Chapter 25

Chemoselective Protein and Peptide Immobilization on Biosensor Surfaces

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Abstract

Site-specific immobilization of proteins and peptides on a sensor surface represents a significant challenge for bioanalytical applications such as surface plasmon resonance (SPR). The most common protocols for covalent protein immobilization usually result in heterogeneous presentation of the ligand at the surface, which can in some instances yield conflicting results with analogous data obtained in solution. Here, we discuss two complementary and generic bioconjugation methods that allow chemoselective immobilization of peptides and proteins via either their C-terminus (native chemical ligation) or their N-terminus (oxime ligation). While the protocols described in this chapter were designed for use in a Biacore instrument, the methods should also be applicable to other SPR instruments and, with slight adjustments, to many other types of bioanalytical applications that rely on protein-functionalized surfaces.

Key words: Biosensor, Protein immobilization, Surface plasmon resonance, Biacore, Native chemical ligation, Oxime ligation

1. Introduction

The immobilization of proteins on surfaces is of practical importance to many areas of the life sciences. The combination of surface plasmon resonance (SPR) with a microfluidic delivery system such as that used in, for example, Biacore instruments is a powerful technology for obtaining kinetic as well as thermodynamic data on biomolecular interactions. However, the site-specific immobilization of proteins and peptides on a sensor surface represents a significant challenge for SPR. The most common protocols for covalent protein immobilization target amine or thiol groups at the protein exterior; consequently, the use of these methods typically results in heterogeneous presentation of the protein at the surface, which can in some cases yield conflicting

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results with analogous data obtained in solution (1). To date, several approaches have been developed for Biacore instruments that allow for more controlled and homogeneous ligand presentation on the chip surface, including the use of biotinylated ligands on streptavidin-functionalized chips, Ni-NTA chips for the immobilization of His-tagged proteins, and the use of antibodies to present the protein ligand in a single orientation. While these methods have proven to be valuable alternatives to direct coupling approaches in certain cases, they all have their own specific drawbacks (2).

In recent years, we as well as others have developed several site-specific covalent immobilization strategies based on the use of bio-orthogonal ligation reactions that were originally developed in the field of peptide chemistry (3). In particular, we explored two complementary and generic bioconjugation methods that allow for the chemoselective immobilization of peptides or recombinant proteins to surfaces via either their C-terminus (native chemical ligation) (4) or their N-terminus (oxime ligation) (5) (Fig. 1). In general, bioconjugation methods targeting the flexible termini of proteins are least likely to interfere with their function. Another attractive property of both conjugation

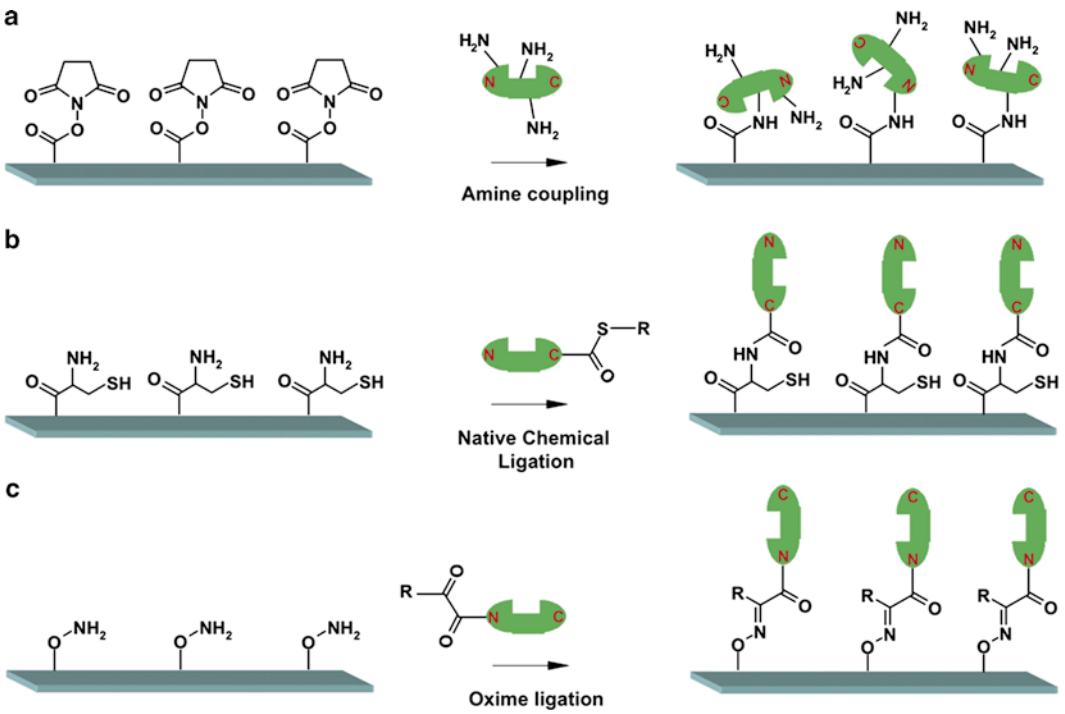

Fig. 1. Classical methods for the immobilization of proteins via reaction with their surface-accessible amines (**a**) result in heterogeneous presentation of the proteins at surfaces. Conjugation using native chemical ligation (**b**) or oxime ligation (**c**) allows homogenous presentation of proteins through chemoselective immobilization via the protein's C- or N-terminus, respectively.

methods is that the surface density of the immobilized ligands is highly controllable and can be increased stepwise at any point in the sensor chip's lifetime. In this chapter, we present detailed protocols for the preparation of SPR chips for use in either native chemical ligation or oxime ligation, the preparation of N- and C-terminally reactive proteins and peptides, and examples of typical applications. While the protocols described herein were originally designed for use in a Biacore instrument, the methods should also be applicable to other SPR instruments and, with slight adjustments, to many other types of bioanalytical applications that rely on protein-functionalized surfaces.

2. Materials

2.1. Synthesis of Thiazolidine-Protected Cysteine Linker (4)

1. *N*-(*tert*-Butyloxycarbonyl)thiazolidine carboxylic acid.
2. *N*-Hydroxysuccinimide (NHS).
3. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU).
4. *N*,*N*-Diisopropylethylamine (DIPEA).
5. *N*,*N*-dimethylformamide (DMF).
6. Diethyl ether.
7. Saturated KCl.
8. Saturated $NaHCO_3$.
9. Na_2SO_4.
10. Mono-*N*-(*tert*-butoxycarbonyl)ethylenediamine was obtained by protecting one amino group of ethylenediamine with *tert*-butyl phenylcarbonate, as described previously in ref. 6.
11. Dichloromethane (DCM).
12. Trifluoroacetic acid (TFA).
13. Ethyl acetate.
14. Brine (saturated NaCl).

2.2. Functionalization of SPR Chips with N-Terminal Cysteines

1. Biacore T100 SPR system (GE Healthcare).
2. Biacore CM5 sensor chips (GE Healthcare).
3. HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.5% (v/v) surfactant P20, pH 7.4) is obtained as a 10× concentrated stock solution from GE Healthcare. After appropriate dilution, the buffer is filtered through 0.2-μm nylon ZapCap-CR filters (Whatman).
4. 1.0 M solution of ethyl-3-[3-dimethylaminopropyl]carbodiimide HCl (EDC) in deionized water.

5. 1.0 M solution of *N*-hydroxysuccinimide (NHS) in deionized water.
6. 250 mM compound **4** (Fig. 3) in 50 mM borate buffer, pH 8.5.
7. Ethanolamine HCl.
8. 250 mM methoxyamine HCl in 50 mM acetate buffer, pH 4.0.

2.3. Synthesis of Peptides with a C-Terminal β-Mercaptopropionic Acid-Leucine Thioester

1. All *tert*-butyloxycarbonyl (*t*-Boc)-protected amino acids were obtained from Novabiochem.
2. 4-Methylbenzhydrylamine hydrochloride (MBHA) resin (0.92 mmol/g loading) (Novabiochem).
3. *S*-Trityl 3-mercaptopropionic acid (Sigma).
4. Triisopropylsilane (Aldrich).
5. HF containing 4% (v/v) *p*-cresol (see Note 1).
6. Reversed-phase high-performance liquid chromatography (HPLC) was performed on a Varian Prostar 320 HPLC system equipped with a VYDAC® protein/peptide C18 column. A gradient of acetonitrile (ACN) in water, both containing 0.1% (v/v) TFA, was used to elute the peptides.

2.4. Synthesis of Recombinant Green Fluorescent Protein with a C-Terminal 2-Mercaptoethanesulfonic Acid Thioester

1. Plasmid pGFPX: pTXB1 vector [New England Biolabs (NEB)] with a green fluorescent protein (GFP) gene cloned into the *Nde*I and *Eco*RI sites present in the multiple cloning site (MCS). This vector encodes GFP fused at its C-terminus to an Mxe intein and a chitin binding domain (7). Other cloning vectors using different intein sequences are also available from NEB.
2. Competent *Escherichia coli* BL21(DE3) cells (Novagen).
3. LB medium (10 g Bacto-peptone, 5 g yeast extract, 10 g NaCl per liter of solution) containing 100 mg/L of ampicillin.
4. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
5. Shaking incubator.
6. High-speed centrifuge.
7. BugBuster® (Novagen) cell lysis buffer containing 1 μL/mL Benzonase® Nuclease (Novagen).
8. 10-mL chitin column.
9. Column buffer: 20 mM sodium phosphate, 0.5 M NaCl, 0.1 mM EDTA, pH 8.0.
10. Cleavage buffer: 200 mM sodium phosphate, 0.5 M NaCl, 0.1 mM EDTA, 50 mM 2-mercaptoethanesulfonic acid (MESNA), pH 6.0.

2.5. Peptide/Protein Ligation to Cysteine-Functionalized SPR Chips

1. SPR chip with N-terminal cysteines (see Subheading 2.2).
2. Peptides or proteins with a C-terminal thioester (see Subheading 2.3 or 2.4).
3. HBS-EP buffer (pH 7.4) containing 50 mM 4-mercaptophenylacetic acid (MPAA) and 10 mM tris(carboxyethyl)phosphine HCl (TCEP).

2.6. Synthesis of t-Boc-Protected Aminooxy Linker (8)

1. Benzyl phenyl carbonate was synthesized according to previously described literature procedures (8).
2. Ethylenediamine.
3. Ethanol.
4. 3 M HCl.
5. 5 M NaOH.
6. *N*-[(*tert*-butyloxycarbonyl)-aminooxy]acetic acid.
7. 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU).
8. Palladium (10%, w/w) on activated carbon (Pd/C catalyst).
9. Hydrogen gas.
10. Celite® 545, diatomaceous earth.

2.7. Functionalization of SPR Chips with Aminooxy Groups

The same materials as listed in Subheading 2.2 are used (with the exception of items 6 and 8).

1. 250 mM compound **8** (Fig. 6) in 50 mM borate buffer, pH 8.5.
2. 1.0 M Phosphoric acid, pH 2.0.

2.8. Synthesis of Peptides or Proteins with an N-Terminal Aldehyde Group

The peptides can be prepared by using either Fmoc- or *t*-Boc-mediated solid-phase peptide synthesis.

1. $NaIO_4$.
2. 10 mM Sodium phosphate buffer, pH 7.0.

2.9. Synthesis of Peptides or Proteins with an N-Terminal Ketone Group

1. Pyridoxal 5′-phosphate (PLP).
2. 50 mM Sodium phosphate buffer pH 6.5.
3. Amicon® Ultra 0.5 mL centrifugal filter devices (5,000 MWCO) (Millipore).
4. The S-protein was purified from RNAse S (grade XII-S) (Sigma) using reversed-phase HPLC as described previously in ref. 9.
5. Protein G′ (Invitrogen).

2.10. Peptide/Protein Ligation to Aminooxy-Functionalized SPR Chips

1. SPR chip with aminooxy groups (see Subheading 2.7).
2. Peptides or proteins with an N-terminal aldehyde or ketone (see Subheading 2.8 or 2.9).
3. 100 mM anilinium acetate, pH 4.5.

3. Methods

Native chemical ligation (NCL) is a chemoselective reaction that occurs spontaneously between a peptide or protein containing a C-terminal thioester and a peptide with an N-terminal cysteine residue under aqueous conditions at neutral pH. The method was originally developed by Dawson et al. in 1994 to allow the chemical synthesis of proteins from smaller peptide fragments obtained by solid-phase peptide synthesis (10). The versatility of NCL was extended by the development of expression systems that use self-cleavable intein domains to generate recombinant proteins with C-terminal thioesters (e.g., IMPACT™ system from New England Biolabs (11)). In other application areas, NCL has been used for the conjugation of recombinant proteins to spectroscopic labels, dendrimers, liposomes, and micelles, as well as the site-specific immobilization of proteins to surfaces and the facile incorporation of unnatural amino acids (7, 12).

In order to apply NCL for use in SPR, we developed a method that allows the functionalization of the SPR chip surface with cysteine residues with a free N-terminus (Fig. 2). Most commercially available SPR chips consist of a gold substrate that is coated with a nonfouling, carboxymethylated dextran layer. This dextran hydrogel surface layer can be readily modified to become reactive toward amine-containing compounds through the activation of the carboxylic acid groups by EDC/NHS treatment. To allow the functionalization of the carboxymethylated dextran layer with cysteine molecules using EDC/NHS coupling, we developed the thiazolidine derivative **4**, which consists of a small diamine linker coupled to a nascent N-terminal cysteine. The thiazolidine ring protects the cysteine during the coupling reaction, but can be readily removed by treatment with 250 mM methoxyamine in buffer at pH 4. These relatively mild conditions are compatible with the Biacore's microfluidics system components, thus allowing deprotection of the cysteine residues to be performed directly within the instrument.

A variety of strategies have been developed to prepare synthetic peptides with C-terminal thioesters. Peptides with thioesters are still most efficiently synthesized using *t*-Boc chemistry (13), although recently, several new methods based on Fmoc chemistry (14) have also been reported. Recombinant full-length proteins

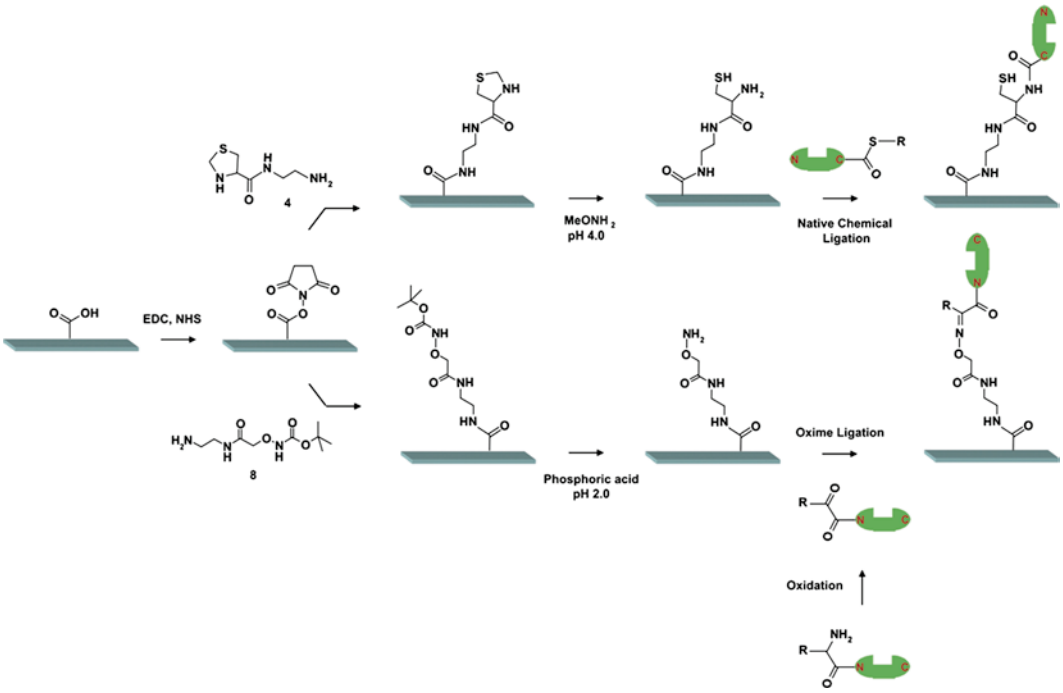

Fig. 2. Synthetic strategies for the generation of cysteine- and aminooxy-functionalized SPR sensor chip surfaces starting from a carboxymethylated dextran-coated gold surface.

with a C-terminal thioester can be obtained using the commercially available IMPACT system from New England Biolabs, in which the gene of interest is cloned into an expression vector that generates a fusion protein with a C-terminal intein domain followed by a chitin domain. The chitin domain allows for the convenient purification of the fusion protein using chitin resins. Treatment with 50 mM MESNA induces the intein-catalyzed cleavage of the fusion protein, and then the protein of interest containing a C-terminal MESNA thioester is eluted from the chitin column. The expression of proteins whose stability depends on the presence of disulfide bonds can be cumbersome using the IMPACT system because of the use of reducing thiols during intein cleavage; however, two methods have recently been reported that allow efficient refolding using a MESNA-diMESNA redox couple (15) or allow efficient expression of the intein-fusion proteins by directing them to the bacterial periplasm (16).

The second bioconjugation method described in this chapter is based on the formation of an oxime bond upon reaction of an aminooxy group with an aldehyde or ketone group. This strategy is complementary to NCL, as it allows chemoselective covalent immobilization of proteins via their N-terminus. The approach

takes advantage of two recent advances in the field of oxime chemistry. Francis and coworkers have shown that treatment of proteins with pyridoxal 5 -phosphate (PLP) allows site-specific oxidation of N-terminal amino acids into N-terminal ketones (17). Unlike the classical oxidation method using $NaIO_4$, which is limited to N-terminal serine residues (18), PLP oxidation can be applied to most N-terminal amino acids, although with varying yields (19). Oxime ligations can thus be applied to a broad range of proteins without the need to introduce additional tags, provided that the N-terminus is not blocked by, for example, acylation. Recently, recombinant procedures have been developed by the Bertozzi group that allow site-specific introduction of formylglycine functionalities at any site within a protein sequence (20). The second important development is the finding by Dawson and coworkers that oxime ligations can be significantly accelerated using aniline as an organocatalyst (21). The latter finding has made oxime ligations one of the most efficient ligation reactions known today.

Similar to the strategy used to generate cysteine-functionalized SPR chip surfaces, a protected aminooxy group linked to a small diaminoethyl spacer (8) can be used to functionalize commercially available carboxymethyl group-containing chips with aminooxy groups (Fig. 2). In the first step of the derivatization procedure, the free amine group of the *t*-Boc-protected aminooxy linker is conjugated to the carboxymethylated dextran surface using standard EDC/NHS chemistry. Deprotection of the aminooxy group in this case requires the removal of the chip from the Biacore instrument and overnight incubation of the chip in 1 M phosphoric acid buffer at pH 2 (22). When catalyzed by aniline, oxime ligations compare favorably to NCL with respect to reaction speed. A second practical advantage of oxime ligations is the absence of redox chemistry, making the method particularly attractive for the immobilization of disulfide bond-containing proteins such as immunoglobulins.

3.1. Synthesis of Thiazolidine-Protected Cysteine Linker (4)

The synthesis of the protected cysteine derivative involves a three-step process as described below (Fig. 3).

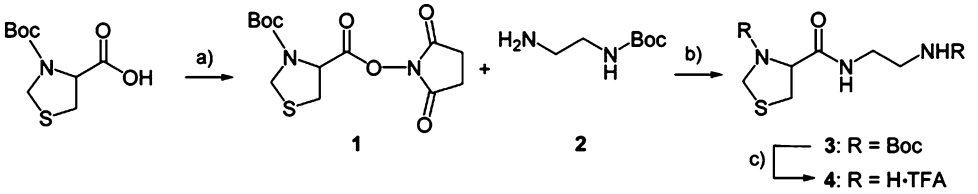

Fig. 3. Synthesis of a thiazolidine-protected cysteine.

3.1.1. Preparation of N-(tert-Butyloxycarbonyl) Thiazolidine-4-Carboxylic Acid N-Hydroxysuccinimidyl Ester (1)

1. Dissolve *N*-(*tert*-butyloxycarbonyl)thiazolidine carboxylic acid (1.00 g, 4.3 mmol), NHS (542 mg, 4.7 mmol), HBTU (1.63 g, 4.3 mmol), and DIPEA (1.66 g, 12.9 mmol) in DMF (10 mL).
2. Stir the reaction mixture for 12 h.
3. Concentrate the reaction mixture to dryness in vacuo and dissolve the residue in ether (100 mL).
4. Wash the ethereal layer with saturated KCl (5×100 mL), saturated $NaHCO_3$ (5×100 mL), and deionized water (10×100 mL).
5. Dry over Na_2SO_4, filter, and concentrate.

The yield is 950 mg (67%). ^{1}H NMR ($CDCl_3$): (3:1 mixture of diastereomers): 5.16 (br, 1H, minor), 4.91 (t, J=5.7 Hz, 1H, major), 4.61 (dd, J=54, 9.2 Hz, 2H, major), 4.54 (dd, J=65, 9.0 Hz, 2H, minor), 3.50 (m, 2H, minor), 3.41 (m, 2H, major), 2.83 (s, 4H, CH_2CH_2), 1.47 (s, 9H).

3.1.2. Preparation of N-(tert-Butyloxycarbonyl) Thiazolidine-4-Carboxylic Acid [2-(N-tert-Butyloxycarbonyl) Aminoethyl]Amide (3)

1. Add a solution of mono-*N*-(*tert*-butoxycarbonyl)ethylenediamine **2** (361 mg, 2.25 mmol) in DCM (2 mL) to a flask that contains **1** (676 mg, 2.05 mmol) and DIPEA (793 mg, 6.14 mmol) in DCM (3 mL).
2. Stir for 6 h.
3. Remove the solvent in vacuo and dissolve the residue in ethyl acetate (50 mL).
4. Extract the product with saturated $NaHCO_3$ (5×50 mL), deionized water (5×50 mL), and brine (50 mL).
5. Dry the organic layer over Na_2SO_4, filter, and concentrate.

The yield is 735 mg (95%). ^{1}H NMR ($CDCl_3$): 6.92 (br, 1H), 4.92 (br, 1H), 4.61 (m, 2H), 4.38 (br, 1H), 3.36 (m, 2H), 3.23 (m, 4H), 1.47 (s, 9H), 1.43 (s, 9H).

3.1.3. Preparation of Thiazolidine-4-Carboxylic Acid (2-Aminoethyl)Amide (4)

1. Dissolve compound **3** (730 mg, 1.94 mmol) in a mixture of DCM and TFA (10 mL, 1:1 (v/v)).
2. Stir for 3 h.
3. Concentrate the mixture in vacuo to dryness and dissolve the residue in deionized water (10 mL).
4. Wash the aqueous layer with DCM (3×25 mL) and lyophilize.

The yield is 330 mg (97%). ^{1}H NMR (d_6-DMSO): 8.64 (s, 1H), 7.83 (br, 3H), 6.0–5.0 (br, 2H), 4.28 (dd, J=15.4, 6.6 Hz, 2H), 4.22 (dd, J=15.8, 6.2 Hz, 1H), 3.34 (m, 2H), 3.25–3.12 (m, 2H), 2.88 (m, 2H).

3.2. Functionalization of SPR Sensor Chips with N-Terminal Cysteines

1. Use HBS-EP (pH 7.4) as the running buffer on a Biacore T100 instrument and set the flow rate to 10 μL/min.
2. Insert a CM5 sensor chip and immobilize the thiazolidine derivative **4** in flow channels 2 and 4 of the Biacore instrument at 25°C according to the procedures described in steps 3–5 below.
3. Inject a solution containing 1.0 M EDC and 1.0 M NHS for 7 min.
4. Inject a solution containing 250 mM of **4** in 50 mM borate buffer (pH 8.5) for 7 min.
5. Inject a solution of ethanolamine HCl (1.0 M) at pH 8.5 for 7 min.
6. Use flow channels 1 and 3 as reference channels, and immobilize ethanolamine HCl according to the procedures described in steps 7–8 below.
7. Inject a solution containing 1.0 M EDC and 1.0 M NHS for 7 min.
8. Inject twice a solution of ethanolamine HCl (1.0 M) at pH 8.5 for 7 min each.
9. Raise the temperature of the chip to 37°C, and set the flow rate in channels 1, 2, 3, and 4 to 5 μL/min.
10. Inject twice a solution containing 250 mM methoxyamine HCl in 50 mM acetate buffer (pH 4.0) for 70 min over all channels.
11. Cool the chip to 25°C and set the flow rate in all channels to 10 μL/min.
12. Regenerate the surface of all channels with 50 mM NaOH in 0.5 M NaCl using 10 pulses of 30 s each (see Note 2).

3.3. Synthesis of Peptides with a C-Terminal β-Mercaptopropionic Acid-Leucine Thioester

1. Preparation of a TAMPAL resin to yield a C-terminal β-Mercaptopropionic Acid-Leucine (MPAL) thioester (13): Activate *t*-Boc-Leu-OH (1.1 mmol) by HBTU (1.0 mmol; 0.5 M in DMF) in the presence of DIPEA (3 mmol) for 3–4 min. Couple *t*-Boc-Leu-OH to 0.25 mmol of 4-methylbenzhydrylamine (MBHA) resin for 10 min. Remove unbound amino acid by rinsing with a DMF flow wash (2×20 s). Remove the *t*-Boc group by a TFA treatment (2×1 min). Perform a second DMF flow wash (2×20 s). Activate 1.1 mmol of *S*-tritylmercaptopropionic acid with 1.0 mmol of HBTU in the presence of 3 mmol DIPEA and couple it for 30 min to the Leu-MBHA resin. Perform a DMF flow wash (2×20 s). Remove the protecting trityl group by the addition of TFA containing 2.5% (v/v) triisopropylsilane and 2.5% (v/v) H_2O. Perform a DMF flow wash (2×20 s).

2. Grow the peptide by manual *t*-Boc-mediated solid-phase peptide synthesis (23): Activate each *t*-Boc-amino acid (1.1 mmol) by HBTU (1.0 mmol) in the presence of DIPEA (3 mmol) for 3–4 min. Couple all activated amino acids for 10 min, except for Arg, Ser, and Asn, which require a coupling time of 20 min. Perform a DMF flow wash (2 × 20 s). Remove the *t*-Boc group by a TFA treatment. Perform a DMF flow wash (2 × 20 s).
3. After synthesis, wash the peptide with DCM and 50% (v/v) MeOH in DCM to remove all DMF and dry in vacuo.
4. Cleave the peptide from the resin and remove the protecting groups from the side chains using HF treatment over 1 h at 0°C with 4% (v/v) *p*-cresol (see Note 1).
5. Precipitate the peptide in ice-cold diethyl ether.
6. Dissolve the pellet in acetonitrile and lyophilize.
7. Purify the peptide using reversed-phase HPLC.

3.4. Synthesis of GFP with a C-Terminal MESNA Thioester

1. Transform *E. coli* BL21(DE3) cells with the pGFPX plasmid.
2. Inoculate 2 mL of LB medium containing 100 mg/L ampicillin with a single colony of transformed bacteria.
3. Incubate overnight in a shaking incubator (225 rpm) at 37°C and transfer the 2-mL seed culture into 200 mL of LB medium containing 100 mg/L ampicillin.
4. Monitor the optical density of the cell culture at $\lambda = 600$ nm using an absorbance spectrophotometer. At $OD_{600} = 0.5$, lower the incubation temperature to 15°C and add 0.3 mM IPTG to induce protein expression.
5. Collect the cells after overnight expression at 15°C (250 rpm) by low-speed centrifugation (~3,000 × *g*), and resuspend the cell pellet in BugBuster (Novagen) lysis buffer containing 1 μL/mL Benzonase Nuclease and incubate for 20 min at 20°C.
6. Centrifuge the cell lysate at 40,000 × *g* for 45 min to obtain a clear supernatant.
7. Load the supernatant onto a 10-mL chitin column equilibrated with column buffer.
8. Wash the column with 10 volumes of column buffer to remove nonbinding and nonspecifically bound proteins.
9. Flush the column with 3 volumes of cleavage buffer, and then close the column outlet and incubate the column overnight at 20°C.
10. Elute the GFP containing a MESNA thioester using 1 volume of cleavage buffer and store at −80°C.

3.5. Peptide/Protein Ligation to Cysteine-Functionalized SPR Chips

1. Incubate the peptide (0.5–5 mM) or protein (0.1 mM) containing a C-terminal thioester in HBS-EP buffer (pH 7.4) containing 50 mM 4-mercaptophenylacetic acid and 10 mM TCEP for 30 min at 25°C.
2. Inject the mixture from step 1 over flow channels 1 and 2 (or 3 and 4) of the Biacore instrument at a flow rate of 5 µL/min. The injection time may be varied in order to obtain surfaces with different immobilization levels. (As an example, the immobilization of a streptavidin-binding peptide (SLLAHPQGGG-MPAL) (24) and a GFP are shown in Figs. 4 and 5, respectively.)

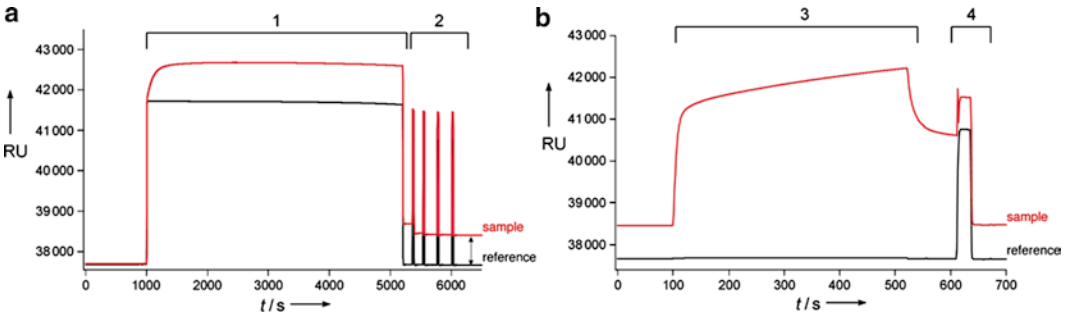

Fig. 4. (**a**) Ligation of a streptavidin-binding peptide to a cysteine-modified CM5 SPR sensor chip: (1) injection of the peptide with an MPAL-thioester in the presence of 50 mM MPAA and 10 mM TCEP; and (2) surface regeneration with 30-s washes with a buffer containing 50 mM NaOH and 500 mM NaCl. (**b**). Binding of streptavidin to the peptide-modified surface: (3) injection of 2 mM streptavidin; and (4) surface regeneration. Reproduced with permission from ref. 4 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA.

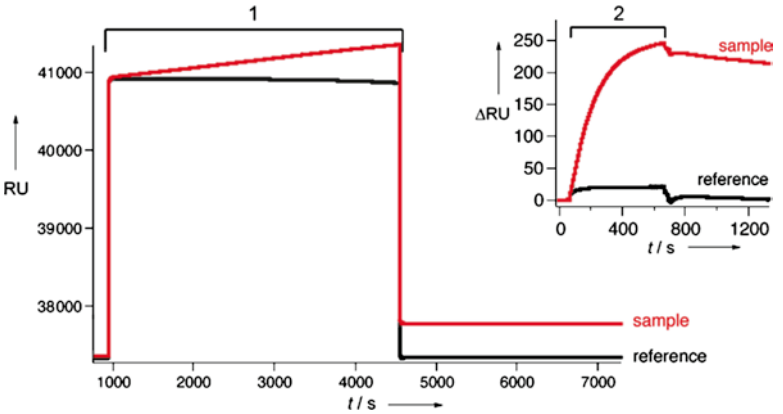

Fig. 5. Immobilization of green fluorescent protein (GFP) to a cysteine-modified CM5 SPR sensor chip. The sample channel was functionalized with cysteines, whereas the reference channel was reacted with ethanolamine. 0.1 mM GFP-MESNA thioester with 50 mM MPAA and 10 mM TCEP was injected over both channels for 1 h (1), followed by injection of buffer only. The difference in the signal response between the sample and reference channels represents the immobilization of 480 resonance units (RU) of GFP. The inset shows the specific binding of anti-GFP antibody JL-8 (Clontech) to the sensor surface of the sample channel (2). Reproduced with permission from ref. 4 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA.

3. Regenerate the surfaces of all the channels using 30-s pulses of an appropriate buffer solution until a stable baseline is reached.
4. Perform binding experiments on the peptide- or protein-modified sensor surface (see Figs. 4 and 5, for examples; also see Note 3).

3.6. Synthesis of t-Boc-Protected Aminooxy Linker (8)

The synthesis of the protected aminooxy derivative involves a three-step process as described below (Fig. 6).

3.6.1. Preparation of (2-Aminoethyl)Carbamic Acid Benzyl Ester (6)

1. Add benzyl phenyl carbonate (1.00 g, 4.4 mmol) to a stirred solution of ethylenediamine (263 mg, 4.4 mmol) in EtOH (17 mL).
2. Stir the reaction mixture for 12 h.
3. Concentrate the reaction mixture in vacuo and dissolve the residue in H_2O (25 mL).
4. Adjust the pH to 3 by the addition of aqueous HCl (3 M) and extract the aqueous layer with DCM (2 × 50 mL).
5. Adjust the pH of the aqueous layer to 11 by the addition of aqueous NaOH (5 M) and extract with DCM (3 × 80 mL).
6. Dry the organic layer over Na_2SO_4, filter, and concentrate.

The yield is 492 mg (58%). ^{1}H NMR ($CDCl_3$): 7.37–7.33 (m, 5H), 5.11 (br s, 3H), 3.26–3.24 (m, 2H), 2.83 (t, 2H, J = 5.9 Hz), 1.38 (br s, 2H).

3.6.2. Preparation of 2-[(tert-Butyloxycarbonyl) Aminooxy]-N[2-(Benzyloxycarbonyl Amino) Ethyl]Acetamide (7)

1. Dissolve **6** (272 mg, 2.7 mmol), *N*-[(*tert*-butyloxycarbonyl) aminooxy]acetic acid (268 mg, 2.7 mmol), HCTU (609 mg, 2.8 mmol), and DIPEA (572 mg, 8.5 mmol) in DMF (5 mL).
2. Stir the reaction mixture for 12 h.
3. Remove the solvent in vacuo and dissolve the residue in ethyl acetate (50 mL).

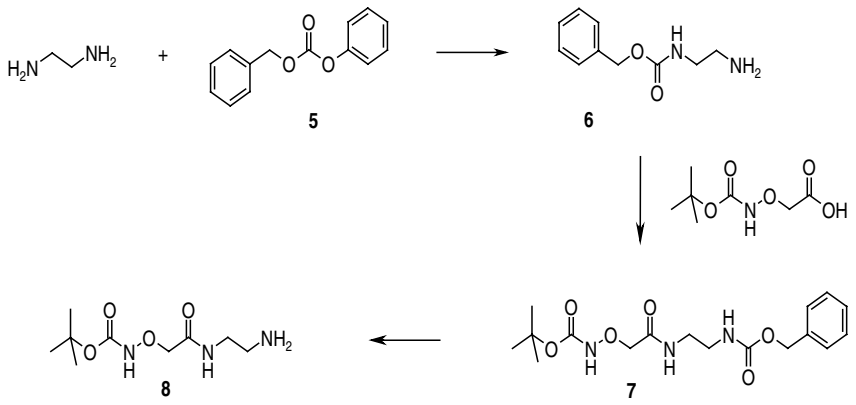

Fig. 6. Synthesis of a protected aminooxy linker.

4. Extract with saturated KCl (3 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL), deionized water (50 mL), and brine (50 mL).
5. Dry the organic layer over Na_2SO_4, filter, and concentrate.

The yield is 418 mg (81%). ^{1}H NMR ($CDCl_3$): 8.21 (br s, 1H), 7.74 (s, 1H), 7.37–7.32 (m, 5H), 5.45 (br s, 1H), 5.11 (s, 2H), 4.34 (s, 2H), 3.45–3.41 (m, 2H), 3.41–3.38 (m, 2H), 1.42 (s, 9H).

3.6.3. Preparation of 2-[(tert-Butyloxycarbonyl) Aminooxy]-N(2-Aminoethyl)Acetamide (8)

1. Add Pd/C catalyst (30 mg) to a solution of **7** (300 mg, 0.82 mmol) in ethanol (25 mL).
2. Degas the suspension and set it under a H_2-atmosphere (1 bar) for 4 h.
3. After exposure to H_2, filter the reaction mixture through Celite® and concentrate in vacuo.
4. Dissolve the residue in deionized water (30 mL), extract with DCM (3 × 30 mL), and lyophilize.

The yield is 170 mg (89%). ^{1}H NMR (d_6-DMSO): 4.17 (s, 2H), 3.35–3.30 (m, 2H), 2.84 (t, 2H, J = 6.2 Hz), 1.42 (s, 9H).

3.7. Functionalization of SPR Sensor Chips with Aminooxy Groups

1. Use HBS-EP (pH 7.4) as the running buffer on a Biacore T100 instrument and set the flow rate to 10 µL/min.
2. Insert a CM5 chip and immobilize the aminooxy derivative **8** in flow channels 2 and 4 at 25°C according to the procedures described in steps 3–5 below.
3. Inject a solution containing 1.0 M EDC and 1.0 M NHS for 7 min.
4. Inject a solution containing 250 mM of **8** in 50 mM borate buffer (pH 8.5) for 7 min.
5. Inject a solution of ethanolamine HCl (1.0 M) at pH 8.5 for 7 min.
6. Use flow channels 1 and 3 as reference channels and immobilize ethanolamine HCl according to the procedures described in steps 7–8 below.
7. Inject a solution containing 1.0 M EDC and 1.0 M NHS for 7 min.
8. Inject twice a solution of ethanolamine HCl (1.0 M) at pH 8.5 for 7 min each.
9. Eject the sensor chip from the Biacore instrument docking area.
10. Incubate the sensor chip overnight in phosphoric acid buffer (1 M) at pH 2.0.
11. Rinse the sensor chip surface with deionized water and re-insert the chip into the Biacore instrument.
12. Regenerate the surface of all channels using 2 × 30-s pulses of HCl (100 mM), NaOH (50 mM), and 0.5% (w/v) SDS at a flow rate of 100 µL/min.

3.8. Synthesis of Peptides or Proteins with an N-Terminal Aldehyde Group

1. Synthesize the peptide using either Fmoc- or *t*-Boc-mediated solid-phase peptide synthesis.
2. Couple an extra serine residue at the N-terminus of the peptide before cleavage.
3. Treat the purified peptide for 5 min with 1.2 equivalents of $NaIO_4$ in 0.01 M sodium phosphate buffer, pH 7.0, at 4°C (18). For proteins that contain a serine or threonine at their N-terminus, this step can be performed to introduce an aldehyde as well.
4. Purify the peptide after 5 min by reversed-phase HPLC.

3.9. Synthesis of Peptides or Proteins with an N-Terminal Ketone Group (see Notes 4 and 5)

1. Dissolve the protein (33 µM) together with PLP (6.7 mM) in sodium phosphate buffer (50 mM, pH 6.5) (17).
2. Incubate overnight at 41°C.
3. Remove the excess PLP remaining via repeated concentration and dilution in 50 mM sodium phosphate buffer (pH 6.5) using Amicon® centrifugal filter devices.

3.10. Peptide/Protein Ligation to Aminooxy-Functionalized SPR Chips

1. Dissolve the peptide (1 mM) or protein (5–100 µM) containing an N-terminal aldehyde or ketone group in 100 mM anilinium acetate, pH 4.5. If the protein is not stable at pH 4.5, then buffers with higher pH values can be used. In the presence of *p*-methoxyaniline as a catalyst, efficient immobilization can still be reached at these pH values (Fig. 7).

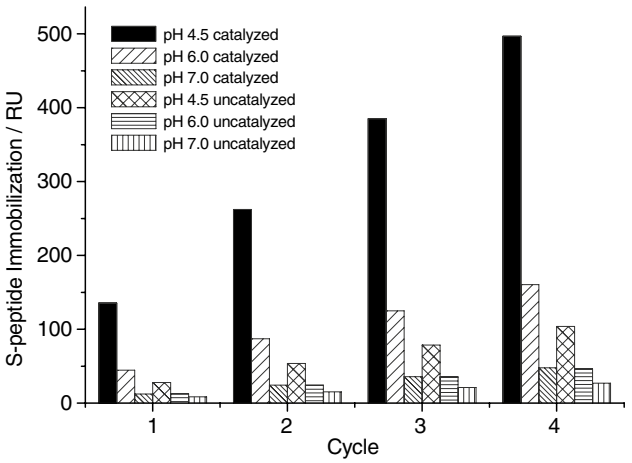

Fig. 7. Efficiency of S-peptide immobilization to SPR sensor chips under various pH conditions and in the presence or absence of catalytic aniline derivatives. Reference-subtracted immobilization levels are shown for an aminooxy SPR sensor chip surface functionalized using sequential injections (4 × 12 s) of 1 mM $NaIO_4$-treated S-peptide in various buffer solutions (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. Reproduced with permission from ref. 5

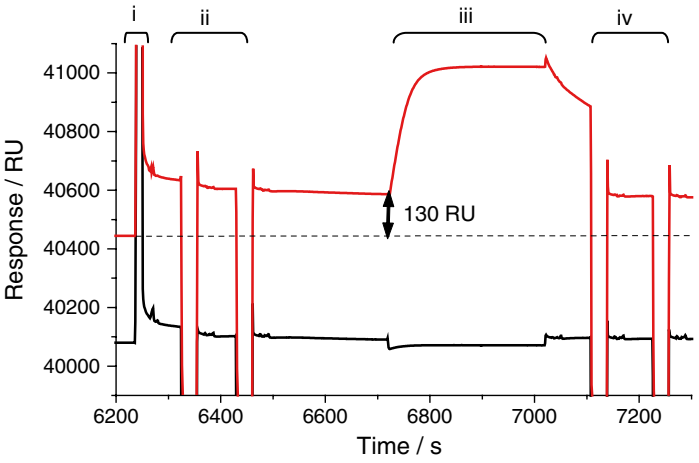

Fig. 8. Surface immobilization of S-peptide to SPR sensor chips using catalyzed oxime ligation. A 12-s pulse of 1 mM $NaIO_4$-treated S-peptide in 100 mM anilinium acetate at pH 4.5 (i) results in 130 RU of immobilized peptide after two regeneration steps using 10 mM glycine, pH 1.5 (ii), subsequent injection of 150 nM S-protein analyte (iii) results in specific binding to the S-peptide-functionalized channel surface. Finally, two regeneration steps using 10 mM glycine (pH 1.5) (iv) were performed to remove the noncovalently bound S-protein analyte from the surface. The sample channel surface (*upper trace*) was functionalized with aminooxy groups, whereas the reference channel surface (*lower trace*) was modified with ethanolamine. Reproduced with permission from ref. 5

2. Inject the mixture from step 1 over flow channels 1 and 2 (or 3 and 4) at a flow rate of 10 µL/min. Vary the injection time to obtain surfaces with different immobilization levels (as examples, the immobilization of the S-peptide (aldehyde-KETAAAKFERQHMDS) (25) and Protein G′ are shown in Figs. 8 and 9, respectively).
3. Regenerate the surfaces of all the channels using 30-s pulses of an appropriate buffer solution until a stable baseline is reached (see Note 6).
4. Perform binding experiments on the peptide- or protein-modified sensor surface (see Note 7).

4. Notes

1. Peptides with a C-terminal thioester prepared via *t*-Boc chemistry require the use of HF for cleavage. *Caution*: HF is an extremely corrosive and poisonous acid, and should be handled with extreme care. Alternatively, these peptides can also be prepared via Fmoc-mediated solid-phase peptide synthesis using, for example, safety-catch resins (14).

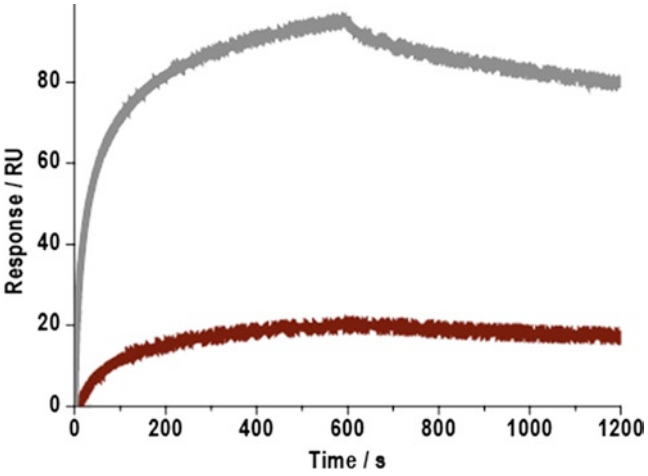

Fig. 9. Comparison of CM5 SPR sensor chips functionalized with 20 RU of protein G′ using oxime ligation (*upper trace*) versus classical amine coupling (*lower trace*). The chip surface obtained using classical amine coupling binds only 17 RU of IgG analyte under saturating concentrations, whereas 130 RU are expected assuming 1:1 binding. The same amount of protein G′ immobilized via oxime ligation showed 97 RU of IgG analyte binding under identical conditions. 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 (pH 4.5) for only 36 s, whereas a 5-min injection of 1 μM protein G′ was required to reach the same immobilization level using classical amine coupling. Reproduced with permission from ref. 5 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA.

2. Sensor chips that were prepared according to the above procedure were used directly in ligation experiments or stored at 4°C in HBS-EP buffer (pH 7.4) until needed. For the chips prepared via native chemical ligation, TCEP (2 mM) was added to the buffer.
3. During binding experiments, we typically use buffers that include 2 mM of TCEP or DTT to prevent oxidation of the surface cysteines. In the absence of these reducing agents, we occasionally observed some baseline drift, which we ascribed to the formation of disulfide bonds. However, these reducing agents may sometimes interfere with proteins whose stability depends on the presence of disulfide bonds. To ensure a stable baseline in the absence of reducing agents, the free sulfhydryl groups can be reacted with DTNB (Ellman's reagent) to form stable disulfide bonds. If required, DTNB protection can be removed by treatment with DTT or TCEP.
4. Unlike the oxidation of N-terminal serines by $NaIO_4$, the oxidation of N-terminal amino acids by PLP is not quantitative. Yields vary between 10 and 80%, and depend strongly on the specific amino acids present. For SPR applications, these nonstoichiometric yields typically do not present a serious

problem, since nonoxidized proteins will not react to the sensor chip surface and will be removed by the flow system. Moreover, proteins containing Q, W, H, P, and K at the N-terminus have been reported to be essentially unreactive toward PLP oxidation (19).

5. The conversion of N-terminal amino acids into N-terminal ketones during PLP oxidation can be quantified by the site-selective attachment of PEG polymers followed by SDS-PAGE analysis (17).
6. Unlike NCL, oxime bond formation is in principle reversible. However, no significant decrease in immobilization levels were observed even after overnight incubation of the chip under a constant flow of buffer, which is consistent with the fact that the oxime bonds are very stable under neutral conditions (26). Partial cleavage of the oxime bond is possible, but only under extreme, nonphysiological conditions such as a constant flow of buffer containing 250 mM methoxylamine and 100 mM anilinium acetate at pH 4.5.
7. Immobilization of protein G can also be performed using C1 chips that lack the dextran layer and are thus more similar to many other non-SPR chip surfaces (5).

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References

1. a) Johnsson, B., Lofas, S., Lindquist, G. (1991) Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal. Biochem.* **198**, 268–277; b) Peluso, P., Wilson, D. S., Do, D., Tran, H., Venkatasubbaiah, M., Quincy, D., Heidecker, B., Poindexter, K., Tolani, N., Phelan, M., Witte, K., Jung, L. S., Wagner, P., Nock, P. (2003) Optimizing antibody immobilization strategies for the construction of protein microarrays. *Anal. Biochem.* **312**, 113–124; c) Kortt, A. A., Oddie, G. W., Iliades, P., Gruen, L. C., Hudson, P. J. (1997) Nonspecific amine immobilization of ligand can be a potential source of error in BIAcore binding experiments and may reduce binding affinities. *Anal. Biochem.* **253**, 103–111.
2. Jonkheijm, P., Weinrich, D., Schroeder, H., Niemeyer, C. M., Waldmann, H. (2008) Chemical strategies for generating protein biochips. *Angew. Chem. Int. Ed.* **47**, 9618–9647.
3. a) Girish, A., Sun, H., Yeo, D. S. Y., Chen, G. Y. J., Chua, T.-K., Yao, S. Q. (2005) Site-specific immobilization of proteins in a microarray using intein-mediated protein splicing. *Bioorg. Med. Chem. Lett.* **15**, 2447–2451; b) Houseman, B. T., Huh, J. H., Kron, S. J., Mrksich, M. (2002) Peptide chips for the quantitative evaluation of protein kinase

activity. *Nat. Biotechnol.* **20**, 270–274; c) Kalia, J., Abbott, N. L., Raines, R. T. (2007) General method for site-specific protein immobilization by staudinger ligation. *Bioconjug. Chem.* **18**, 1064–1069; d) Camarero, J. A., Kwon, Y., Coleman, M. A. (2004) Chemoselective attachment of biologically active proteins to surfaces by expressed protein ligation and its application for "protein chip" fabrication. *J. Am. Chem. Soc.* **126**, 14730–14731.

4. Helms, B., van Baal, I., Merkx, M., Meijer, E. W. (2007) Site-specific protein and peptide immobilization on a biosensor surface by pulsed native chemical ligation. *ChemBioChem* **8**, 1790–1794.
5. Lempens, E. H. M., Helms, B. A., Merkx, M., Meijer, E. W. (2009) Efficient and chemoselective surface immobilization of proteins by using aniline-catalyzed oxime chemistry. *ChemBioChem* **10**, 658–662.
6. Jones, D. S. Coutts, S. M., Gamino, C. A., Iverson, G. M., Linnik, M. D. Randow, M. E., Ton-Nu, H.-T., Victoria, E. J. (1999) Multivalent thioether-peptide conjugates: B cell tolerance of an anti-peptide immune response. *Bioconjug. Chem.* **10**, 480–488.
7. van Baal, I., Malda, H., Synowsky, S. A., van Dongen, J. L. J., Hackeng, T. M., Merkx, M., Meijer, E. W. (2005) Multivalent peptide and protein dendrimers using native chemical ligation. *Angew. Chem. Int. Ed.* **44**, 5052–5057.
8. Pittelkow, M., Lewinsky, R., Christensen, J. B. (2002) Selective synthesis of carbamate protected polyamines using alkyl phenyl carbonates. *Synthesis* **15**, 2195–2202.
9. Lempens, E. H. M., van Baal, I., van Dongen, J. L. J., Hackeng, T. M., Merkx, M., Meijer, E. W. (2009) Noncovalent synthesis of protein dendrimers. *Chem. Eur. J.* **15**, 8760–8767.
10. a) Dawson, P. E., Muir, T. W., Clark-Lewis, I., Kent, S. B. H. (1994) Synthesis of proteins by native chemical ligation. *Science* **266**, 776–779; b) Johnson, E. C. B., Kent, S. B. H. (2006) Insights into the mechanism and catalysis of the native chemical ligation reaction. *J. Am. Chem. Soc.* **128**, 6640–6646.
11. Muir, T. W., Sondhi, D., Cole, P. A. (1998) Expressed protein ligation: a general method for protein engineering. *Proc. Natl. Acad. Sci. USA* **95**, 6705–6710.
12. a) Reulen, S. W. A., Brusselaars, W. W. T., Langereis, S., Mulder, W. J. M., Breurken, M., Merkx, M. (2007) Protein-liposome conjugates using cysteine-lipids and native chemical ligation. *Bioconjug. Chem.* **18**, 590–596; b) Reulen, S. W. A., Dankers, P. Y. W., Bomans, P. H. H., Meijer, E. W., Merkx, M. (2009) Collagen targeting using protein-functionalized micelles: the strength of multiple weak interactions. *J. Am. Chem. Soc.* **131**, 7304–7312.
13. Hackeng, T. M., Griffin, J. H., Dawson, P. E. (1999) Protein synthesis by native chemical ligation: expanded scope by using straightforward methodology. *Proc. Natl. Acad. Sci. USA* **96** 10068–10073.
14. a) Ingenito, R., Bianchi, E., Fattori, D., Pessi, A. (1999) Solid phase synthesis of peptide C-terminal thioesters by Fmoc/t-Bu chemistry. *J. Am. Chem. Soc.* **121**, 11369–11374; b) Blanco-Canosa, J. B., Dawson, P. E. (2008) An efficient Fmoc-SPPS approach for the generation of thioester peptide precursors for use in native chemical ligation. *Angew. Chem. Int. Ed.* **47**, 6851–6855.
15. Bastings, M. M. C., van Baal, I., Meijer, E. W., Merkx, M. (2008) One-step refolding and purification of disulfide-containing proteins with a C-terminal MESNA thioester. *BMC Biotechnology* **8,** 76.
16. Reulen, S. W. A., van Baal, I., Raats, J. M. H., Merkx, M. (2009) Efficient, chemoselective synthesis of immunomicelles using single-domain antibodies with a C-terminal thioester. *BMC Biotechnology* **9**, 66.
17. Gilmore, J. M., Scheck, R. A., Esser-Kahn, A. P., Joshi, N. S., Francis, M. B. (2006) N-terminal protein modification through a biomimetic transamination reaction. *Angew. Chem. Int. Ed.* **45**, 5307–5311.
18. Geoghegan, K. F., Stroh, J. G. (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine. *Bioconjug. Chem.* **3**, 138–146.
19. Scheck, R. A., Dedeo, M. T., Iavarone, A. T., Francis, M. B. (2008) Optimization of a biomimetic transamination reaction. *J. Am. Chem. Soc.* **130**, 11762–11770.
20. a) Carrico, I. S., Carlson, B. L., Bertozzi, C. R. (2007) Introducing genetically encoded aldehydes into proteins. *Nat. Chem. Biol.* **3**, 321–322; b) Rush, J. S., Bertozzi, C. R. (2008) New aldehyde tag sequences identified by screening formylglycine generating enzymes in vitro and in vivo. *J. Am. Chem. Soc.* **130**, 12240–12241.
21. Dirksen, A., Hackeng, T. M., Dawson, P. E. (2006) Nucleophilic catalysis of oxime ligation. *Angew. Chem. Int. Ed.* **45**, 7581–7584.
22. Li, B., Bemish, R., Buzon, R. A., Chiu, C. K.-F., Colgan, S. T., Kissel, W., Le, T., Leeman, K. R., Newell, L., Roth, J. (2003) Aqueous phosphoric acid as a mild reagent for

deprotection of the tert-butoxycarbonyl group. *Tetrahedron Lett.* **44**, 8113–8115.

23. Schnolzer, M., Alewood, P., Jones, A., Alewood, D., Kent, S. B. (1992) In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int. J. Peptide Protein Res.* **40**, 180–93.
24. Bastings, M. M. C., Helms, B. A., van Baal, I., Hackeng, T. M., Merkx, M, Meijer, E. W. (2011) From phage display to dendrimer display: insights into multivalent binding **133**, doi:10.1021/ja110700x.
25. a) Richards, F. M., Vithayathil, P. J. (1959) Preparation of subtilisin-modified ribonuclease and the separation of the peptide and protein components. *J. Biol. Chem.* **234**, 1459-1465; b) Raines, R. T. (1998) Ribonuclease A. *Chem. Rev.* **98**, 1045–1065.
26. Kalia, J., Raines, R. T. (2008) Hydrolytic stability of hydrazones and oximes. *Angew. Chem. Int. Ed.* **47**, 7523–7526.