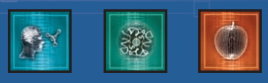
Ligand immobilization using thiol-disulphide exchange



Abstract

This Application Note describes an immobilization procedure based on thiol-disulphide exchange, providing a valuable complement to the established amine coupling reaction. Thiol coupling is particularly useful for acidic ligands. The method is exemplified by immobilization of acidic peptides from the interleukin-2 receptor, the acidic carcinoembryonic antigen (CEA) and antibodies against β2-microglobulin. The last-named example provides a direct comparison of the thiol and amine coupling methods.

Introduction

Biacore's surface plasmon resonance (SPR) technology is a complete system for real-time biomolecular interaction analysis [1-4]. One of the interacting components (the ligand) is immobilized on the surface of a sensor chip and the other component (the analyte) is injected in solution over the chip under continuous flow conditions.

The established procedure for ligand immobilization links primary amine groups in the ligand to derivatized carboxyl groups on the sensor chip surface [5]. This Application Note describes an immobilization method based on thiol- disulphide exchange, a valuable alternative to amine coupling in many situations.

Two variants of thiol-based coupling are available, depending on whether the thiol group is on the ligand or the surface matrix. For both methods, Biacore AB offers PDEA Thiol Coupling Reagent (Figure 1).

Ligands can be immobilized via intrinsic thiol groups or via groups introduced by derivatization of carboxyl or amino groups (Table 1).

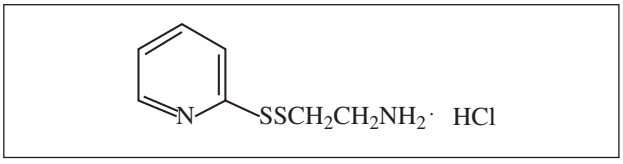


Figure 1
PDEA Thiol Coupling Reagent (Product no. BR-1000-58).

Method:	*Amine*	*Thiol*
Functional groups		
-SH	-	+
$-NH_2$	+	+ (after modification)
-COOH	-	+ (after modification)
Biomolecules		
acidic ligands	-	+
neutral ligands	+	(+)
basic ligands	+	(+)

Table 1
Coupling chemistry for different functional groups and types of ligand. + recommended, (+) acceptable, - unsuitable.

BIACORE

Peptide sequence		*pI*
SP-8	Ala Val Asn Gly Thr Ser Gln Phe Thr **Cys**	5.3
SP-9	**Cys** Leu Glu Thr Leu Thr Pro Asp Thr Gln Tyr Glu	2.8

Table 2. IL-2 receptor peptides used in the work described. Residues containing thiol groups are marked with bold face.

Reagents

HBS-EP	10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.05% Surfactant P20
SP-8	Peptide SP-8, 50 µg/ml in 10 mM Na-formate, pH 4.0
SP-9	Peptide SP-9, 200 µg/ml in 10 mM Na-citrate, pH 2.5

For detailed instructions regarding preparation of reagent solutions, see Instrument Handbook

Immobilization protocol

Time (s)	Event	Comments
0	HBS, flow 5 µl/min	Start cycle
180	Mix NHS + EDC 1:1 Inject 10 µl	Activate surface
420	Inject 15 µl PDEA	Modify surface
780	Inject 35 µl peptide	Couple peptide
1320	Inject 20 µl cysteine/ NaCl	Deactivate excess reactive groups
1620	–	End cycle

Table 3. Protocol for immobilizing IL-2 receptor peptides on Sensor Chip CM5 by thiol coupling.

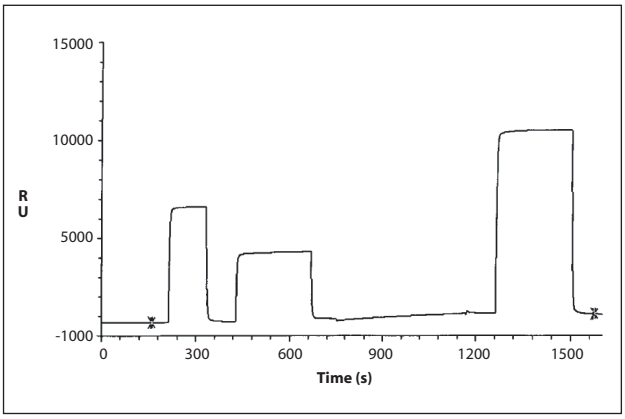


Figure 2. Typical sensorgram showing the immobilization of IL-2 receptor peptides on Sensor Chip CM5.

Surface	Serum SP-8 Ra2	Serum SP-9 Ra5
Peptide SP-8	1244	135
Peptide SP-9	44	4700
Sensor Chip CM5 Control	7	135

Table 4. Binding of rabbit antisera to immobilized IL-2 receptor peptides and to unmodified Sensor Chip CM5. Binding levels are expressed in resonance units.

Modifying carboxyl groups with PDEA has the added advantage in the immobilization of acidic ligands that the isoelectric point of the ligand is increased.

This favours electrostatic preconcentration of ligand on the sensor chip surface in the coupling procedure. Common ligands for which thiol coupling is appropriate include:

- Acidic proteins for which amine coupling is inefficient
- Peptides and other small ligands where the number of amine groups is limited
- Other biomolecules, where amine coupling is empirically found to be unsatisfactory.

Immobilizing interleukin-2 receptor peptides by thiol coupling

Interleukin-2 (IL-2) is a growth factor involved in amplifying the proliferative response of T-cells [6]. In the work described here, two peptides derived from the IL-2 receptor, one of which was acidic (pI<3), were were immobilized on a Sensor Chip CM5 via thiol groups in the peptides. Rabbit polyclonal antibodies were then passed over the immobilized ligand and binding was analyzed.

Materials and methods

IL-2 receptor peptides and rabbit sera containing antibodies against specific peptides were a gift from Biotest Pharma, Germany. Other materials were from Biacore AB.

Immobilization of IL-2 receptor peptides

Conditions for immobilizing the peptides via thiol groups are summarized in Table 3, and a typical sensorgram is shown in Figure 2. The amount of peptide immobilized (about 300 RU) corresponds to ≈250 fmole/mm^2 .

Antibody binding

Immobilized peptides were challenged with 5 µl rabbit serum diluted 1/10 in HBS-EP buffer. Buffer flow was maintained at 5 µl/min throughout. After each cycle, the surface was regenerated with 10 µl 0.1 M HCl. No significant change in baseline signal or antibody binding capacity was observed after 10 cycles of regeneration.

Results

Each rabbit serum was injected over each thiol-immobilized IL-2 peptide, and over an unmodified Sensor Chip CM5 as a control. The results are summarized in Table 4. The sera bound specifically to both IL-2 peptides. Binding to the non-antigenic peptide was comparable with background binding. The immobilized peptides thus retain biologically relevant specific binding properties.

Immobilizing carcinoembryonic antigen (CEA) by thiol coupling

Carcinoembryonic antigen (CEA) is an acidic embryonic cell surface marker that is also expressed in carcinomas [7]. The low isoelectric point (< 3.0) makes it difficult to immobilize CEA on Sensor Chip CM5 with amine coupling. Thiol coupling of PDEA-modified CEA is a tested, alternative approach.

Materials and methods

Purified CEA from human carcinoma metastases, and purified anti-CEA monoclonal antibodies and all other materials were from Pharmacia Diagnostics AB.

Modification of CEA with PDEA

CEA (1 mg) and PDEA (5.5 mg) were dissolved in 1 ml 0.1 M MES buffer pH 5.0 and cooled on ice. 20 µl 0.4 M EDC in water were added and allowed to react on ice for 1 h. Low molecular weight reagents were removed by gel filtration on a NAP-10 column (Amersham Biosciences). The degree of modification determined spectrophotometrically was 2.8.

Immobilization of CEA

Conditions for immobilizing the modified CEA on Sensor Chip CM5 are described in Table 5, and a typical sensorgram is shown in Figure 3. The amount of CEA immobilized (about 5000 RU) corresponds to the equivalent of ≈28 fmol/mm^2.

Paired antibody binding

Pairs of MAbs were tested for simultaneous binding to immobilized CEA by injecting 20 µl of one MAb followed by 20 µl of the second MAb. HBS flow was maintained at 5 µl/min throughout.

After injection of the second MAb, the surface was regenerated with 15 µl 0.1 M HCl. No significant change in baseline signal or antibody binding capacity was observed after 16 cycles of surface regeneration.

Results

The ability of pairs of antibodies to bind simultaneously to antigen defines separate epitopes. Figure 4 shows the results of paired antibody experiments tests for 4 antibodies binding to thiol-coupled CEA. Three distinct epitopes were identified, consistant with published mapping data for CEA [7]. These results demonstrate that thiol-coupling of PDEA-modified ligand is a viable approach to immobilization of CEA.

Ligand

CEA	PDEA-modified CEA, 200 µg/ml in 10 mM Na-citrate pH 3.0

Biacore immobilization protocol

Time (s)	Event	Comments
0	HBS-EP, flow 5 µl/min	Start cycle
180	Mix NHS + EDC 1:1 Inject 10 µl	Activate surface
420	Inject 15 µl cystamine	Introduce surface disulphides
780	Inject 15 µl DTE	Reduce surface disulphides to thiols
960	Inject 35 µl CEA	Couple CEA
1440	Inject 20 µl PDEA/NaCl	Deactivate excess reactive groups
1800	–	End cycle

Table 5. Protocol for immobilizing PDEA-modified CEA on Sensor Chip CM5 by thiol coupling.

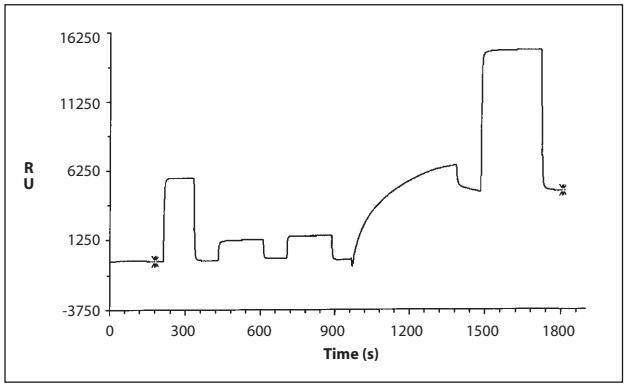


Figure 3. Typical sensorgram showing the immobilization of PDEA-modified CEA on Sensor Chip CM5. Refer to Table 5 for a description of the procedure.

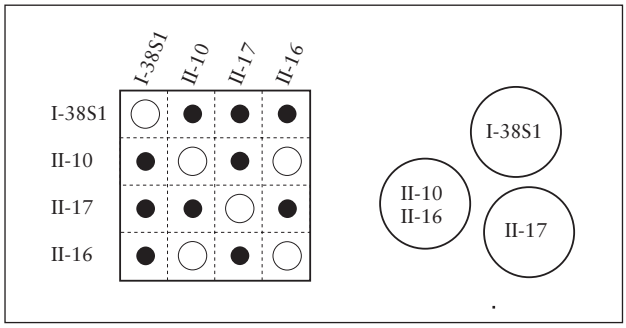
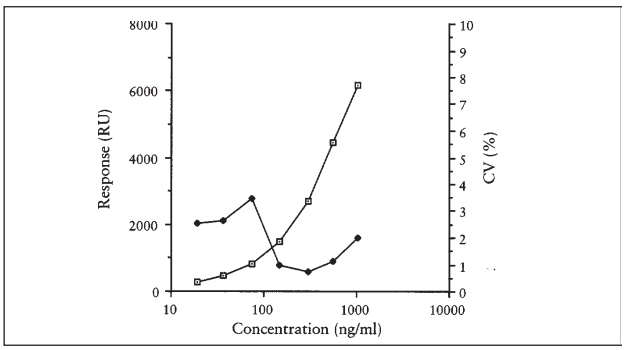


Figure 4. Paired antibody binding to immobilized CEA.
Left: Matrix showing mutual binding patterns.
Right: Interpretation of the binding patterns as a two-dimensional surface-like map.



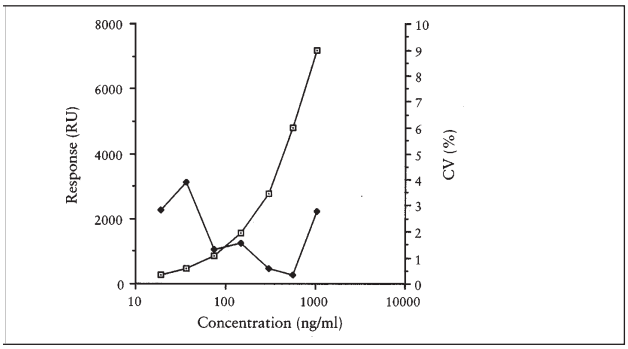


Figure 5. Standard curves for the β2μ assay using MAbs immobilized by thiol coupling (top) and amine coupling (bottom). Precision profiles from five-fold repeated analyses are included.

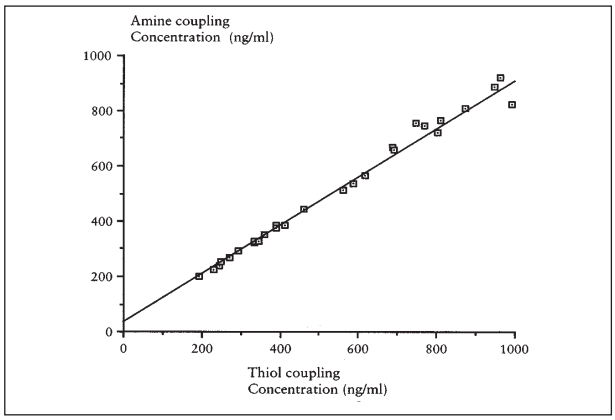


Figure 6. Correlation between β2μ determination in 30 serum samples using thiol-coupled SPDP-MAb and amine-coupled native MAb as first antibody.

Comparison of amine- and thiol-coupled antibodies in a sandwich assay for β2-microglobulin

Determination of the concentration of β2-microglobulin (β2μ) in serum using an immunometric method is an established Biacore application [8]. Here, the same assay is tested using a capturing antibody, immobilized by thiol coupling.

Materials and methods

Monoclonal anti-β2μ, the Ig fraction of polyclonal anti-β2μ and purified β2μ were from Pharmacia Diagnostics AB. SPDP was from Amersham Biosciences. Other materials were from Biacore AB.

Modification of anti-β2μ with SPDP

1 ml anti-β2μ MAb (1 mg/ml in 0.15 M NaCl) was mixed with 0.5 ml 0.3 M phosphate buffer pH 7.5 and 17 μl 2.5 mM SPDP in ethanol, and allowed to react for 2 h at room temperature. Low molecular weight reagents were removed by gel filtration. The degree of modification determined spectrophotometrically was 2.1.

Immobilization of anti-β2μ

SPDP-modified anti-β2m in 75 mM Na-acetate pH 4.5 was immobilized on Sensor Chip CM5 using the conditions described for CEA (Table 5). The amount of anti-β2m immobilized (about 10,000 RU) corresponds to approximately ≈70 fmol/mm^2.

Determination of β2μ

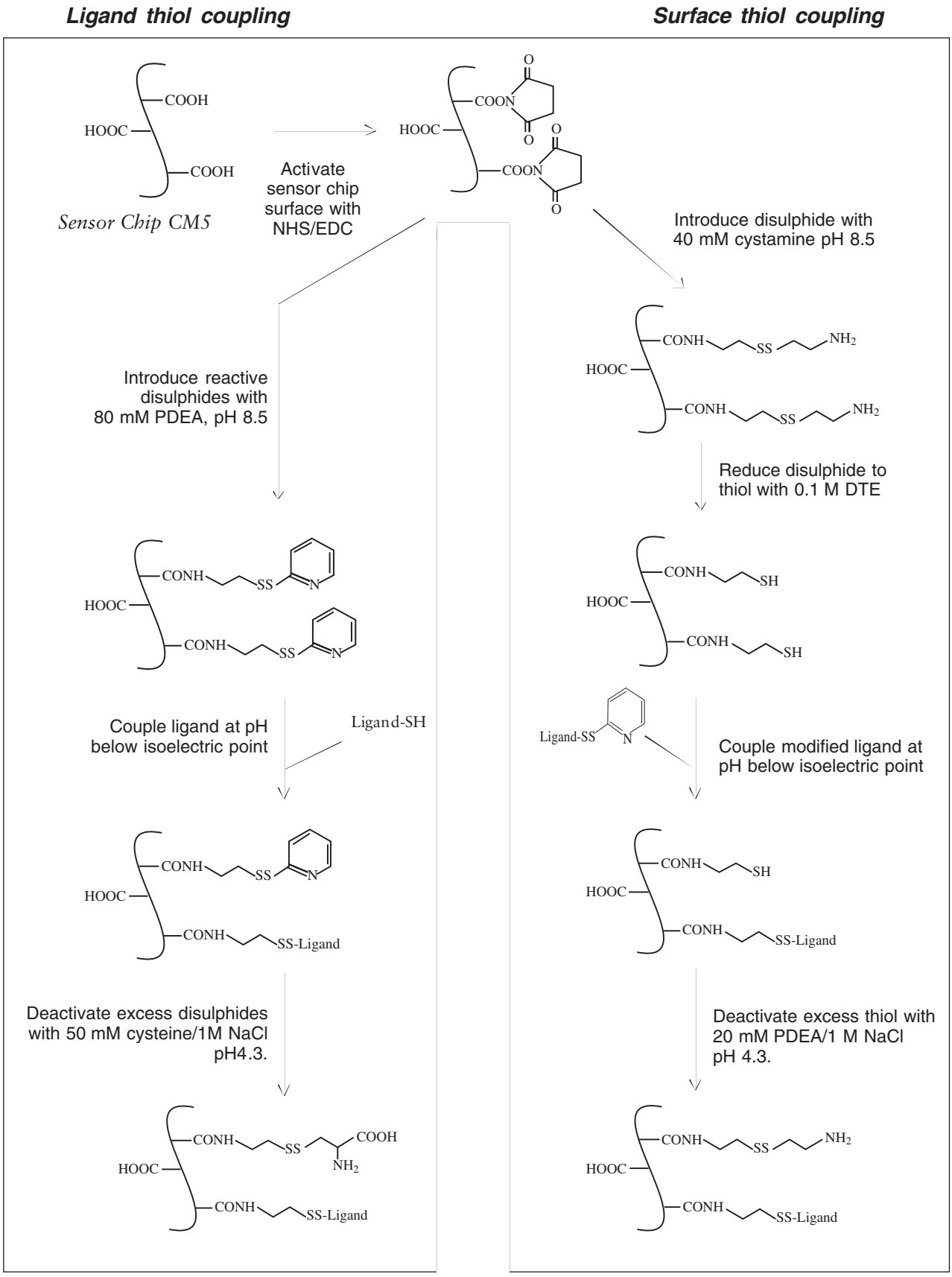
To determine β2μ concentrations, 5 μl samples were injected over the immobilized MAb, followed by 4 μl Ig fraction of polyclonal anti-β2μ antibodies at 2.5 mg/ml in 10 mM HEPES buffer pH 7.5. Buffer flow was maintained at 5 μl/min throughout. The second antibody response reflects the concentration of β2μ in the sample.

Results

To evaluate thiol-coupled antibodies in the β2μ assay, SPDP-modified MAb was immobilized via surface thiol groups and the same unmodified MAb was immobilized via amine groups. Standard curves for 8 known concentrations of β2μ in HBS for the two methods are shown in Figure 5. The curves are practically identical, with precision better than 5% CV over the whole range. Figure 6 shows that correlation is excellent between thiol- and amine-coupled MAbs, for measurements on 30 serum samples diluted to give a β2μ concentration ranging from 100 to 1100 ng/ml. Correlation between the real-time Biacore assay and independent measurements using RIA has been shown previously.

These tests show that performance of the β2μ assay using thiol- and amine-coupled MAbs is equivalent.

Chemistry of thiol-disulphide exchange coupling



References

[1] Fägerstam, L., Techniques in Protein Chemistry II, ed. J. J. Villafranca, Academic Press, New York, p. 65 (1991).

[2] Jönsson,U. et al., Biotechniques 11, (1991).

[3] Kretschmann, E. and Raether, H., Z. Naturforschung, Teil A 23, 2135 (1968).

[4] Liedberg, B., Nylander, C. and Lundström, I., Sensors and Actuators 4, 299 (1983).

[5] Johnsson, B., Löfås, S and Lindqvist, G. Analyt. Biochem. 198, 268 (1991).

[6] Roitt, I, Essential Immunology, Blackwell Scientific Publications, Oxford, 7th ed. (1991).

[7] Hammarström, S. et al., Cancer Research 49, 4852 (1989).

BIACORE

www.biacore.com

BR-9000-73 February 2002