays. This way of immobilization does not necessarily require purification of tagged proteins from crude cell lysates, further reducing the time and effort involved in immobilization. The minimal number of intermediate steps in the immobilization procedure minimizes the risk for undesired heterogeneity on the surface. The compatibility with high-throughput procedures and the simple incubation steps make leucine-zipper mediated immobilization ideally suited for applications which involve a multitude of proteins to be analysed, like multiplex iSPR.

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