Efficient and Chemoselective Surface Immobilization of Proteins by Using Aniline-Catalyzed Oxime Chemistry

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The immobilization of proteins on surfaces is important in many areas of life sciences, ranging from surface plasmon resonance analysis of specific protein–protein and protein–drug interactions to the screening of entire proteomes on protein microarrays. While protein chips potentially offer many of the same advantages as DNA chips, their development has been hampered by a lack of immobilization methods that are at the same time chemoselective and generally applicable. Protein immobilization methods that target amine or thiol groups at the protein exterior are widely used, but typically result in heterogeneous conjugation at multiple sites on the protein, thus offering limited possibilities to control the activity of the immobilized protein. In recent years several site-specific immobilization strategies have been developed based on bioorthogonal ligation reactions such as native chemical ligation, Staudinger ligation or the Diels–Alder reaction. While these methods typically result in a homogenous surface representation of proteins, their routine application has been hampered by the need to introduce a peptide tag or non-natural amino acid into the protein of interest. In addition, the slow reaction rates of some of these ligations require long incubation times and/or the use of high protein concentrations. Here we present an efficient protein-immobilization method based on catalyzed oxime ligation that is both chemoselective and broadly applicable to proteins that possess a freely accessible N terminus. Surface plasmon resonance (SPR) was chosen to demonstrate the scope of the new immobilization strategy, because it allows direct and real-time detection of the amount of immobilized ligand and characterization of the binding properties. The strength of the new method is illustrated by the generation of homogenous protein 'G' surfaces, which can be used in antibody microarrays.

Our approach takes advantage of two recent advances in the field of oxime chemistry (Scheme 1). The first innovation is the site-specific introduction of N-terminal ketones into proteins by using oxidation with pyridoxal 5'-phosphate (PLP) optimized by Francis and co-workers. Although this reaction is not quantitative, and the yield also depends on the nature of the N-terminal amino acid, PLP oxidation is unique in that it can be applied to almost any protein, provided the N-terminal residue is not blocked. A second important finding is that oxime ligations can be significantly accelerated by using aniline as a small-molecule organocatalyst. Here we show that aniline-catalyzed oxime ligations allow very efficient immobilization of native proteins and peptides to surfaces modified with alkoxyamines without loss of activity (Scheme 1). While attachment of PLP-oxidized proteins to an aminooxy functionalized surface has been reported recently, the present study is the first comprehensive study that demonstrates the broad scope and remarkable efficiency of oxime ligation for surface immobilization, in particular when catalyzed by aniline.

Commercially available carboxylate-functionalized SPR chips were modified with alkoxyamine groups via a newly developed bifunctional linker (1, Scheme 1). The surface-bound carboxylic acid groups were activated inside the Biacore instrument with EDC and NHS, followed by injection of the protected aminooxy derivative 1 (250 mM) in 50 mM borate buffer at pH 8.5. The remaining activated esters on the chip were quenched with 2-ethanolamine. Compound 1 was prepared from commercially available Boc-protected aminooxyacetic acid and Cbz-protected ethylenediamine followed by catalytic hydrogenation (Scheme S1 in the Supporting Information). Deprotection of 1 on the chip was achieved by taking the chip out of the Biacore instrument and incubating it overnight in 1 M phosphoric acid buffer at pH 2. Regeneration of the surface with successive treatments of 100 mM HCl, 50 mM NaOH, and 0.5 % SDS was performed to remove noncovalently bound organics from the surface. At the end of this procedure, about 160 responsive units of the aminooxy-derivative were immobilized in the sample channel of the biosensor chip (160 RUs ~160 pg mm⁻² ~1 μmol m⁻²). A reference surface functionalized with 2-ethanolamine was prepared to allow correction for buffer effects.

First the efficiency of aniline-catalyzed oxime ligation on these aminooxy-functionalized chips was studied by using S-peptide as a model peptide. The S-peptide (SKETAAKFERQH~) was prepared from commercially available carboxylate-functionalized SPR chips was used to immobilize the S-peptide (SKETAAKFERQHRNase S upon binding to S-protein, was prepared by Fmoc-mediated solid-phase peptide synthesis. In this case, introduction of an N-terminal glyoxyl group was achieved by oxidation of an N-terminal serine residue by NaOCl, a method that results in essentially complete conversion to the aldehyde. A 12 s injection of 1 mM oxidized S-peptide in 100 mM anilinium acetate, pH 4.5, resulted in as much as 130 RUs of immobilized peptide. Subsequent pulses of 12 s resulted in the additional immobilization of 130 RUs per injection; this showed that the reaction is very reproducible and that the immobilization level is highly tuneable (Figure 1). Importantly, no immobilization was observed in the reference channel under any of the conditions tested.

Since some proteins are not stable at pH 4.5, we also tested the efficiency of the oxime ligation at higher pH (Figure 1B).
Although the rates at higher pH are threefold (pH 6) or tenfold (pH 7) lower, oxime ligations are still relatively efficient at these pH values in the presence of p-methoxyaniline as a catalyst, and more than sufficient for typical BIAcore applications. Furthermore, because aldehydes are known to be more reactive in oxime ligations than ketones, we also tested the immobilization efficiency using an S-peptide containing an N-terminal ketone (Figure S1). The ketone-functionalized peptide showed an eightfold decrease in activity compared to the glyoxyl-functionalized peptide. However, this rate of immobilization is still orders of magnitude faster than many other ligation reactions. Furthermore, no significant decrease in immobilized S-peptide was observed after overnight incubation of the chip under a constant flow of running buffer, thus confirming a recent study that showed that oxime bonds are very stable at neutral pH.

Incubation of the immobilized S-peptide with 250 mM methoxylamine in 100 mM anilinium acetate at pH 4.5 for one hour resulted in a 60% loss of immobilized S-peptide, showing that bond cleavage is only possible under these nonphysiological conditions.

To confirm that the binding properties of the S-peptide were unperturbed by the oxime ligation, the binding of S-protein was studied over a concentration range of 1 to 250 nM at different peptide densities (Figure S2). Fitting to a 1:1 binding model yielded a $K_D$ of 2.7 nM, a value that is similar to those in previous reports on other immobilization strategies or independent techniques.

To verify that the oxime ligation proceeds with similar efficiency and specificity for larger, folded proteins we tried immobilizing the S-protein instead of the S-peptide. The native S-protein contains an N-terminal serine and, like the S-peptide, can be quantitatively oxidized with NaIO$_4$. To prevent oxidation of sensitive residues, such as Met, Trp or Cys, the reaction was carried out with 1.2 equiv of NaIO$_4$ for 5 min at 4°C. Injection of 100 mM oxidized S-protein for 5 min over an aminooxy chip resulted in 587 RU of immobilization, thus making catalyzed oxime ligation several orders of magnitude more efficient than protein immobilization by native chemical ligation.

These results show that the aniline-catalyzed oxime ligation is a very efficient method for surface immobilization. Injection times of a few seconds at millimolar concentrations of oxidized peptide yield immobilization levels that are sufficient for any type of kinetic or thermodynamic quantification of a biomolecular interaction. The surface density of ligands can be tuned easily by sequential injections, something that is not possible with the commonly used amine coupling.

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Scheme 1. Strategy for the site-specific immobilization of peptides and proteins on a biosensor chip surface by using aniline-catalyzed oxime chemistry. N-terminal aldehydes or ketones can be introduced into proteins either by oxidation of N-terminal amino acids with pyridoxal 5'-phosphate (R$^1$ = R$^2$ = amino acid side chain) or by oxidation of N-terminal serine (R$^1$ = CH$_2$OH, R$^2$ = H) or threonine (R$^1$ = CHCH$_2$OH, R$^2$ = H) residues with NaIO$_4$. 

Figure 1. Surface immobilization of S-peptide by catalyzed oxime ligation. A) A 12 s pulse of 1 mM NaIO4-treated S-peptide in 100 mM anilinium acetate pH 4.5 (i) results in 130 RU of immobilized peptide after two regeneration steps with 10 mM glycine pH 1.5 (ii). Subsequent injection of 150 mM S-protein (iii) results in specific binding to the S-peptide functionalized channel. Finally, two regeneration steps with 10 mM glycine pH 1.5 (iv) were performed to remove the noncovalently bound S-protein from the surface. The sample channel is shown in red and the reference channel in black. B) Efficiency of peptide immobilization at various pH values and in the presence or absence of aniline derivatives as catalysts. Reference-subtracted immobilization levels are shown of an aminoxy biosensor surface functionalized by sequential injections (4×12 s) of 1 mM S-peptide in various buffers (100 mM anilinium acetate, pH 4.5; 100 mM anisidine in HBS-EP, pH 6.0; 100 mM anisidine in HBS-EP, pH 7.0; 100 mM ammonium acetate, pH 4.5; HBS-EP, pH 6.0; or HBS-EP, pH 7.0) at room temperature.

Next, we tested whether proteins obtained by PLP oxidation of their N-terminal residue are also efficiently immobilized on aminoxy-functionalized chips. PLP oxidation has been reported to result in only partial conversion to the N-terminal ketone (10–80%).[4] For our application this is not expected to present a problem, as nonoxidized protein will not react and will be removed under the flow conditions during immobilization. Overnight incubation of the model protein glutathione S-transferase (GST) with 10 mM pyridoxal 5’-phosphate in phosphate buffer at pH 6.5 resulted in 22% conversion of the N-terminal methionine into the corresponding ketone (Figure S4). Injection of only 5 μM GST for 1 min in 100 mM anilinium acetate at pH 4.5 was sufficient to generate 150 RU of immobilized GST, thus confirming the remarkable efficiency of aniline-catalyzed oxime ligation in protein-surface immobilization. No immobilization was observed in the reference channel, and again no decrease in immobilized GST was observed after overnight incubation of the GST-immobilized chip under a constant flow of running buffer. To confirm that the immobilized GST could engage in specific interactions, a binding experiment was performed with a single domain antibody against GST. Fitting of the steady-state response levels to a one-site binding model gave an overall $K_D$ of $184 \pm 10 \text{ nM}$ (Figure S5), which is in agreement with ELISA experiments performed previously in our group (unpublished results).

After showing the generality of the method, we tested it with a highly relevant problem from the field of antibody microarrays, in that we directly compared the performance of oxime ligation with that of classical amine coupling in the generation of antibody-functionalized chips. Protein G’ binds to the Fc parts of mammalian immunoglobulin (IgG) and is frequently used as a docking protein to ensure homogenous surface presentation of IgGs. However, the potential gain in binding capacity that results from homogeneous IgG presentation has been shown to be severely attenuated by random immobilization of protein G’ itself.[11] Indeed, immobilization of 20 RU of protein G’ by classical amine coupling results in a chip surface that binds only 17 RU of IgG at saturating concentrations of IgG (250 nM; Figure 2). Assuming a 1:1 binding ratio between protein G’ and IgG, 130 RU of IgG are expected; it follows that only 13% of protein G’ immobilized in this fashion is capable of binding IgG. In contrast, the same amount of protein G’ immobilized by oxime ligation showed 97 RU of IgG binding, thus demonstrating that most of the protein G’ immobilized in this way was able to bind IgG (Figures 2 and S6A). Note that immobilization of 20 RU of protein G’ required incubation with 5 μM PLP-treated protein G’ (27% of the N-terminal methionines were oxidized to a ketone) in 100 mM anilinium acetate at pH 4.5 for only 36 s, whereas a five-minute injection of 1 μM protein G’ was required to reach the same immobilization level when using amine coupling. The immobilization of protein G’ was also performed on Biacore C1 chips, which lack the dextran layer and are more similar to classical chip surfaces. Although longer injection times were needed to obtain reasonable immobilization levels (due to the fact that fewer aminoxy groups can be introduced onto C1 chips than onto CM5 chips) similar kinetic parameters were obtained (Figure S6B). We anticipate that oriented protein G’ surfaces prepared by our new immobilization strategy lead to increased sensitivity and are therefore ideal for incorporation into antibody microarrays used for measuring the concentrations of low-abundance proteins in complex biological mixtures.

In conclusion, aniline-catalyzed oxime ligation provides a fast, selective and broadly applicable method for protein and peptide immobilization. The chemoselectivity of the method ensures the generation of homogenous protein surfaces and results in fully active proteins with dissociation constants that are similar to those obtained by other techniques. Provided the protein’s N terminus is accessible and not blocked by post-translational modifications, the method presented here can be applied to many proteins without recombinant modification, with the exception of proteins containing Q, W, H, P, or K as an N-terminal residue.[6] In those cases and for proteins in which
the N terminus is functionally important, recombinant procedures have recently become available that allow site-specific introduction of ketone functionalities at any place in a protein.\[12\] We therefore expect that aniline-catalyzed oxime ligation will become the method of choice for surface immobilization of proteins and peptides in a wide variety of protein-based analytical applications.

**Experimental Section**

**Chip modification**: All experiments were performed on a BIAcore T100 (GE Healthcare) with HBS-EP at pH 7.4 as the running buffer. A flow rate of 10 \(\mu\)L min\(^{-1}\) was used unless otherwise stated. Aminoxy derivative 4 was immobilized by standard amine coupling in flow cells 2 and 4 of a CMS (or C1) sensor chip by using the Wizard software tool for immobilization. Amine coupling involved activation of the surface by using a 7 min injection of an equimolar mixture of EDC and NHS (1.0 mm) followed by a 7 min injection of 1 (250 mm) in borate buffer (50 mm, pH 8.5). Then ethanolamine-HCl (1.0 mm) was injected over the surface for 7 min to block unreacted sites. Ethanolamine-HCl was immobilized in reference channels 1 and 3 by using a similar approach. Deprotection of the \(tBoc\) groups was achieved by ejecting the chip out of the BIAcore and placing it overnight in phosphoric acid buffer (1 mm, pH 2). The next day, the chip surface was rinsed with deionized water followed by regeneration with 2 x 30 s pulses of HCl (100 mm), NaOH (50 mm) and 0.5 % SDS over all channels at a flow rate of 100 \(\mu\)L min\(^{-1}\). Chips that were prepared according to the above procedure were used directly in ligation experiments, or were stored at 4 °C in HBS-EP buffer at pH 7.4 until needed.

**NaIO\(_4\) oxidation**: An N-terminal aldehyde was introduced into the S-peptide or S-protein by treatment with 1.2 equiv of NaIO\(_4\) in sodium phosphate buffer (0.01 mm, pH 7.0) at 4 °C. After 5 min, the reaction was quenched by addition of ethylene glycol and purified by reversed-phase HPLC.

**PLP oxidation**: GST/protein G\(^{\prime}\) (33 mm) was dissolved together with pyridoxal 5'-phosphate (6.7 mm) in phosphate buffer (50 mm, pH 6.5) and incubated overnight at 41 °C. To quantify the conversion of the PLP oxidation, site-selective attachment of PEG polymers was performed, followed by SDS-PAGE analysis. Thereto, a 5 kDa PEG-alkoxyamine was synthesized according to literature procedures\[13\] and ligated to the N-terminal ketone, showing 22 % conversion in case of GST (Figure S3) and 27 % conversion for protein G\(^{\prime}\).

**Peptide/protein ligation to aminoxy-modified chips**: Peptides (1 mm) or proteins (5–100 mm) were dissolved in anilinium acetate (100 mm) or proteins (5–100 mm) were dissolved in anilinium acetate (100 mm, pH 4.5) and injected over flow cells 1 and 2 (or 3 and 4) at a flow rate of 10 \(\mu\)L min\(^{-1}\). Subsequent regeneration with glycine (10 mm, pH 1.5) yielded the peptide- or protein-modified surface ready for binding experiments.

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![Figure 2. Binding responses of protein G\(^{\prime}\)-modified surfaces to a saturating concentration of IgG (600 s injection of 250 nm IgG followed by 600 s dissociation). 20 RUs of protein G\(^{\prime}\) (\(R=CH_2CH_2SCH_3\)) were immobilized either via oxime chemistry (gray line) or conventional amine coupling (red line).](image-url)
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