BIACORE®

Sensor Surface Handbook

BIACORE

Biacore®
Sensor Surface Handbook

Contents

1. Introduction

1.1 Principles of Biacore technology

Biacore systems exploit the phenomenon of surface plasmon resonance (SPR) to monitor the interaction between molecules in real time. The technology involves attaching one interacting partner to the surface of a sensor chip, and then passing sample containing other interaction partner(s) over the surface. Binding of molecules to the sensor surface generates a response which is proportional to the bound mass, and changes in amount bound can be detected down to a few picograms or less per square millimeter on the sensor surface, corresponding to concentrations in the picomolar to nanomolar range in the bulk sample solution. Binding events are followed in real time and a range of interaction characteristics can be determined. Among the questions that can be addressed with Biacore are:

The specificity of biomolecular interactions, investigated by testing the extent of binding between different pairs of molecules.

The kinetics and affinity of an interaction, investigated by analyzing the time curve and level of binding in terms of molecular interaction models.

The concentration of specific molecules present in the sample, investigated by measuring the level of response obtained from the sample.

1.2 Biacore terminology

Biacore systems monitor the interaction between two molecules, of which one is attached to the sensor surface and the other is free in solution. The following terms are used in the context of Biacore assays:

The interaction partner attached to the surface is called the *ligand* (Figure 1-1). (The term "ligand" is applied here in analogy with terminology used in affinity chromatography contexts, and does not imply that the surface-attached molecule is a ligand for a cellular receptor.)

The *analyte* is the interaction partner that is passed in solution over the immobilized ligand (Figure 1-1).
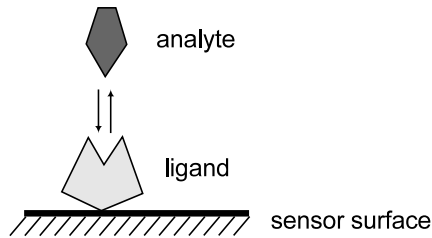
Figure 1-1. *The ligand is the interaction partner that is attached to the sensor surface. The analyte is free in solution and binds to the immobilized ligand.*

Analysis is performed by injecting sample over the surface in a carefully controlled fashion. The sample is carried in a continuous flow of buffer, termed **running buffer**.

Regeneration is the process of removing bound analyte from the surface after an analysis cycle without damaging the ligand, in preparation for a new cycle.

Response is measured in **resonance units** (RU). The response is directly proportional to the concentration of biomolecules on the surface.

A **sensorgram** is a plot of response against time, showing the progress of the interaction (Figure 1-2). This curve is displayed directly on the computer screen during the course of an analysis.

A **report point** records the response on a sensorgram at a specific time averaged over a short time window, as well as the slope of the sensorgram over the window. The response may be absolute (above a fixed zero level determined by the detector) or relative to the response at another specified report point (Figure 1-2).
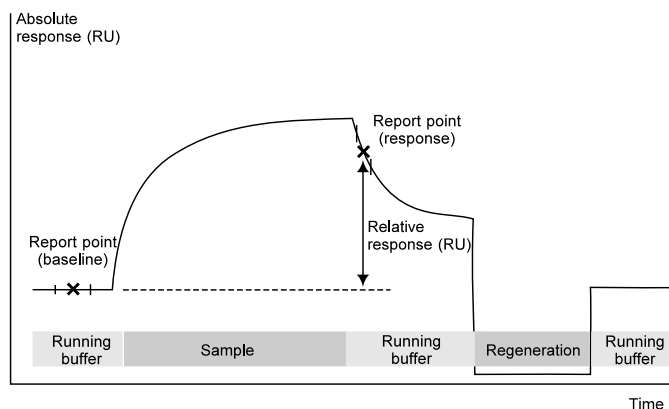
Figure 1-2. *Schematic illustration of a sensorgram. The bars below the sensorgram curve indicate the solutions that pass over the sensor surface.*

1.3 Components of Biacore systems

1.3.1   The SPR detection system

SPR is a phenomenon that occurs in thin conducting films at an interface between media of different refractive index. In Biacore systems, the media are the glass of the sensor chip and the sample solution, and the conducting film is a thin layer of gold on the sensor chip surface.

Under conditions of total internal reflection, light incident on the reflecting interface leaks an electric field intensity called an ***evanescent wave field*** across the interface into the medium of lower refractive index, without actually losing net energy. The amplitude of the evanescent field wave decreases exponentially with distance from the surface, and the effective penetration depth in terms of sensitivity to refractive index is about 20% of the wavelength of the incident light.
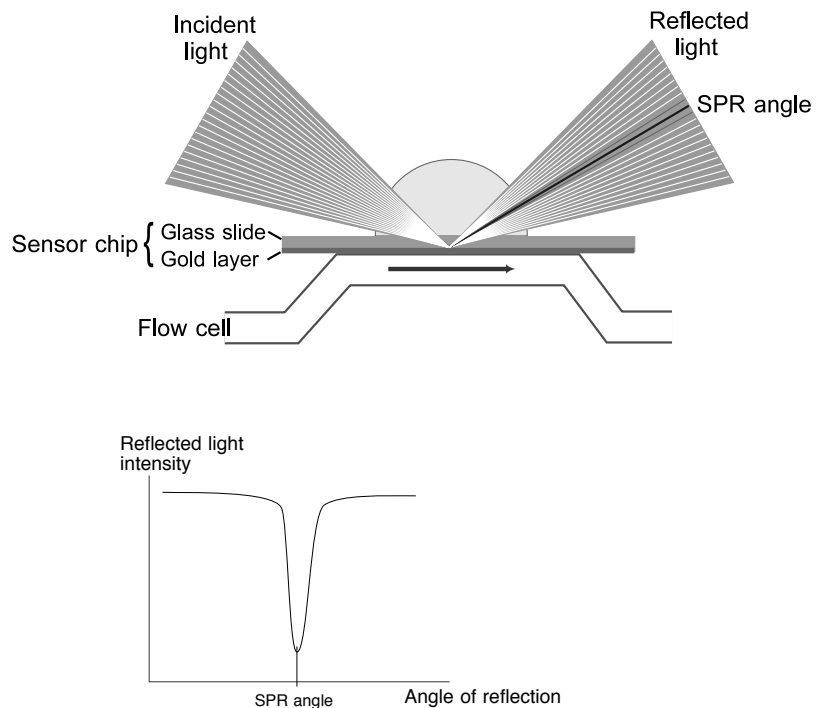
Figure 1-3. *The SPR principle.*

At a certain combination of angle of incidence and energy (wavelength), the incident light excites plasmons (electron charge density waves) in the gold film. As a result, a characteristic absorption of energy via the evanescent wave field occurs and SPR is seen as a drop in the intensity of the reflected light (Figure 1-3).

Because the evanescent wave field penetrates the solution, conditions for this resonance effect are very sensitive to the refractive index of the solution within the effective penetration depth of the evanescent field. Changes in solute concentration at the surface of the sensor chip cause changes in the refractive index of the solution which can be measured as changes in the SPR conditions. The penetration depth of the evanescent wave determines the thickness of the solution layer where refractive index changes are monitored: only refractive index changes close to the surface affect the SPR signal.

To achieve total internal reflection of the light at the interface between the sensor chip and the solution, the glass side of the sensor chip is pressed against a semi-cylindrical glass prism, using a silicone ***opto-interface*** to ensure good optical contact (see Figure 1-5). Light from a light-emitting diode (wavelength about 800 nm) is focused in a wedge on to the sensor surface, covering a fixed range of incident and reflected angles. The effective penetration depth of the evanescent wave under these conditions is of the order of 150 nm.

SPR arises in principle in any thin conducting film under the conditions described, although the wavelength at which resonance occurs and the shape of the energy absorption profile differ with different conducting materials. Gold is used in Biacore sensor chips because it combines favorable SPR characteristics with stability and a high level of inertness in biomolecular interaction contexts.

Note: There are several commercial systems available on the market for monitoring biomolecular interactions in real time without labels. Some of these, like Biacore, use SPR detection, while others exploit wave guide resonance or resonant mirror technology. Although these technologies are superficially similar and both detect mass changes at the surface through changes in refractive index, the physical principles are different and sensor surfaces designed for one technology cannot be used with the other.

1.3.2   *The sensor surface*
The sensor chip is at the heart of Biacore's technology: the chip provides the physical conditions necessary to generate the SPR signal, and the interaction being studied occurs on the surface of the chip.

The specificity of a Biacore analysis is determined through the nature and properties of the molecule attached to the sensor surface. Biomolecules may be attached to the surface of the sensor chip using three different approaches (see Figure 1-4):

  Covalent immobilization, where the molecule is attached to the surface through a covalent chemical link.

High affinity capture, where the molecule of interest is attached by non-covalent interaction with another molecule (which in turn is usually attached using covalent immobilization).

Hydrophobic adsorption, which exploits more or less specific hydrophobic interactions to attach either the molecule of interest or a hydrophobic carrier such as a lipid monolayer or bilayer to the sensor chip surface.
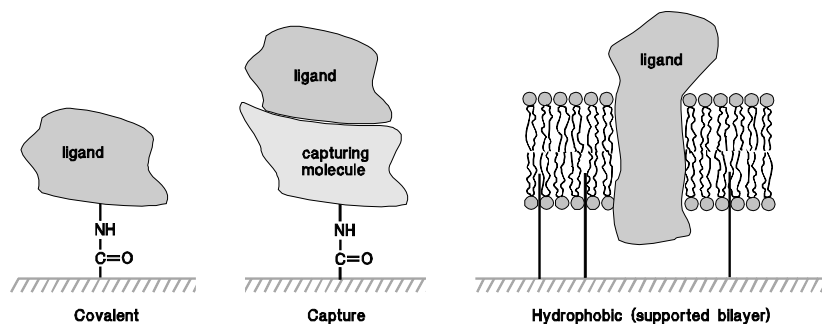
Figure 1-4. *Three approaches for attaching biomolecules to the sensor chip surface.*

These approaches are discussed in more detail in Chapters 3 – 5.

1.3.3　The microfluidic system

The interaction being studied takes place on the gold-covered side of the sensor chip, opposite from the side where the light is reflected. Sample containing analyte is supplied in a controlled fashion to the sensor surface through a microfluidic system. The sensor surface itself forms one wall of a flow cell which is an integral part of the microfluidic system (Figure 1-5).
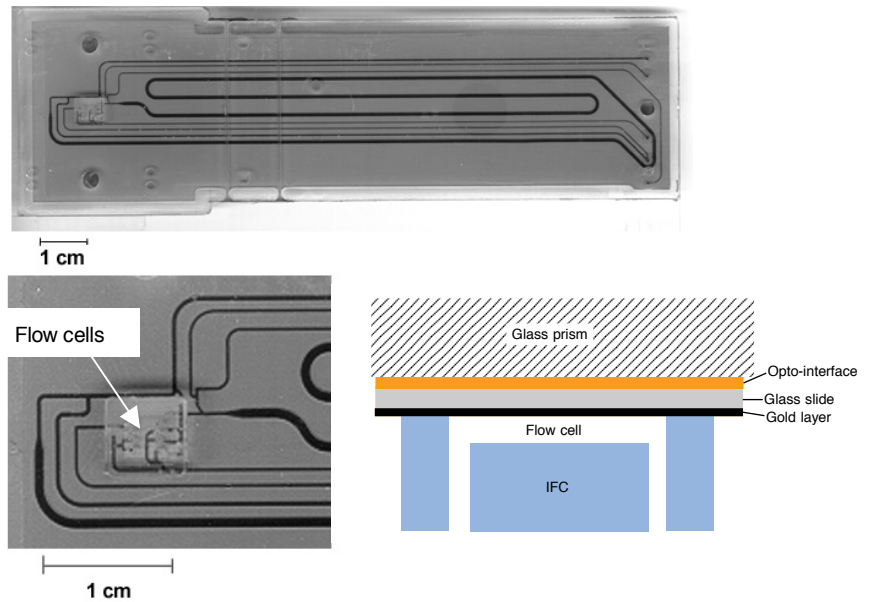
Figure 1-5. *Microfluidic system and flow cell. Top: Photograph of the IFC used in Biacore 3000. Bottom left: Close-up of the flow cell area on the IFC. The sensor chip is pressed on to the flow cell block shown in this close-up. Bottom right: Diagrammatic section through the flow cell, showing how the IFC channels are pressed against the sensor surface to form the flow cell.*

The microfluidic system consists of a series of channels and valves in a plastic block, the Integrated Microfluidic Cartridge (IFC). The flow cells are formed by pressing the sensor chip against a set of open channels on the surface of the IFC, so that the chip can easily be exchanged. Delivery of sample and buffer to the flow cells is precisely controlled by the pump system and the valves in the IFC. In this way, a continuous flow of liquid is maintained over the sensor surface throughout an analysis, switching between buffer and sample with minimum disturbance or dispersion of the sample boundary. This high degree or precision in sample delivery is important for reproducibility of assay procedures, and also provides the controlled conditions necessary for interpreting kinetic data obtained from the interaction studies. More information on the details of the flow system in Biacore systems may be found in the respective Instrument Handbooks.

1.4   Scope of this handbook

This Handbook provides a general guide to the design and use of sensor surfaces for Biacore analyses, and covers:

Properties of the sensor surface

Approaches to immobilizing ligand

Practical aspects of ligand immobilization

Regeneration of the sensor surface

Sensor chip storage and re-use

Aspects of sensor chip usage that are specific to individual instruments are described in the respective instrument and/or methodology handbooks.

1.5   Additional information

More information about Biacore systems, sensor chips and applications may be found in the following sources:

Instrument and methodology handbooks for the respective systems

Biacore Concentration Analysis Handbook, describing how to design, develop and perform concentration measurements using Biacore's SPR-based biosensor technology

Biacore Advisor Series products, which are interactive multimedia presentations describing the technology and its applications

The Biacore web site at www.biacore.com provides extensive and up-to date information about Biacore products, including a searchable database of literature references.

2. Sensor surface properties

2.1 The sensor surface

The sensor chip in Biacore has two essential features:

A glass surface covered with a thin gold layer, creating the conditions required for generating an SPR signal which is the basis for detection of biomolecular interaction. This feature is common to all Biacore sensor chips.

A coating of some sort on the gold layer, providing a means for attaching ligand and an environment where the interaction being studied will occur. The coating does not affect the SPR effect, and varies between different types of chips. Uncoated chips are also available for users wishing to prepare their own customized surfaces for special applications.

The sensor chip itself is mounted on a plastic carrier to facilitate handling. The carrier is in turn held in a protective plastic sheath (Figure 2-1). Different sheath and carrier formats are used in the two major instrument platforms currently offered: the formats differ in size and construction of the carrier and sheath, but the properties of the sensor surface itself are unaffected.
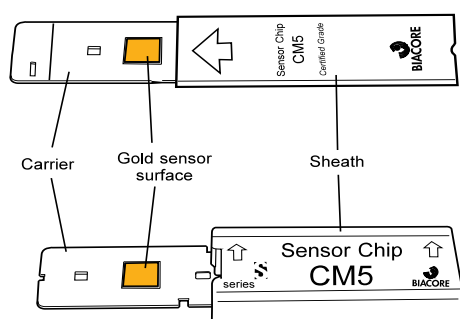
Figure 2-1. *Sensor chip and carrier construction (top: Classic format, bottom: Series S format).*

The gold layer and surface coating on the sensor surface are stable under a wide range of conditions, including extremes of pH and moderate concentrations of many organic solvents. Once the ligand has been immobilized, the resistance of the sensor surface to various agents and conditions is determined primarily by the properties of the attached ligand.

2.1.1 The gold layer

The glass surface of the sensor chip is coated with a uniform thin layer of gold (approximately 50 nm thick) to provide the conditions for surface plasmon resonance. SPR arises under conditions of total internal reflection at a thin film of many conducting materials. The characteristics of the phenomenon in terms of angle and wavelength of the incident light and the shape of the reflectance minimum vary according to the nature and thickness of the conducting film. Gold gives a well-defined reflectance minimum at easily handled visible light wavelengths, and is also amenable to covalent bonding of surface matrix layers (see below) at the same time as the metal is largely inert in physiological buffer conditions. Reliable SPR detection requires a carefully controlled and uniformly thin gold layer on the glass surface, and Biacore sensor chips are prepared under rigorous quality control.

2.1.2 The surface coating

Unmodified gold is in general not a suitable surface environment for biomolecular interactions, and the gold on almost all Biacore sensor chips is covered with a covalently bonded monolayer of alkanethiol molecules. This serves to protect the biological samples from contact with the gold and at the same time provides a means of attachment of a surface matrix which further enhances the usefulness of the surface in biomolecular contexts. Modification of the gold by attachment of the alkanethiol layer does not impair the SPR properties of the surface.

The alkanethiol layer presents a strongly hydrophobic surface environment which is exploited directly in Sensor Chip HPA, designed for work with lipid monolayers (see Section 2.2.3). On most sensor chips, however, the surface is covered with a matrix of carboxymethylated dextran, a flexible unbranched carbohydrate polymer forming a thin surface layer approximately 100 nm thick on Sensor Chip CM5. The dextran matrix close to the surface is equivalent in concentration to an aqueous solution of about 2% dextran, and imparts several important properties to the sensor surface:

> It provides a hydrophilic environment favorable to most solution-based biomolecular interactions.

> It provides a defined chemical basis for covalent attachment of biomolecules to the surface using a wide range of well-defined chemistries (see Chapter 4).

> The negatively charged carboxyl groups allow electrostatic concentration of positively charged molecules from solution, enabling efficient immobilization from dilute ligand solutions (see Section 4.1.2).

It increases the surface capacity for ligand immobilization many-fold in comparison with a flat surface.

It extends the region where interactions occur to encompass a thin layer with a thickness of the same order of magnitude as the penetration depth of the evanescent wave.

Dextran is a relatively inert molecule in the context of most biomolecular interactions, and the flexibility of the unbranched polymer chains allows the "surface-attached" biomolecules to move with relative freedom within the surface layer. Dextran-based surface matrices thus provide an excellent environment for many biomolecular interactions.

2.2   The range of Biacore sensor chips

Biacore offers a wide selection of different sensor surfaces, to fill the varied application requirements for SPR technology. This section gives an overview of the available sensor chip types. Essential features of the different sensor chips are summarized in the table below.

Sensor Chip	Surface type	Uses
Sensor Chip CM5	CM-dextran	General purpose
Sensor Chip CM4	CM-dextran with lower carboxymethylation level than CM5	For low immobilization levels and reduced non-specific binding
Sensor Chip CM3	CM-dextran with shorter dextran matrix than CM5	For large ligand molecules and particles
Sensor Chip C1	Flat carboxylated surface, no dextran matrix	For applications where dextran interferes
Sensor Chip SA	CM-dextran with immobilized streptavidin	For capture of biotinylated ligands
Sensor Chip NTA	CM-dextran with immobilized NTA	For capture of poly-histidine tagged ligands
Sensor Chip L1	CM-dextran with lipophilic modification	For capture of liposomes and supported lipid bilayers
Sensor Chip HPA	Flat hydrophobic surface	For capture of lipid monolayers
Sensor Chip Au	Plain gold surface	For surface interaction studies and custom design of surface chemistry
SIA Kit Au	Plain gold surface, not mounted on chip carrier	For surface modifications outside Biacore systems

2.2.1 CM-series sensor chips

CM-series sensor chips carry a matrix of carboxymethylated dextran covalently attached to the gold surface (Figure 2-2). Ligands can be attached to the dextran matrix using a variety of chemical methods, exploiting common functional groups on the ligand such as amino, thiol, and aldehyde groups. The chemistry and procedures for ligand immobilization are described in Chapter 4.

The dextran matrix is flexible, allowing relatively free movement of attached ligands within the surface layer.
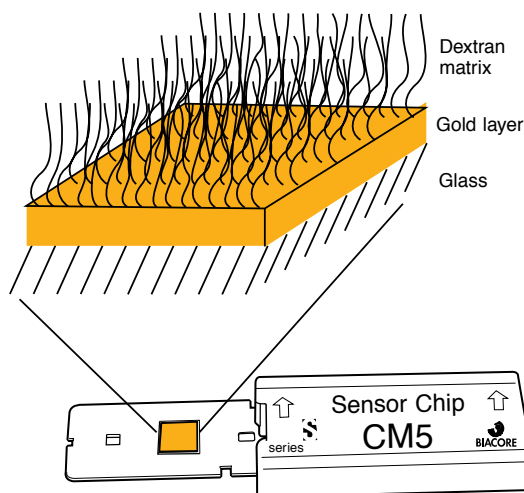
Figure 2-2. *Schematic illustration of the structure of the sensor chip surface on CM-series chips.*

Sensor Chip CM5

Sensor Chip CM5 is the most commonly used general purpose chip, and provides a high surface capacity for immobilizing a wide range of ligands from small organic molecules to proteins, nucleic acids and carbohydrates. The surface matrix on Sensor Chip CM5 extends about 100 nm from the gold surface under physiological buffer conditions.

Sensor Chip CM4

Sensor Chip CM4 is similar to Sensor Chip CM5 but has a lower degree of carboxymethylation (about 30% of that of Sensor Chip CM5), resulting in reduced immobilization capacity and also lower surface charge density. The lower charge can help to reduce non-specific binding of positively charged molecules to the surface, so that this chip is well suited to applications such as ligand fishing where the analyte may be present in a complex mixture such as cell extract or culture medium. The lower immobilization capacity of Sensor Chip CM4 can also be advantageous for kinetic analysis experiments, where low ligand immobilization levels are recommended.

Sensor Chip CM3
The dextran matrix on Sensor Chip CM3 has the same molar carboxymethylation level as Sensor Chip CM5, but the matrix consists of shorter dextran chains, reducing steric effects when working with large molecules (molecular weight above about 1,000,000 daltons) and particles such as viruses and whole cells. The immobilization capacity of the surface is about 30% of that on Sensor Chip CM5, and Sensor Chip CM3 can (like CM4) be advantageous for kinetic analyses.

2.2.2 Sensor chips for capture applications

Sensor Chip SA
The surface of Sensor Chip SA carries a dextran matrix to which streptavidin has been covalently attached. Streptavidin is a tetrameric protein with a high affinity for biotin (equilibrium dissociation constant $K_D \approx 10^{-15}$ M), so that the surface is prepared for high affinity capture of biotinylated ligands. The interaction of biotin with streptavidin is so strong that the biotinylated ligand cannot normally be removed to regenerate the streptavidin surface, and capture of a biotinylated ligand on Sensor Chip SA is in practical terms almost equivalent in stability to covalent immobilization of the ligand.

Sensor Chip SA is particularly suited to work with nucleic acid ligands, since controlled biotinylation of nucleic acids is a well-established procedure. Biotin may however also be introduced into proteins, carbohydrates and lipids, and biotinylation followed by capture on Sensor Chip SA often provides a convenient alternative for ligands that are difficult to immobilize covalently.

Sensor Chip NTA
Sensor Chip NTA has a dextran surface matrix with immobilized nitrilotriacetic acid (NTA)[1], providing a means for capturing polyhistidine-tagged ligands through metal chelation. The NTA molecule chelates metal ions such as Ni^{2+}, leaving coordination sites free that can bind to polyhistidine tags on recombinant proteins. The surface is easily regenerated with EDTA, which removes the metal ion and releases the his-tagged ligand.

The stability of polyhistidine binding to chelated nickel ions varies considerably with the micro-environment of the tag in the protein. The usefulness of metal chelation capture thus varies from case to case, but the approach is simple to test and very convenient for ligands where the capturing interaction is sufficiently stable.

---

[1] Nitrilotriacetic acid (NTA) is manufactured by QIAGEN GmbH and used under license from F. Hoffmann-LaRoche Ltd and QIAGEN GmbH.

2.2.3   Sensor chips for hydrophobic ligands

Sensor Chip HPA
Sensor Chip HPA presents a flat hydrophobic surface consisting of long-chain alkanethiol molecules attached directly to the gold film. There is no dextran or other matrix on the surface.

This sensor chip is designed for work with lipids and membrane-associated molecules. Injected lipids form a monolayer that covers the hydrophobic surface, with the hydrophilic part of the lipids oriented towards the solution (see Figure 3-6). Molecules associated with membrane surfaces can be incorporated into the monolayer and used as ligands for interaction analysis.

Sensor Chip L1
Sensor Chip L1 has a surface matrix of carboxymethyl dextran similar to that on Sensor Chip CM5, to which lipophilic residues have been covalently attached. The chip is designed for direct capture of lipid vesicles and liposomes through insertion of the lipophilic residues into the membrane (see Figure 3-7). The bilayer structure of the membrane is retained, and under favorable conditions forms a supported bilayer covering the sensor chip surface.

Sensor Chip L1 provides an alternative approach to Sensor Chip HPA for working with membrane-associated ligands, and is particularly suitable for work with transmembrane proteins.

2.2.4   Sensor chips for special applications

Sensor Chip C1
Sensor Chip C1 has a flat carboxylated surface with no dextran matrix. The carboxyl groups support the same immobilization chemistries as the dextran matrix on CM-series chips, but the surface of Sensor Chip C1 lacks the hydrophilic character of the dextran matrix and the immobilization capacity is greatly reduced (typically to about 10% of that on Sensor Chip CM5 under comparable conditions).

The chip can be valuable for work with very bulky ligands such as viruses and whole cells. In addition, the lack of a flexible dextran matrix on the surface means that attached ligands are not free to move within the surface layer, and avidity effects seen with multivalent analytes are reduced.

Sensor Chip Au and SIA Kit Au
Sensor Chip Au offers a plain gold surface mounted on the chip carrier for creation of customized surfaces. Unmounted gold surfaces are also

available with a separate chip carrier in SIA Kit Au, for preparation of surfaces using procedures that the plastic chip carrier does not withstand.

Spin-coating and other techniques for creation of self-assembled monolayers can be used in preparation of customized surfaces. The interested reader is referred to the literature on surface coating and surface interaction studies for more details: the techniques are not discussed further in this handbook.

3. Surface preparation strategies

Measurements with Biacore are based on interaction of analyte in solution with ligand attached to the sensor chip. Molecules can be attached to the chip surface using several techniques, including covalent immobilization using a variety of chemical methods, high affinity capture to a specific capturing molecule and adsorption of lipid mono- or bilayers.

This chapter gives an overview of the methods available for attaching the ligand to the sensor chip surface. Detailed procedures are described in the next chapter.

3.1   Covalent immobilization

Covalent immobilization to the dextran matrix on the sensor surface is a commonly used approach for attaching the ligand to the surface, and is also the method of choice for immobilizing capturing molecules for capture methods (Section 3.2). The carboxymethyl dextran on Sensor Chip CM5, other CM-series chips and Sensor Chip L1, as well as the carboxyl groups directly attached to the surface of Sensor Chip C1, provide a foundation for a range of covalent immobilization chemistries. The most common of these are:

Amine coupling, exploiting primary amine groups on the ligand after activation of the surface with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS).

Thiol coupling, exploiting thiol-disulfide exchange between thiol groups and active disulfides introduced on either the ligand or the surface matrix.

Aldehyde coupling, using the reaction between hydrazine or carbohydrazide groups introduced on the surface and aldehyde groups obtained by oxidation of carbohydrates in the ligand.
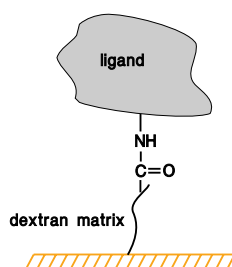

Figure 3-1. *Covalently immobilized ligand is linked to the surface through a covalent chemical bond. This illustration represents amine coupling.*

Covalent immobilization generally results in stable attachment of the ligand to the surface under the buffer conditions normally used for interaction analysis and surface regeneration. Regeneration of the surface removes bound analyte at the end of each analysis cycle but leaves the ligand attached to the surface. Some covalent coupling methods may have limited stability under certain conditions: thiol-disulfide exchange coupling, for example, is not suitable for attaching ligands that are to be studied in the presence of reducing agents.

Although covalent immobilization involves chemical modification of the ligand which can potentially affect the analyte-binding activity, most ligands can be immobilized by one or another chemistry without losing activity. In cases where covalent attachment does result in loss of ligand activity or is unsuitable for other reasons, capturing provides an alternative approach (see below).

The site of immobilization on the ligand molecule – and therefore the orientation of the immobilized ligand – cannot usually be determined with approaches like amine coupling that address multiple targets in the ligand (most proteins contain several available amine groups). Thiol coupling will often give more homogeneous ligand immobilization since thiol groups are generally less common than amines. Protein engineering can be used to introduce single thiol groups into ligands that lack thiols in their naturally occurring form. Capturing approaches can provide an alternative if homogeneous orientation of the ligand is important for the experimental design.

3.2   Capturing approaches

Capturing approaches rely on interaction with a capturing molecule to bind the ligand to the sensor surface. The capturing molecule is itself normally immobilized on the surface using covalent chemistry. The interaction between the ligand and capturing molecule should have a high affinity (slow dissociation rate) so that ligand does not bleed off the surface during the course of an analysis cycle. In most cases, regeneration of the surface involves removal of ligand from the capturing molecule together with any bound analyte, and fresh ligand is captured as the first step in each analysis cycle (Figure 3-2).

Response

Time
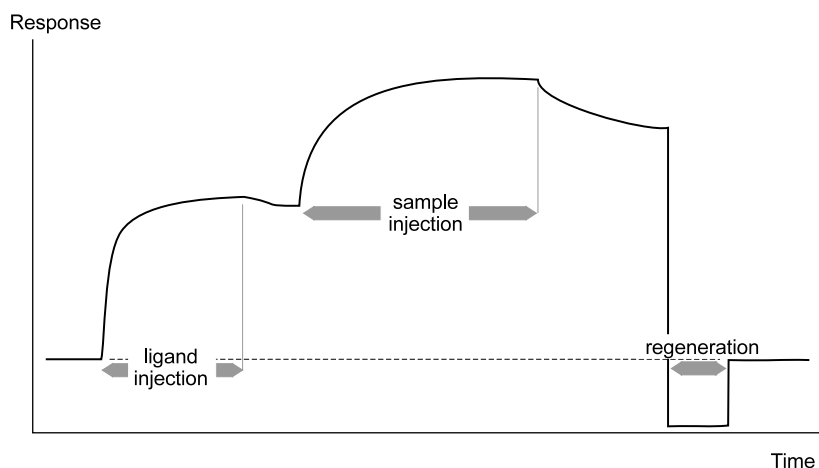
Figure 3-2. *Typical analysis cycle using the capturing approach.*

Capturing approaches may be preferable to covalent immobilization in a number of respects:

   The ligand is usually not modified in any way, provided that the capturing interaction does not introduce conformational changes in the ligand. The approach can be valuable for ligands that are easily inactivated by chemical coupling methods.

   Ligand is attached to the surface in a specific orientation (determined by the location of the binding site for the capturing molecule) so that attachment to the surface does not introduce heterogeneity in the ligand population.

   Capturing provides a micro-scale affinity purification of the ligand, allowing immobilization of specific ligands from complex mixtures such as cell extracts or cell culture media.

   Capturing allows the same surface to be used for analysis of interactions involving different ligands, for example in studies of panels of antibodies, since fresh ligand is captured for each cycle.

On the other hand, capturing approaches consume more ligand when fresh material has to be captured for each analysis cycle, and the maximum attainable analyte binding capacity is usually lower with capture than with direct immobilization of the ligand. In addition, stringent demands are placed on the tightness of the capturing interaction for applications like kinetic determination where dissociation of captured ligand during the analysis cycle complicates evaluation of the results. The advantages of capturing however outweigh these considerations in a wide variety of application situations.

The commonest capturing approaches (described in the following sections) are streptavidin- or avidin-biotin capture, antibody-based capture, and capture of tagged proteins. Capturing may however in principle be applied to any ligand for which a high affinity interactant is available that binds independently of the analyte.

3.2.1 Streptavidin-biotin capture

Sensor Chip SA provides a sensor surface with covalently attached streptavidin, designed for capture of biotinylated compounds. The affinity of streptavidin (or avidin) for biotin is extremely high, with an equilibrium dissociation constant of about 10^{-15} M, and dissociation of biotinylated ligands from the surface of Sensor Chip SA is generally negligible during the course of a Biacore analysis. Reagents and methods for introducing biotin into a range of molecules including nucleic acids, lipids, proteins and carbohydrates are readily available, and capture of biotinylated ligands on Sensor Chip SA is usually a simple and reliable procedure.
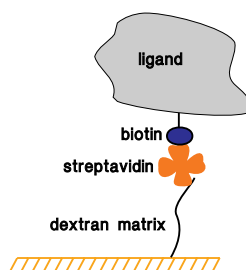
Figure 3-3. *Biotinylated ligands can be captured by high affinity binding to streptavidin on the surface.*

Capture of biotinylated ligands on a streptavidin surface is a special case of the capturing approach, since the streptavidin-biotin interaction is so tight that the capturing is essentially irreversible. Removal of the ligand in the regeneration step is not usually feasible, and regeneration is normally directed towards removing bound analyte while leaving the biotinylated ligand on the surface. In this respect, streptavidin-biotin capture is more similar to covalent attachment than to other capturing approaches.

3.2.2   *Antibody-based capture*

Monoclonal or polyclonal antibodies are widely-used tools in biotechnology contexts, and specifically tailored high affinity antibodies are commercially available for many antigens.
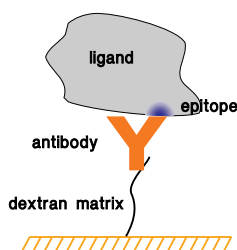
Figure 3-4. *Antibody-based capture relies on the specific binding between an immobilized antibody and the antigenic epitope on the ligand.*

Antibodies offer a number of advantages as capturing molecules in Biacore, in addition to their commercial availability. They are readily immobilized on the sensor surface using amine coupling without compromising their antigen-binding capacity, and most monoclonal antibodies are easily regenerated at low pH using glycine-HCl (see Chapter 6). In addition, the high selectivity of most antibodies for their specific antigens makes them powerful tools for affinity capture of ligands from impure samples. The availability of monoclonal antibodies directed against chosen epitopes on the antigen also enables ligands to be captured in controlled orientations.

3.2.3   *Capturing tagged recombinant proteins*

Recombinant proteins are often tagged for purposes of identification and affinity purification, and the same principle can be exploited for capture of recombinant proteins on the sensor surface in Biacore. Poly-histidine is a commonly used tag that can chelate with Ni^{2+} ions in complex with nitrilotriacetic acid (NTA), providing a convenient approach for capturing his-tagged constructs on Sensor Chip NTA. High affinity antibodies are also available directed against poly-histidine and other commonly used tags, and these can be immobilized on the surface for capture of the tagged proteins.
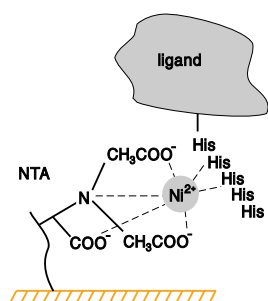
Figure 3-5. *Nickel ions form a chelation bond between NTA immobilized on the surface and poly-histidine tags on the ligand.*

Another commonly used recombinant tag is glutathione-S-transferase (GST), which may be captured on an anti-GST antibody surface. Antibodies against GST that are suitable for capturing purposes are available from Biacore. Other examples of recombinant tags that may be used for capturing are the FLAG and c-myc epitopes.

The specificity of tag-based capture is valuable in work with recombinant proteins in crude extracts or partially purified samples, since the tag interaction serves the double role of identification and affinity purification of the ligand. This enables characterization of the interaction properties of the recombinant protein directly in crude cell extracts or culture medium, without extensive investment in time and expense to purify the protein.

3.3 Hydrophobic attachment

Membrane-associated proteins present special problems for study in aqueous environments, particularly in cases where water-soluble fragments do not adequately mimic the function of the intact protein. Two essentially distinct approaches, based respectively on lipid monolayers and bilayers, are available for retaining a hydrophobic environment for ligands attached to the sensor chip surfaces.

3.3.1   Monolayer attachment
Sensor Chip HPA lacks a carboxymethylated dextran matrix, so that the hydrophobic alkanethiol layer covering the gold film (see Section 2.1.2) is directly exposed to the solution. Adsorption of lipids from micelles or liposomes to this surface creates a lipid monolayer with the hydrophobic lipid tails oriented towards the gold film and the hydrophilic heads towards the aqueous sample. This structure can support interaction studies with membrane-associated ligands that are introduced into the lipid preparation before adsorption to the surface (Figure 3-6).
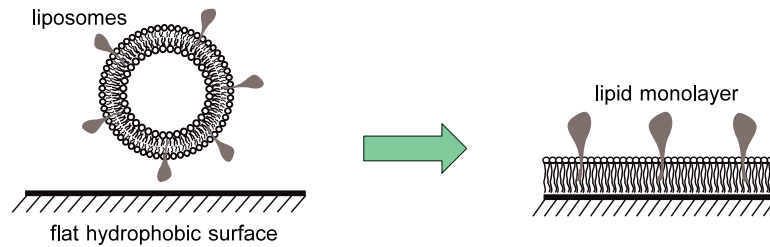
Figure 3-6. *Adsorption of liposomes on Sensor Chip HPA results in the formation of a lipid monolayer covering the chip surface.*

While this approach provides a stable environment for ligands associated with a membrane surface or partially inserted into the membrane, it is not suitable for more deeply inserted or trans-membrane proteins since the surface represents only half the membrane structure. Lipid bilayers can be supported in various ways on the surface for studies that require complete membrane structures, discussed in the next section.

3.3.2   Bilayer attachment

Several approaches are available for attachment of intact membrane structures with associated ligands to the sensor surface. Proteins incorporated into liposomes may be directly immobilized on the sensor chip surface. Liposomes may be prepared with a specific antigenic component or with biotinylated lipids, allowing capture of the liposomes with immobilized antibody or streptavidin respectively. Alternatively, Sensor Chip L1 may be used: this surface carries hydrophobic structures on the dextran matrix that can insert into liposomes and serve to attach the membranes to the dextran matrix (Figure 3-7).
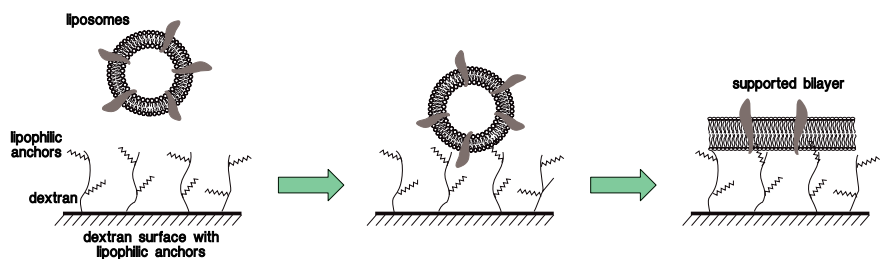
Figure 3-7. *Liposomes attach to the hydrophobic anchors in the dextran matrix on Sensor Chip L1 and can fuse to form a supported lipid bilayer.*

A variant of this approach, called on-surface reconstitution (OSR), involves immobilization of detergent-solubilized transmembrane protein on the carboxymethylated dextran matrix of Sensor Chip L1, followed immediately by replacement of the detergent by lipid to reconstitute membrane structures around the immobilized protein.

Under suitable conditions, this will result in formation of a supported lipid bilayer separated from the surface of the sensor chip by an aqueous region (Figure 3-8).
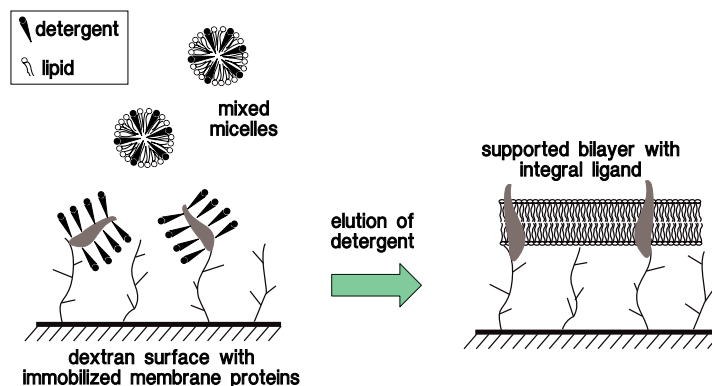
Figure 3-8. *On-surface reconstitution of supported lipid bilayers with integrated membrane proteins. (This is a simplified representation: see Section 5.4 for a more detailed description of the steps involved in on-surface reconstitution.)*

3.4   Immobilizing small molecules

Some assay formats, in particular inhibition assays, involve immobilization of small molecules on the sensor surface. The general principles for immobilizing small molecules are the same as for macromolecules, but there are a number of differences in practice:

Small molecules do not in general offer the same diversity of potential attachment sites as macromolecules. In some cases, a suitable amine or thiol group may be present in the native molecule: in others it may be necessary to introduce such a group through modification of the molecule.

The chemical procedure of immobilizing a small molecule involves a greater risk of adversely affecting its binding to the interaction partner, since the chemical modification affects a larger proportion of the molecular structure.

In many cases, it can be advantageous to introduce the coupling group on a spacer arm so that the immobilization reaction is kept at a distance from the functional molecule.

Electrostatic pre-concentration (see Section 4.1.2) is usually ineffective for small molecules. High concentrations (typically 5–10 mM) are used in immobilization to compensate for this effect.

Capturing approaches cannot normally be used for small molecules, since they require that the molecule in question carries independent binding sites for the capturing molecule and the analyte. It may however in some cases be feasible to construct a bifunctional entity where one moiety binds to the capturing molecule and the other interacts with the analyte, for instance by linking the ligand to a biotin moiety.

Note: The Surface Prep unit supplied with some Biacore systems enables immobilization of ligand outside the detector and IFC system of the instrument. This eliminates potential carry-over and contamination problems associated with high ligand concentrations, and is recommended for immobilization of low molecular weight ligands.

3.5   Techniques for stabilizing the surface

For many applications, it is important that the ligand remains stably attached to the surface so that the sensorgram baseline is constant and the analyte binding capacity does not decrease. For covalently immobilized ligands, this stability should hold over the time-scale of a complete experiment: for captured ligands which are renewed for each cycle, the stability requirement applies only over the duration of one cycle.

Covalent immobilization using amine coupling generally results in completely stable bond holding the ligand on the sensor surface. Other covalent coupling methods also give stable attachment under appropriate conditions (for example thiol coupling is stable as long as reducing agents are not included in the buffer).

Multimeric ligands can present special problems, since covalent attachment may involve only one or a few of the subunits in the multimer, leaving the others free to dissociate from the surface. This is seen as a spontaneous decrease in the baseline, often associated with a loss of analyte binding capacity (when the dissociating subunits are essential for analyte binding). Treatment of the surface with a short injection of EDC/NHS after ligand immobilization can help to stabilize such surfaces, by cross-linking the ligand subunits either to each other or to the dextran matrix in situ on the sensor surface. The cross-linking treatment should be short (less than 30 seconds, in some cases as short as 5 seconds): longer treatments can lead to loss of ligand activity. Follow the cross-linking step with an injection of ethanolamine to inactivate any remaining succinimide esters.

Non-covalently captured ligand will always dissociate from the surface to some degree during the course of the analysis, with the extent of the dissociation determined by the dissociation rate constant for the capturing interaction under the particular conditions used. Provided that the dissociation rate is reasonably low, this can usually be ignored

since fresh ligand is captured for each analysis cycle. (A dissociation rate constant of 1.5×10^{-4} s^{-1} results in a loss of about 5% of the ligand during a 5-minute analysis cycle.) If a capturing molecule with a sufficiently slow dissociation cannot be found and dissociation of ligand is a serious issue, capturing followed by cross-linking on the surface may still be exploited as an approach for combining the affinity purification advantage of the capturing approach with immobilization of the captured ligand on the surface. Ligand which is captured and then cross-linked cannot however be removed by regeneration.

3.6   Strategy for surface preparation

3.6.1   How much ligand should be immobilized?

The amount of ligand to immobilize for any application depends in the first place on the relative molecular weights of the ligand and analyte and on the sensitivity of the Biacore system. Since the SPR response is directly proportional to the mass concentration of material at the surface, the analyte binding capacity of a given surface is related to the amount of ligand immobilized as follows (assuming a 1:1 binding stoichiometry):

$$\text{analyte binding capacity (RU)} = \frac{\text{analyte MW}}{\text{ligand MW}} \cdot \text{immobilized ligand (RU)}$$

For example, if the ligand molecular weight is 100,000 daltons and the analyte molecular weight is 50,000 daltons, immobilizing 5,000 RU of ligand will give a theoretical analyte binding capacity of 2,500 RU assuming that the ligand is 100% active. Note that this is the theoretical binding capacity, which can strictly be reached only at infinite analyte concentrations and infinite contact time. In practice, the maximum observed response (the effective binding capacity) is also affected by the activity of the ligand, the kinetics of analyte binding and limitations on the maximum contact time and available analyte concentration. The theoretical binding capacity can however be useful as a guide to how much ligand to immobilize and also as a reference for assessing the activity of the surface (see Section 7.1).

General guidelines for choosing a suitable immobilization level differ according to the purpose of the analysis:

For concentration assays with macromolecular ligands and analytes, immobilize as much ligand as is reasonably possible. For protein ligands of average molecular size (of the order of 50,000–150,000 daltons), immobilization levels of 10,000–20,000 RU are typical. The analyte binding response at a given sample concentration is directly related to the level of immobilized ligand,

Biacore Sensor Surface Handbook
version AA

so that a high immobilization level will enable measurements at lower analyte concentrations. In addition, high levels of immobilized ligand ensure rapid binding of analyte and favor mass-transport limited binding (discussed further in the Biacore Concentration Analysis Handbook), making concentration measurements less dependent on the affinity of ligand for analyte.

If the ligand is large and the analyte is small, responses will generally be low so that the sensitivity of a direct concentration assay may be limited even at high ligand immobilization levels (20,000 RU or higher). Concentration assays for small molecules are generally better designed as inhibition assays to overcome this limitation (see the Biacore Concentration Analysis Handbook).

If the ligand is small and the analyte is large, it may be necessary to moderate the amount of ligand immobilized in order to avoid steric crowding effects at high analyte concentrations. This is also true for assays that use capturing or enhancement reagents, where a multi-molecular complex is built up on the sensor surface during the course of an assay. If several of the components are large, high levels of immobilized ligand can result in crowding and steric hindrance between binding molecules at a subsequent stage in the assay, limiting the observed response.

For kinetic measurements, it is generally recommended to use the lowest amount of immobilized ligand that will give an acceptably measurable response. This reduces limitations on binding rates imposed by mass transport of the analyte to the surface, and can improve the performance of the kinetic measurements (these considerations are discussed more fully in the Biacore Advisor Kinetics multimedia guide). Because of the mass dependence of the SPR response, more ligand must generally be immobilized for analysis of low molecular weight analytes. The guiding factor in planning the amount of ligand to immobilize is the expected analyte binding levels, not the absolute immobilization level of ligand.

3.6.2 Choice of sensor surface

The choice of sensor surface is dictated by the nature and demands of the application, although there is considerable flexibility in the choice for most applications. General guidelines are given here:

For general purpose applications where the ligand or capturing molecule is immobilized by standard covalent techniques, Sensor Chip CM5 is the first choice.

Applications that show significant levels of non-specific binding of positively charged molecules may benefit from Sensor Chip CM4, with a lower degree of carboxymethylation and thus a reduced net

charge. With plasma or serum samples, however, the reverse may be true: Sensor Chip CM5 has been observed to give lower non-specific binding than CM4 with these samples. Bear in mind that the immobilization capacity of Sensor Chip CM4 is lower than that of CM5.

Kinetic measurements are best performed with the lowest manageable immobilization level. The reduced capacity of Sensor Chip CM4 and Sensor Chip CM3 can help to keep immobilization levels low.

The shorter dextran matrix on Sensor Chip CM3 can be an advantage in experiments involving large molecular aggregates, virus particles and whole cells.

If interference from the dextran matrix itself is suspected, Sensor Chip C1 offers a low-capacity surface with no dextran matrix.

Sensor Chip SA and Sensor Chip NTA provide ready-to-use surfaces for capture of biotinylated ligands and his-tagged ligands respectively.

Lipid monolayers can be studied on Sensor Chip HPA. Immobilized liposomes and supported lipid bilayers can be formed using a variety of techniques on Sensor Chip L1.

Customized surfaces for surface interaction studies can be prepared on Sensor Chip Au and on the chips included in SIA Kit Au. These products are not generally suitable for use directly in biomolecular interaction studies since proteins show a strong tendency to adsorb to and denature on the unmodified gold surface.

3.6.3 Choice of immobilization method

For most protein ligands, amine coupling is the simplest approach, although it is not necessarily the most effective. For some proteins, thiol coupling can give better yields of immobilized ligand. Acidic proteins which are not efficiently pre-concentrated in their native state (see Section 4.1.2) can be modified with PDEA for surface thiol coupling: this reaction modifies carboxyl groups on the protein, raising the isoelectric point of the protein and facilitating immobilization.

In cases where amine or thiol coupling is not satisfactory, either because yields are too low or because the coupling procedure inactivates the protein, biotinylation followed by capture on a streptavidin surface can often provide an alternative approach. Biotinylation can be performed using mild reaction conditions. If a specific high-affinity interactant such as an antibody is available, capturing through this interaction may be preferable since modification of the ligand with biotin is avoided. Many glycoproteins,

which may be difficult to immobilize by other methods can also be successfully captured on Sensor Chip SA after biotinylation.

Capture through streptavidin-biotin interaction is also the method of choice for immobilizing nucleic acids, which are easily biotinylated and which are generally not amenable to amine or thiol coupling chemistry. In addition, capturing methods are much less dependent on electrostatic pre-concentration, which is inefficient for nucleic acids.

Some biomolecules, notably carbohydrates and glycoconjugates, may be successfully immobilized using aldehyde chemistry after oxidation of *cis*-diols in the ligand to aldehydes.

4. Covalent immobilization and capturing methods

This chapter gives a more detailed description of the conditions and methods used for covalent attachment of macromolecular ligands or capturing molecules to the sensor surface. Some suggestions for covalent immobilization of small molecules are given in Appendix A, and corresponding aspects of hydrophobic adsorption are considered in Chapter 5.

4.1   Conditions for immobilization

4.1.1   Temperature

Surface activation and ligand attachment is normally performed at 25°C unless the ligand is temperature-sensitive. Immobilization at lower temperatures may require prolonged contact times for surface activation and ligand immobilization. Using higher temperatures may in some cases help to increase the immobilization level obtained.

Conditions specified in this Handbook apply for immobilization at 25°C unless otherwise stated. If immobilization is performed at a different temperature, conditions may need to be adjusted.

4.1.2   Buffer pH and ligand pre-concentration

Immobilization of macromolecular ligands is usually performed from reasonably dilute ligand solutions (10–50 µg/ml or less), and result in immobilization levels that correspond to a concentration in the dextran matrix of the order of 10–20 mg/ml or more[1]. This is achieved through *electrostatic pre-concentration* of ligands in the dextran matrix.

At pH values above about 3.5, the carboxymethylated dextran on the sensor chip surface is negatively charged, and electrostatic attraction provides an efficient means for concentrating positively charged

---

[1] An SPR response of 1,000 RU corresponds approximately to a surface concentration of 1 ng/mm^2 for an average protein ligand on Sensor Chip CM5. If the thickness of the dextran matrix is taken to be 100 nm, this is equivalent to a volume concentration in the dextran matrix of 10 mg/ml.

ligands on the surface. This mechanism works well for most proteins and for several other kinds of biomolecules.

The primary requirement for this electrostatic pre-concentration on the surface is that the pH of the ligand solution should lie between 3.5 and the isoelectric point of the ligand, so that the surface and the ligand carry opposite net charges (Figure 4-1). In addition, the electrostatic interactions involved in pre-concentration are favored by low ionic strength in the coupling buffer. In general, covalent immobilization of proteins is best performed from solutions in 10 mM buffer (e.g. sodium acetate) at pH 4–5.5. A range of ready-to-use buffers for protein immobilization is available from Biacore. The extent of pre-concentration of ligand on an unactivated sensor chip under different buffer conditions can be measured to determine the optimum conditions of pH and ionic strength for immobilization (Section 4.1.5).
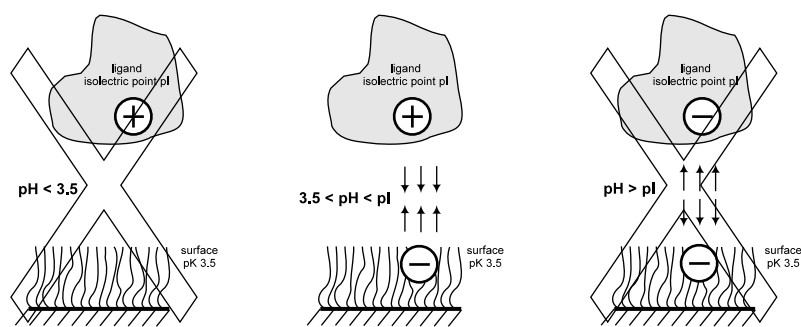

Figure 4-1. *Ligand is concentrated on the surface through electrostatic attraction when the pH lies between the isoelectric point of the ligand and the pK_a of the surface. If the pH is too low or too high, ligand will not be concentrated on the surface.*

Some ligands (for example highly acidic proteins and nucleic acids) are negatively charged even at low pH values and cannot be efficiently pre-concentrated on carboxymethyl dextran surfaces. Modification of the ligand can in some cases help to reduce the acidity: for example introduction of active disulfides for thiol coupling (Section 4.5.1) may raise the isoelectric point of proteins sufficiently to allow pre-concentration. High affinity capture (Section 3.2) is an alternative immobilization approach that does not depend on electrostatic pre-concentration: attachment of biotinylated nucleic acids to streptavidin-coated surfaces is one example. Capturing interactions can be exploited in buffers of physiological ionic strength or higher to reduce the effects of electrostatic repulsion of acidic ligands.

4.1.3   Other buffer conditions

For many proteins, coupling in 10 mM acetate buffer pH 5 works well. If you need to use other conditions, bear the following considerations in mind:

The buffer pH should be at least 0.5–1 unit below the isoelectric point of the ligand. For ligands with isoelectric point above pH 7, the buffer pH may be increased to 5.5 or 6. The optimum pH can be determined experimentally (Section 4.1.5).

The ionic strength should be low (recommended 10–20 mM monovalent cations).

Buffer components containing primary amine groups and other strong nucleophilic groups (e.g. Tris, sodium azide) must be avoided for amine coupling, since these will compete with the ligand for activated esters on the sensor chip surface. Thiol coupling must be performed in the absence of reducing agents.

4.1.4   Ligand concentration

Ligand solutions for immobilization are usually quite dilute (typically 10–50 µg/ml for most proteins) provided that efficient pre-concentration can be achieved. Higher concentrations may be needed for proteins that for reasons of low isoelectric point or other factors do not pre-concentrate efficiently on the surface. The best concentration will vary according to the nature of the ligand, the type of sensor surface and the requirements of the application.

4.1.5   Method for determining coupling conditions

While the general recommendation to immobilize proteins at pH 5 works adequately in many cases, the coupling conditions frequently need to be optimized for best results. In order to determine suitable coupling conditions without permanently modifying the sensor chip surface, inject ligand in coupling buffer over a surface that has not been activated with EDC/NHS, using a contact time of 2 minutes. Pre-concentration of the ligand on the surface will be seen as an increase in response, and will give an indication of whether the conditions are suitable.

A procedure for determining a suitable coupling pH is outlined below. An analogous procedure may be used to test other aspects of the coupling conditions.

1. Prepare ligand solutions in the different coupling buffers to be tested. As a general rule, cover the pH range 4–5.5 in steps of 0.5 pH units for initial scouting.

2. Inject the ligand solution, using a contact time of 2 minutes. Use a higher ionic strength buffer at pH slightly above neutral (e.g. HBS, available from Biacore) as running buffer, so that the non-covalently bound ligand is washed off the surface after each

injection. Start at the highest pH to reduce the risk of aggregation or precipitation of the ligand on the surface.

3. Set report points just before the start and end of the injection to determine the level of electrostatically bound ligand.

4. Inject a short pulse of 1 M ethanolamine-HCl pH 8.5 (included in the Amine Coupling Kit) or 50 mM NaOH over the surface to remove the last traces of electrostatically bound ligand.

5. Repeat steps 2–4 with ligand in different buffers and/or at different concentrations, adjusting the conditions in accordance with the solutions.
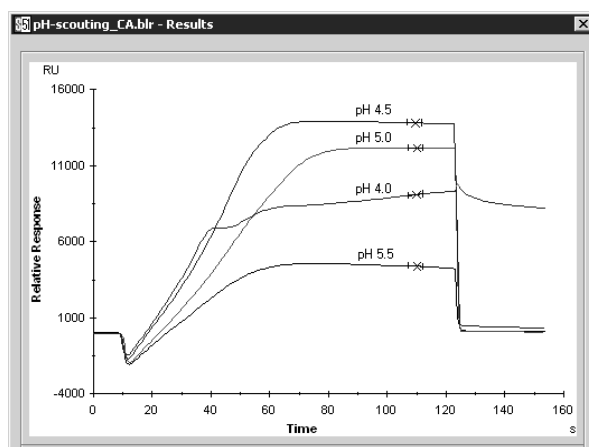

Figure 4-2. *An example of pre-concentration scouting results. Binding increases as the pH is reduced from 5.5 to 4.5. At pH 4.0, the sensorgram is irregular and bound material does not dissociate from the surface at the end of the injection, indicating that the protein is aggregating or denaturing. The optimum pH for this protein is 4.5. (Illustration from the pH scouting wizard in Biacore S51 Control Software.)*

In assessing the results of pre-concentration tests, bear in mind that pre-concentration is generally more efficient at lower pH values, but the amine coupling chemistry requires uncharged amine groups and is therefore more efficient at higher pH. Choose the highest pH conditions that give adequate pre-concentration.

Many protein ligands tend to aggregate or precipitate at low pH. This is often visible as irregularities in the sensorgram (see Figure 4-2): avoid using buffers in which this behavior is observed.

If you do not obtain satisfactory electrostatic pre-concentration at any pH, try increasing the ligand concentration and if possible reducing the ionic strength of the buffer. If this does not help, you may need to use a different coupling approach.

4.2 General immobilization procedures

The general pattern of steps involved in immobilizing ligand is the same for essentially all covalent immobilization methods:

1. Activate the surface by injection of appropriate reagents.

2. Inject the ligand solution.

3. Inject reagent to deactivate remaining active groups on the surface and remove non-covalently bound ligand.

There may be additional steps depending on the properties of the ligand, the type of sensor surface and the details of the chemistry used. Different Biacore systems provide varying levels of software wizard support for ligand immobilization.

4.2.1   Preparing solutions

For most immobilization approaches, the surface is activated with a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (see Section 4.3). Reagent solutions should be freshly prepared and mixed shortly before use. The efficiency of immobilization will be reduced if the solutions are not fresh.

Many protein ligands exhibit limited stability in the low ionic strength, low pH buffers typically used for covalent immobilization, and ligand solutions should be prepared in immobilization buffer as shortly as possible before use. If ligand is diluted into immobilization buffer from a stock solution, make sure the dilution factor is sufficiently high so that the ionic strength and pH of the immobilization buffer are not significantly affected. Rapid buffer exchange using desalting columns (e.g. NAP-10 from Amersham Biosciences or microspin techniques) is often a preferable alternative to dilution from stock solutions.

4.2.2   Evaluating covalent immobilization

Judge the results of immobilization in terms of the amount of ligand remaining on the surface at the end of the immobilization procedure, measured as the response relative to the baseline before surface activation (Figure 4-3). Measure the amount of ligand attached after final deactivation of the surface (which also serves to remove non-covalently bound ligand). The amount bound before the deactivation step can provide useful information for troubleshooting purposes (see Section 7.2).
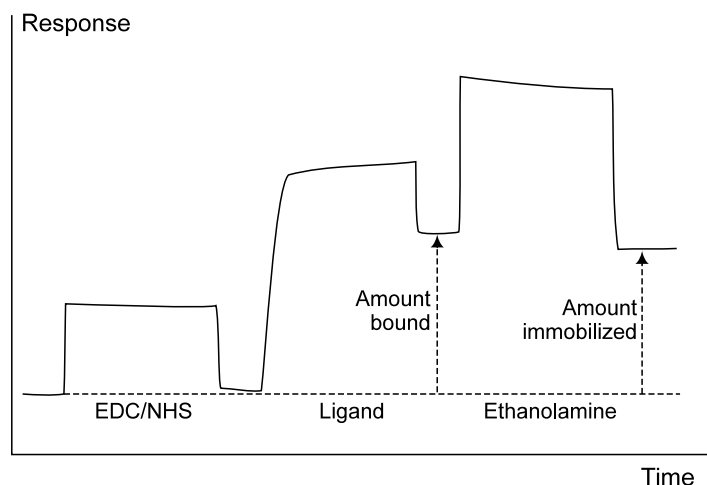
Figure 4-3. *Sensorgram from a typical amine coupling in Biacore 3000, illustrating the distinction between the amount of ligand bound and the amount immobilized.*

Surface activation itself results in a small change in response (of the order of 100–400 RU for activation with EDC/NHS), but this may usually be ignored in measuring the amount of ligand immobilized. Exact values for the amount of ligand immobilized are generally not critical: a more relevant measure of the level of immobilization is the capacity of the surface for binding analyte.

When the ligand is a small molecule, the response that derives from immobilized ligand is low, and the results of immobilization can only be assessed accurately in terms of the analyte binding capacity of the surface.

4.2.3   Testing the surface after immobilization
A newly prepared surface should always be tested for analyte binding capacity by injection of analyte. The amount of immobilized ligand only gives an indication of the *potential* analyte binding capacity: the real capacity may be lower due to inactive starting material or partial loss of ligand activity, or higher due to unexpected multiple binding sites or non-specific binding.

When the immobilization procedure for the ligand is previously untested (because the ligand is new or a new chip or immobilization chemistry is being used), testing analyte binding capacity may be done as a prelude to regeneration scouting (Section 6.1.4). For preparation of a new chip using previously established immobilization procedures, a routine test of analyte binding capacity is always recommended to confirm that the ligand is active.

4.3   Activating the surface

Regardless of the chemistry used to attach the ligand to the sensor surface, the first step in almost all covalent immobilization procedures is activation of the carboxyl groups on the surface with a mixture of 0.2 M EDC and 0.05 M NHS to give reactive succinimide esters (Figure 4-4).
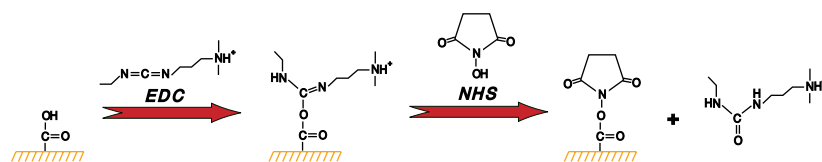

Figure 4-4. *Activation of carboxymethyl dextran with EDC/NHS. The dextran chains are omitted from this and similar illustrations for simplicity.*

The succinimide esters react spontaneously with amine and other nucleophilic groups, allowing direct immobilization of molecules containing such groups. Amine coupling (Section 4.4) is the commonest form of this kind of immobilization reaction for proteins and for a range of other molecules.

Other groups can be introduced on to the dextran matrix once it has been activated with EDC/NHS. One commonly used example is the introduction of reactive disulfides that can then be used in a thiol-disulfide exchange reaction to immobilize thiol-containing ligands (ligand thiol coupling, Section 4.5.1). Another is the introduction of hydrazide groups which can react with *cis*-diols obtained by oxidation of aldehyde-containing molecules (Section 4.6).

The well-defined chemistry of the dextran matrix and of EDC/NHS activation allows alternative approaches to covalent immobilization to be devised for situations where the more standard methods are not suitable.

4.4   Amine coupling

4.4.1   Chemistry

Amine coupling chemistry is the most widely applicable approach for attaching biomolecules covalently to the sensor surface. With this method, carboxyl groups on the surface of the sensor chip surface are first activated with a mixture of EDC and NHS to give reactive succinimide esters. Ligand is then passed over the surface and the esters react spontaneously with primary amine groups or other nucleophilic groups to link the ligand covalently to the dextran matrix (Figure 4-5).
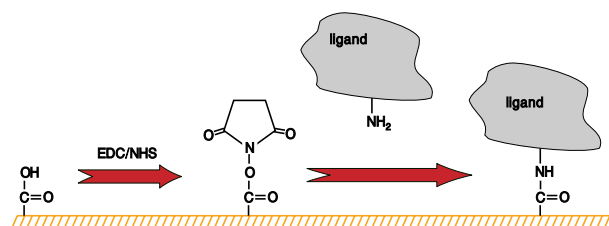
Figure 4-5. *Amine coupling of ligands to the sensor surface.*

Most proteins contain several amine groups so that efficient attachment can be achieved without seriously affecting the biological activity of the ligand. In some instances, however, amine coupling may involve groups at or near the active site or binding site of the ligand, with the result that attachment is accompanied by loss of activity. In such cases, the ligand can be attached using alternative coupling chemistry or a capturing approach.

4.4.2   Required solutions

EDC, NHS and ethanolamine are in cluded in the Amine Coupling Kit from Biacore.

EDC	0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water
NHS	0.1 M N-hydroxysuccinimide in water
Ethanolamine	1 M ethanolamine-HCl pH 8.5
Ligand	Typically 20–50 µg/ml in immobilization buffer

4.4.3   Immobilization procedure

Injection	*Recommended conditions*
1. EDC/NHS (activate the surface)	Flow rate 5–10 µl/min Contact time 6–10 min
2. Ligand (immobilize ligand)	Flow rate 5–10 µl/min Contact time 5–10 min
3. Ethanolamine (deactivate excess reactive groups)	Flow rate 5–10 µl/min Contact time 6–7 min

The recommended conditions are for typical, general purpose immobilization requirements. Ligand concentration and contact time

are the major factors that can be used to control the amount of ligand immobilized. The flow rate for the ligand injection may be reduced to conserve ligand solution if long contact times are required. Recommended flow rates and contact times for optimal immobilization may vary between different Biacore systems.

4.4.4 Results

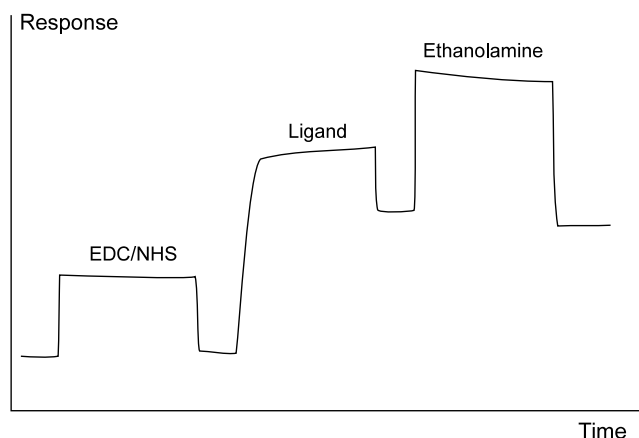A schematic sensorgram for amine coupling is shown in Figure 4-6.

Figure 4-6. *Schematic sensorgram showing the results of amine coupling.*

4.5 Thiol coupling

Thiol coupling can help to immobilize ligands in a defined orientation, since the there are often fewer potential attachment sites than with amine coupling, and in many cases is reduced to one single site. Thiol coupling can also be a valuable approach if the ligand is inactivated by amine coupling as a result of the presence of an active amine group in the analyte binding site. Surface thiol coupling, where active disulfide groups are introduced on to the ligand molecule, is also valuable for acidic proteins, since the introduction of reactive disulfides by substitution of carboxyl groups with PDEA raises the isoelectric point of the protein, improving the electrostatic pre-concentration properties (see Section 4.1.2).

One approach to thiol coupling utilizes exchange reactions between thiols and active disulfide groups. The active disulfide moiety may be introduced either on the dextran matrix (to exchange with a thiol group on the ligand, referred to as the ***ligand thiol*** approach) or on the ligand molecule (to exchange with a thiol group introduced on the dextran matrix, referred to as the ***surface thiol*** approach). A

recommended reagent for introducing active disulfide groups is 2-(2-pyridinyldithio)ethaneamine (PDEA, Figure 4-7). Ligand and surface thiol approaches are described in Sections 4.5.1 and 4.5.2 respectively.
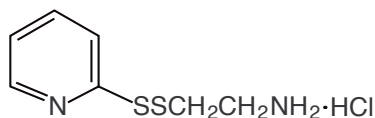
SSCH$_2$CH$_2$NH$_2$·HCl

Figure 4-7. *PDEA Thiol coupling reagent, 2-(2-pyridinyldithio)ethaneamine hydrochloride.*

An alternative approach relies on reaction of thiol groups on the ligand with maleimide reagents introduced on to the dextran matrix (Section 4.5.3).

4.5.1   Ligand thiol coupling

Chemistry
The ligand thiol coupling approach introduces reactive disulfide groups into the dextran matrix on the sensor chip. Coupling occurs through thiol-disulfide exchange with native or introduced thiol groups on the ligand.
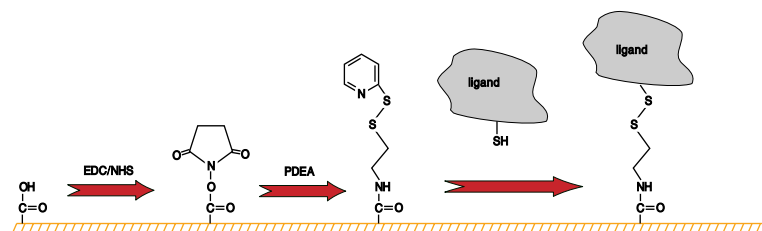
Figure 4-8. *Ligand thiol coupling of ligands to the sensor surface.*

Required solutions
Reagents for both surface and ligand thiol coupling methods are available in Thiol Coupling Kit from Biacore. PDEA is also available as a separate product from Biacore.

EDC	0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water
NHS	0.1 M N-hydroxysuccinimide in water
PDEA	80 mM 2-(2-pyridinyldithio)ethaneamine in 0.1 M sodium borate pH 8.5. Use within 30 minutes of preparation.
Cysteine/NaCl	50 mM cysteine and 1 M NaCl in 0.1 M sodium acetate, pH 4.0
Ligand	Typically 20–50 µg/ml in immobilization buffer

Immobilization procedure

Injection	Recommended conditions
1. EDC/NHS (activate the surface)	Flow rate 5–10 µl/min Contact time 2 min
2. PDEA (introduce reactive disulfide groups)	Flow rate 5–10 µl/min Contact time 4 min
4. Ligand (immobilize the ligand)	Flow rate 5–10 µl/min Contact time 6–7 min
5. Cysteine/NaCl (deactivate excess reactive groups)	Flow rate 5–10 µl/min Contact time 4 min

Recommended flow rates and contact times for optimal immobilization may vary between different Biacore systems.

Results
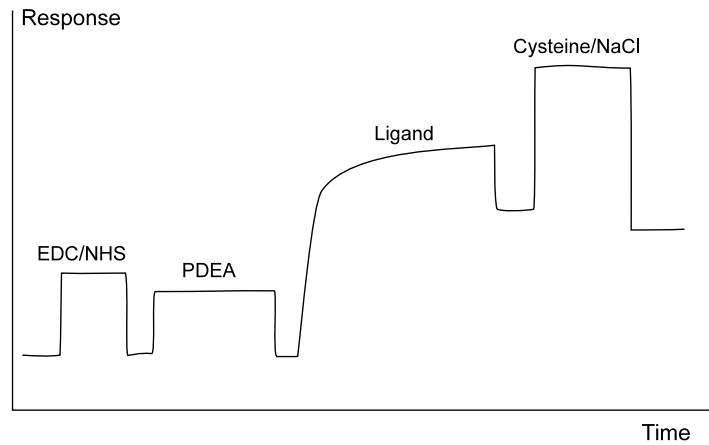A schematic sensorgram for ligand thiol coupling is shown in Figure 4-9.

Figure 4-9. *Schematic sensorgram showing the results of ligand thiol coupling.*

Additional ligand thiol methods
Thiol groups for ligand thiol immobilization can be introduced into ligands by modification of e.g. amine groups with Traut's reagent[1].

4.5.2 Surface thiol coupling

Chemistry
The surface thiol coupling approach introduces thiol groups into the dextran matrix on the sensor chip and reactive disulfides into the ligand. Coupling occurs through thiol-disulfide exchange.
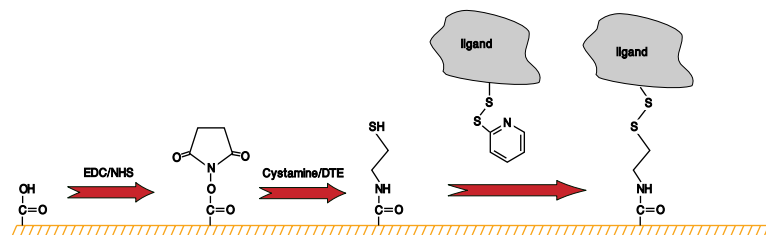

Figure 4-10. *Surface thiol coupling of ligands to the sensor surface.*

---

[1] Ghosh, S. S. *et al.* (1990) *Bioconjugate Chem.* **1**, 71-76.

Modification of the ligand with PDEA
Follow the steps below to modify a protein ligand with PDEA.
Volumes can be scaled down if required.

1.   Prepare a solution of 0.5 mg ligand in 0.5 ml 0.1 M morpholino-
      ethanesulfonic acid (MES) buffer pH 5.0 at 25°C.

2.   Add 0.25 ml 15 mg/ml PDEA in 0.1 M MES buffer pH 5.0 (final
      PDEA concentration in the mixture 22 mM).

3.   Add 25 µl 0.4 M EDC (final EDC concentration 13 mM).

4.   Mix and incubate for 10 minutes at 25°C. (If the ligand tends to
      deteriorate at room temperature, the modification may be
      performed on ice for 1 hour.)

5.   Remove the excess reagents on a buffer exchange device
      equilibrated with a suitable buffer (e.g. NAP-10 column from
      Amersham Biosciences).

Under these conditions, PDEA reacts with carboxyl groups on the
ligand (Figure 4-11). In addition to introducing a reactive disulfide
into the ligand, this has the effect of raising the isoelectric point which
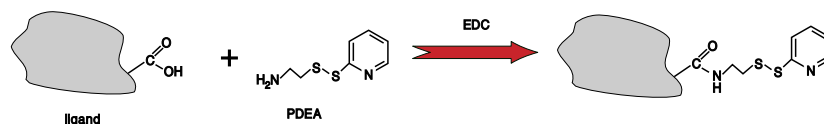can be an advantage in immobilization of acidic proteins.

Figure 4-11. *Reaction with PDEA introduces reactive disulfide groups on to
carboxyl residues in the ligand.*

Determining the degree of modification
For proteins modified with PDEA, the degree of modification can be
determined approximately by reduction of the disulphide bond and
spectrophotometric estimation of the thiopyridone released
(absorbance maximum 343 nm).

1)   Measure the absorbance of the modified protein at 280 nm (A_{280})
      and 343 nm ($A1_{343}$).

2)   To 1 ml protein solution, add 50 µl 100 mM DTE in water. Mix
      and allow to react for a few minutes at room temperature.

3)   Measure the absorbance again at 343 nm ($A2_{343}$). Calculate the
      degree of modification as follows:

$$c_{TP} = \frac{A2_{343} - A1_{343}}{\varepsilon_{343,TP}\,l}$$

$$A_{280,prot} = A_{280} - (c_{TP}\varepsilon_{280,TP}\,l)$$

$$c_{prot} = \frac{A_{280,prot}}{\varepsilon_{280,prot}\,l}$$

$$\text{molar degree of modification} = \frac{c_{TP}}{c_{prot}}$$

where

c_{TP}	is the molar concentration of thiopyridone
c_{prot}	is the molar concentration of protein
$A_{280,prot}$	is the contribution of the protein to the absorbance at 280 nm
$\varepsilon_{343,TP}$	is the molar extinction coefficient for thiopyridone at 343 nm (= 8.08×10^3 M^{-1}cm^{-1})
$\varepsilon_{280,TP}$	is the molar extinction coefficient for thiopyridone at 280 nm (= 5.1×10^3 M^{-1}cm^{-1})
$\varepsilon_{280,prot}$	is the molar extinction coefficient for the protein at 280 nm (determined separately or available from the literature)
l	is the path length of the spectrophotometer cell in cm.

Immobilization - required solutions
Reagents for both surface and ligand thiol coupling methods are available in Thiol Coupling Kit from Biacore. PDEA is also available as a separate product from Biacore.

EDC	0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water
NHS	0.1 M N-hydroxysuccinimide in water
Cystamine	40 mM cystamine dihydrochloride in 0.1 M sodium borate pH 8.5
DTE	0.1 M dithioerythritol or dithiothreitol in 0.1 M sodium borate pH 8.5
PDEA/NaCl	20 mM 2-(2-pyridinyldithio)ethaneamine and 1 M NaCl in 0.1 M sodium acetate pH 4.0
Ligand	Typically 20–50 μg/ml in immobilization buffer

Immobilization procedure

Injection	Recommended conditions
1. EDC/NHS (activate the surface)	Flow rate 5–10 µl/min Contact time 2 min
2. Cystamine (introduce disulfide groups)	Flow rate 5–10 µl/min Contact time 3 min
3. DTE (reduced disulfides to thiols)	Flow rate 5–10 µl/min Contact time 3 min
4. Ligand (immobilize the ligand)	Flow rate 5–10 µl/min Contact time 6–7 min
5. PDEA/NaCl (deactivate excess reactive groups)	Flow rate 5–10 µl/min Contact time 4 min

Recommended flow rates and contact times for optimal immobilization may vary between different Biacore systems.

Results
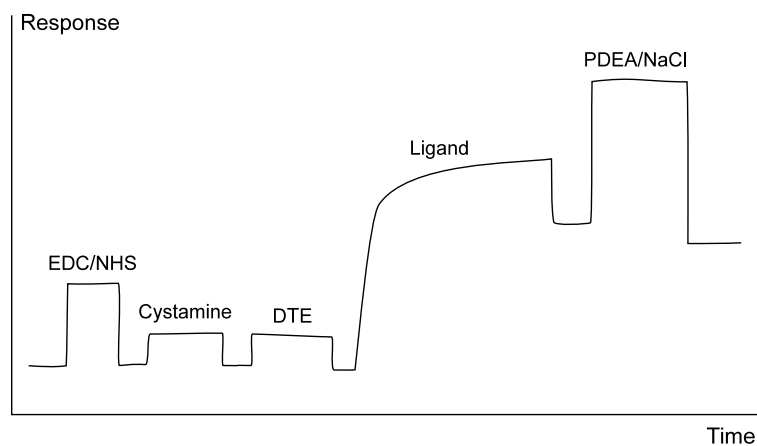A schematic sensorgram for surface thiol coupling is shown in Figure 4-12.

Figure 4-12. *Schematic sensorgram showing the results of surface thiol coupling.*

Additional surface thiol methods
The surface thiol approach can be applied to any ligand into which a reactive disulfide or maleimide group is introduced. Examples include modification of amine groups with heterobifunctional reagents that are

activated with NHS, such as N-succinimidyl-3-[2-pyridyldithio]-propionate (SPDP) and 4-succinimidyloxycarbonyl-methyl-α-[2-pyridyldithio]-toluene (SMPT), and modification of aldehydes (created by oxidation of *cis*-diols, see Section 4.6) with N-[ε-maleimidocaproic acid]hydrazide (EMCH).

4.5.3   Maleimide coupling

Ligand immobilization by thiol-disulfide exchange is not suitable for experiments where the surface is exposed to reducing agents or high pH, since the coupling bond is unstable under these conditions. An alternative approach that exploits thiol groups on the ligand is coupling mediated by maleimide reagents, resulting in a thioether bond between the ligand and the dextran matrix. Protocols for coupling on Sensor Chip CM5 with two maleimide reagents (Figure 4-13), N-[ε-maleimidocaproic acid]-hydrazide (EMCH) and N-[γ-maleimidobutyryloxy]sulfo-succinimide ester (sulfo-GMBS) are suggested below.
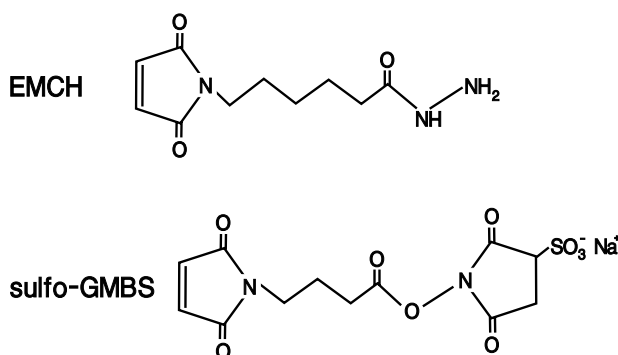

Figure 4-13. *Reagents for maleimide coupling. Top, EMCH: bottom sulfo-GMBS.*

EMCH coupling
EMCH is first coupled to the surface via the terminal hydrazide group after activation of the dextran matrix with EDC/NHS. Thiol groups on the injected ligand then react with the maleimide group to form a stable thioether linkage (Figure 4-14).
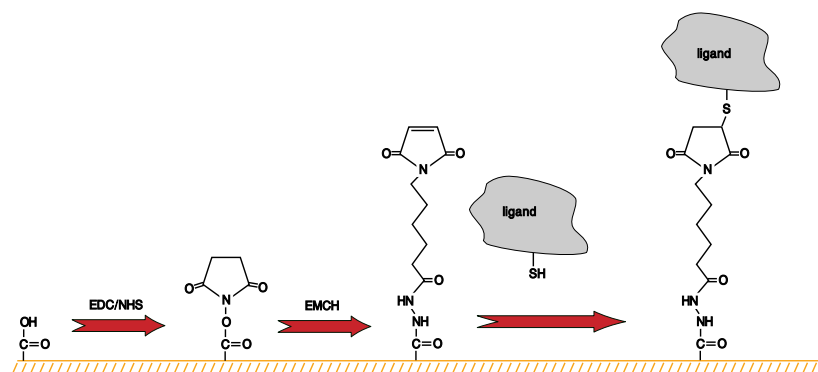
Figure 4-14. *Maleimide coupling using EMCH.*

Required solutions
EDC and NHS are available in the Amine Coupling Kit from Biacore.
Sodium borate buffer containing 1 M NaCl is also available from
Biacore.

EDC	0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water
NHS	0.1 M N-hydroxysuccinimide in water
Ethanolamine pH 7*	1 M ethanolamine-HCl in 0.1 M sodium phosphate pH 7.0
EMCH**	50 mM EMCH in 10 mM sodium borate containing 1 M NaCl pH 8.5
Cysteine/NaCl	50 mM cysteine and 1 M NaCl in 0.1 M sodium acetate, pH 4.0
Ligand	Typically 20–50 µg/ml in immobilization buffer

* It is important that the ethanolamine is at neutral pH. Do not use
the ethanolamine pH 8.5 that is included with the Amine Coupling Kit
since this will destroy the maleimide reagent on the dextran matrix.

** EMCH is sparingly soluble in aqueous buffers. The solution should
be centrifuged for 1–2 minutes at 10–20,000g before use. An
alternative reagent that gives comparable results for most ligands and
is more soluble in aqueous buffers is N-[β-maleimidopropionic acid]-
hydrazide (BMPH).

Immobilization procedure

Injection	Recommended conditions
1. EDC/NHS (activate the surface)	Flow rate 5–10 µl/min Contact time 6–7 min
2. EMCH (introduce maleimide groups)	Flow rate 5–10 µl/min Contact time 3 min
3. Ethanolamine pH 7 (deactivate excess reactive groups)	Flow rate 5–10 µl/min Contact time 3 min
4. Ligand (immobilize the ligand)	Flow rate 5–10 µl/min Contact time 6–7 min
5. Cysteine/NaCl (deactivate excess maleimide groups)	Flow rate 5–10 µl/min Contact time 4 min

Recommended flow rates and contact times for optimal immobilization may vary between different Biacore systems.

Results

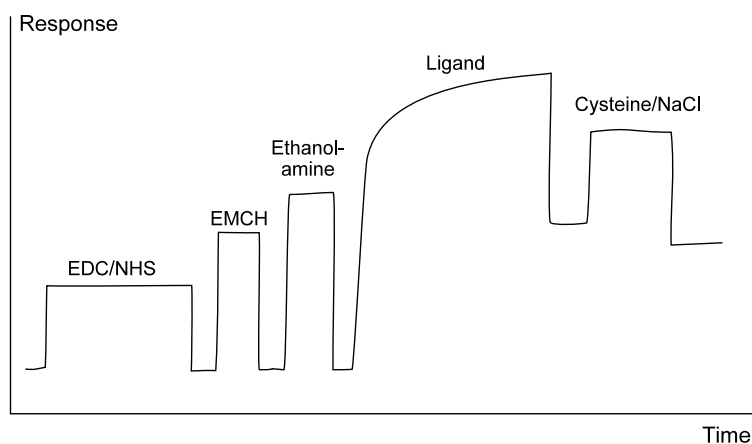A schematic sensorgram for maleimide coupling using EMCH is shown in Figure 4-15.

Figure 4-15. *Schematic sensorgram showing the results of maleimide coupling using EMCH.*

Sulfo-GMBS coupling

Ethylenediamine is coupled to the surface using amine coupling chemistry to provide a surface with amine functionality. Sulfo-GMBS reacts with the amine groups to form a surface with maleimide function that can be used to attach thiol-containing ligands (Figure 4-16). Note that the amine surface is not stable and should be derivatized with sulfo-GMBS directly after preparation.
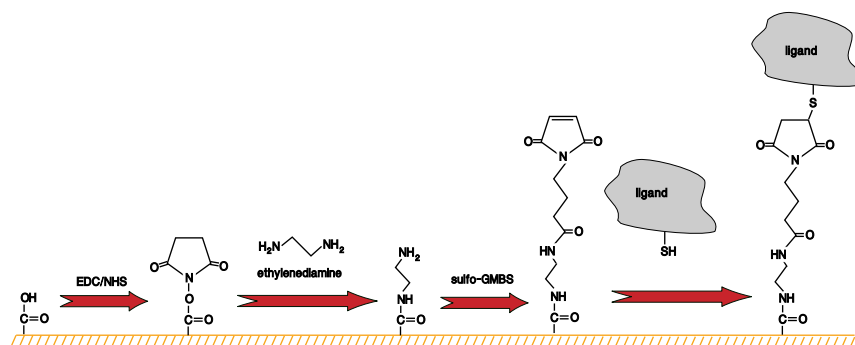

Figure 4-16. *Maleimide coupling using sulfo-GMBS.*

Required solutions

EDC and NHS are available in the Amine Coupling Kit from Biacore.

EDC	0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water
NHS	0.1 M N-hydroxysuccinimide in water
Ethylenediamine	0.1 M ethylenediamine in 0.1 M sodium borate pH 8.5
Sulfo-GMBS	50 mM sulfo-GMBS in 0.1 M sodium borate pH 8.5
Cysteine/NaCl	50 mM cysteine and 1 M NaCl in 0.1 M sodium acetate, pH 4.0
Ligand	Typically 20–50 µg/ml in immobilization buffer

Immobilization procedure

Injection	Recommended conditions
1. EDC/NHS (activate the surface)	Flow rate 5–10 µl/min Contact time 6–7 min
2. Ethylenediamine (introduce amine groups)	Flow rate 5–10 µl/min Contact time 6–7 min
3. Sulfo-GMBS (introduce maleimide groups)	Flow rate 5–10 µl/min Contact time 4 min
4. Ligand (immobilize the ligand)	Flow rate 5–10 µl/min Contact time 6–7 min
5. Cysteine/NaCl (deactivate excess maleimide groups)	Flow rate 5–10 µl/min Contact time 4 min

Recommended flow rates and contact times for optimal immobilization may vary between different Biacore systems.

Results
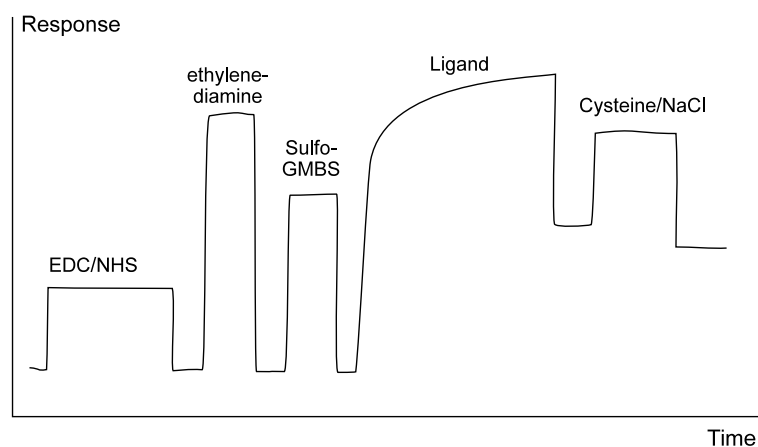A schematic sensorgram for maleimide coupling using sulfo-GMBS is shown in Figure 4-17.

Figure 4-17. *Schematic sensorgram showing the results of maleimide coupling using sulfo-GMBS.*

4.6   Aldehyde coupling

4.6.1   Chemistry

Ligands containing aldehyde groups (either native or introduced by oxidation of *cis*-diols) can be immobilized after activating the surface with hydrazine or carbohydrazide. The chemistry of aldehyde coupling is summarized in Figure 4-18.
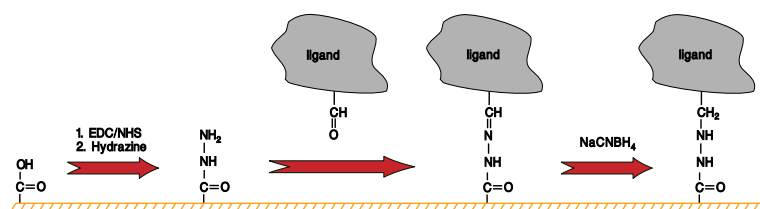

Figure 4-18. *Aldehyde coupling of ligands to the sensor surface.*

Aldehyde coupling provides an alternative approach for immobilizing glycoproteins and other glycoconjugates. The method is particularly suitable for ligands containing sialic acid, since these residues are very easily oxidized to aldehydes using sodium periodate.

4.6.2   Oxidizing the ligand

Ligands containing *cis*-diols are oxidized with sodium metaperiodate to introduce aldehyde groups before immobilization.

Oxidation of *cis*-diols to aldehydes with periodate is a simple and well characterized method of introducing aldehyde groups into monosaccharide residues[1]. This method is suitable for the majority of polysaccharides and glycoconjugates.

Materials
100 mM sodium acetate buffer pH 5.5 (buffer A)
10 mM sodium acetate buffer pH 4.0 (buffer B)
50 mM freshly prepared sodium metaperiodate in buffer A
NAP-5 column (Amersham Biosciences) or other desalting column
Distilled water (MilliQ® quality)

_____

[1] O'Shannessy, D. J. and Wilchek, M. (1990) *Analyt. Biochem*. **191**, 1-8.

Procedure
Prepare a cold solution of the ligand to be oxidized in buffer A at 1 mg/ml, then add 1/50 volume of sodium metaperiodate solution (final periodate concentration 1 mM). Incubate for 20 minutes on ice.

Stop the reaction by desalting the mixture on a desalting column eluted with buffer B. Store the oxidized ligand in the refrigerator.

The degree of oxidation is most easily estimated by testing immobilization to a hydrazide-activated sensor chip surface. Unoxidized ligand is preferably used as negative control. If the results indicate insufficient oxidation, increase the time of oxidation, the periodate concentration or the oxidation temperature.

Enzymatic oxidation of terminal galactose and *N*-acetyl-D-galactose residues with galactose oxidase is an alternative method applicable to many glycoproteins[1]. In many cases, penultimate galactose residues can be exposed by treatment with neuraminidase before being oxidized with galactose oxidase.

4.6.3 Required solutions
EDC, NHS and ethanolamine are included in Amine Coupling Kit from Biacore.

EDC	0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water
NHS	0.1 M N-hydroxysuccinimide in water
Hydrazine* or carbohydrazide	5 mM hydrazine or carbohydrazide in water
Ethanolamine	1 M ethanolamine-HCl pH 8.5
Cyanoboro-hydride	0.1 M sodium cyanoborohydride in 0.1 M sodium acetate pH 4.0
Ligand	Typically 20–50 µg/ml in immobilization buffer

*Warning
Hydrazine is extremely toxic. Carbohydrazide is recommended as an alternative reagent.

---

[1] Avigad, E., Amaral, D., Asensio, C. and Horecker, B.L. (1962) *J. Biol. Chem.* **237**, 2736.

4.6.4   Immobilization procedure

Injection	Recommended conditions
1. EDC/NHS (activate the surface)	Flow rate 5–10 µl/min Contact time 3 min
2. Hydrazine or carbohydrazide (introduce hydrazide groups)	Flow rate 5–10 µl/min Contact time 6–7 min
3. Ethanolamine (deactivate excess reactive groups)	Flow rate 5–10 µl/min Contact time 6–7 min
4. Ligand (immobilize the ligand)	Flow rate 5–10 µl/min Contact time 6–7 min
5. Cyanoborohydride (stabilize the bond)	Flow rate 2 µl/min Contact time 20 min

Recommended flow rates and contact times for optimal immobilization may vary between different Biacore systems.

4.6.5   Results
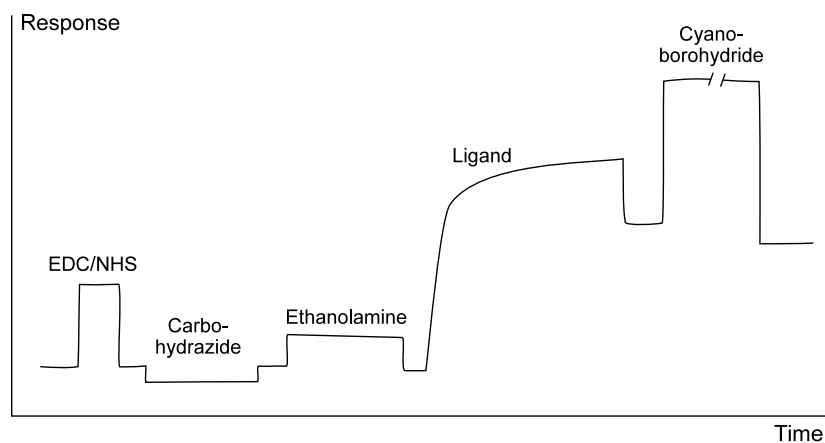A schematic sensorgram for aldehyde coupling is shown in Figure 4-19.

Figure 4-19. *Schematic sensorgram showing the results of aldehyde coupling.*

4.7   Ligand modification for capturing

For capturing approaches, the capturing molecule is normally attached to the sensor surface using one of the covalent methods described above. Ligand is then captured by a simple injection of ligand solution in a suitable buffer. The amount of captured ligand may sometimes be regulated by adjusting the ligand concentration and contact time, although regulation of the amount of immobilized capturing molecule is generally a more reliable approach.

Several capturing approaches rely on interaction of the capturing molecule with the unmodified ligand or with a ligand that has been modified using recombinant techniques to introduce a suitable capturing site. Capturing on Sensor Chip SA is an exception to this generalization, since biotin is usually introduced into the ligand by chemical techniques in vitro.

4.7.1   Biotinylation for streptavidin-biotin capture

A range of methods and commercial reagents for ligand biotinylation is available: the choice of method will depend largely on the nature of the ligand. Reagents with long spacer arms can be used to separate the biotin residue from the ligand molecule and reduce steric hindrance effects in immobilization and analysis.

Substitution levels around 1 biotin residue per ligand molecule are recommended for capture on streptavidin. In general, the conditions recommended for biotinylation of proteins with commercial reagents tend to give higher substitution levels, resulting in multi-point attachment of the ligand to the surface with consequent impairment of assay performance. If the reagent supplier does not provide guidelines for regulating the level of substitution, try using lower reagent concentration and/or shorter reaction times.

It is important that excess biotinylation reagent is removed from the ligand before capture on streptavidin. Desalting columns are generally suitable for macromolecular ligands. More selective chromatographic techniques such as higher resolution gel filtration or reversed phase chromatography can be used for smaller ligands.

Several methods are available for determining the level of ligand biotinylation. Spectrophotometric methods based on displacement of bound chromophores from avidin by the biotinylated ligand (e.g. the HABA method using 4'-hydroxyazo-benzene-2-carboxylic acid[1]) are generally easy to perform but have low sensitivity and require relatively large quantities of biotinylated ligand. More sensitive assays based on e.g. enzymatic, chemiluminescent or fluorescent detection are

---

[1] Cifonelli, J. A. (1968), *Carbohydrate Research* **8**, 233.

valuable when the amount of ligand is limited. Detailed descriptions of a variety of methods may be found in the literature[1].

---

[1] See for example *Methods in Enzymology* vol. 4 (1988).

5. Hydrophobic adsorption methods

This chapter describes procedures for attaching lipid membranes and vesicles to the sensor chip surface.

5.1 Monolayer adsorption on Sensor Chip HPA

The gold film on Sensor Chip HPA is covered with a flat layer of long-chain alkanethiol molecules presenting a hydrophobic surface suitable for adsorption of polar lipid monolayers. Ligand molecules can be embedded in or associated with the lipid monolayer.

5.1.1 Preparation for use

The hydrophobic surface on Sensor Chip HPA is inherently "sticky" and tends to adsorb a wide range of molecules and particles, and also to bind microscopic air bubbles from buffers. It is essential for successful use of Sensor Chip HPA that instruments are kept scrupulously clean and that buffers are thoroughly degassed. Running buffer must be detergent-free.

Instrument cleaning and maintenance

Ensure that the Biacore instrument is properly maintained and cleaned, and that all traces of detergent used in other assays are removed before docking Sensor Chip HPA. The following cleaning procedure is recommended as a routine for all instruments. Run the procedure with a blank Sensor Chip CM5 or Maintenance Chip docked in the instrument:

1. Run **Desorb** followed by **Sanitize** according to the instrument maintenance instructions.

2. Switch to distilled water as running buffer using the buffer change procedures recommended for the instrument.

3. Allow the instrument to run on **Standby** or on a low continuous flow rate overnight with distilled water.

4. Switch to the (detergent-free) running buffer for the experiment, again using the recommended buffer change procedure.

If the instrument has recently been used with samples known to be difficult to clean out of the flow system, it may be necessary to run

several **Desorb** and **Sanitize** cycles before washing with water and switching to running buffer.

Keep the instrument properly maintained according to the recommended procedures, and try to avoid switching between Sensor Chip HPA experiments and assays on other sensor chips that require detergent in the running buffer.

Buffers and other solutions
Make sure all buffers and regeneration solutions are properly filtered and thoroughly degassed. Detergents should not be used.

Degassing is particularly important if experiments are run at temperatures above 25°C.

5.1.2   Liposome preparation and adsorption

Preparing liposomes
Liposomes for adsorption on Sensor Chip HPA should be prepared in running buffer, using standard liposome preparation techniques[1]. Small liposomes, such as those obtained by extrusion through a 50 nm pore size filter, will adsorb faster to the surface than liposomes with larger diameters and give better coverage of the sensor surface. Membrane-associated proteins may be included with the liposomes[2], although incorporation of transmembrane proteins is not appropriate since the lipids adsorb to the sensor surface in a monolayer (half-membrane) structure (see Figure 3-6).

Preparing the sensor surface
To minimize adsorption of unwanted material from the air and running buffer on to the sensor chip surface, the times between opening the sealed chip package, docking the chip in the instrument and coating the surface with lipids should be kept as short as possible. Do not open the package or dock the chip until the liposomes are prepared and ready for use.

Follow the procedure below to coat the sensor chip surface with a lipid monolayer:

1.   Wash the surface with a 5-minute injection of 40 mM octyl glucoside (n-octyl β-D-glucopyranoside) in water.

---

[1] See for example Hope, M. J. *et al* (1985) *Biochim. Biophys. Acta* **812,** 55-65.

[2] Cooper, M. A., Hansson, A., Löfås, S. and Williams, D. H. (2000) *Analyt. Biochem.* **277**, 196-205.

2. Inject the liposome preparation at a low flow rate (2–10 µl/min), starting directly after the octyl glucoside wash. A liposome concentration of 0.5 mM with respect to phospholipids is usually sufficient. The process of coating the sensor chip surface usually takes 0.5–3 hours depending on lipid composition, liposome size and temperature. Small liposomes adsorb faster than larger ones. At temperatures below the phase transition temperature (T_c) for the lipid mixture, adsorption may be slow and the lipids may not form a monolayer on the surface. Adsorption is seen as a steady increase in response, which flattens out as the surface coverage approaches completion. It is important to cover the surface as completely as possible with lipids (see below). Maximum responses reached are usually in the region of 1500–2000 RU: if the response is significantly lower, this may be an indication that surface coverage is inadequate.

3. At this stage, lipids may be present on the surface in a variety of forms, including partially fused liposomes and multi-layered structures. Wash the surface with a short injection (0.5–1 minutes) of 10–100 mM NaOH to remove these loosely bound structures if the baseline is unstable.

Testing the surface
The tendency of the hydrophobic surface on the sensor chip to bind proteins indiscriminately is largely eliminated when the surface is covered by a lipid monolayer (which presents the polar hydrophilic side of the monolayer to the solution).

To get an indication of the extent of surface coverage, perform a 5-minute injection of bovine serum albumin (BSA) or an application-specific negative control protein at 0.1 mg/ml in running buffer. An uncoated surface washed with octyl glucoside will typically bind about 1000 RU of BSA, whereas a surface fully covered with dimyristoyl phosphatidylcholine (DMPC) or palmitoyloleoyl phosphatidylcholine (POPC) will bind less than 100 RU.

Injecting BSA or another irrelevant protein prior to the assay can help to reduce unwanted binding of sample components to the surface by blocking any exposed hydrophobic areas on the sensor chip.

5.2   Liposome attachment on Sensor Chip L1

Sensor Chip L1 has a surface coated with carboxymethyl dextran that is partially modified with lipophilic substituents, giving the surface amphiphilic properties. The surface provides sites for attachment of lipid membranes (through insertion of the lipophilic substituents into the membrane) while at the same time maintaining the hydrophilic surface properties and covalent immobilization potential of CM-series sensor chips.

5.2.1   Preparations for use

The substituted surface matrix on Sensor Chip L1 does not have the same "sticky" characteristics as the hydrophobic surface of Sensor Chip HPA, and demands for cleanliness and instrument maintenance are less rigorous than with Sensor Chip HPA. It is however important that all traces of detergent are removed from the flow system and that buffers are detergent-free. If the instrument has been used recently with detergent-containing buffers, a cleaning routine such as that described in Section 5.1.1 is recommended.

5.2.2   Liposome preparation and adsorption

Preparing liposomes
Liposomes for adsorption on Sensor Chip L1 should be prepared in running buffer, using standard liposome preparation techniques. Unlike adsorption to Sensor Chip HPA, the rate of adsorption to Sensor Chip L1 is not significantly affected by liposome size. Both membrane-associated and transmembrane proteins may be incorporated into the liposomes.

Preparing the sensor surface
Follow the procedure below to attach liposomes to the sensor surface:

1. Wash the surface with repeated (at least 2) short injections of detergent such as 20 mM CHAPS (3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate) or 40 mM octyl glucoside in water, or with 2:3 isopropanol:50 mM NaOH. It is important to wash the needle and flow system (including the sample line in the IFC) thoroughly.

2. Inject the liposome preparation at a low flow rate (2–10 µl/min). A liposome concentration of 0.5 mM with respect to phospholipids is usually sufficient. Liposome adsorption is usually adequate within a few minutes, although injections of an hour or more can be required to obtain maximum coverage. Adsorption is seen as an increase in response, which flattens out as the surface coverage approaches completion.

    Formation of a stable liposome layer is favored by high flow rates and contact times longer than 3 minutes for the liposome injection and a wait period (with buffer flow) after the injection.

3. For surfaces prepared with pure liposomes, a short injection of 10–100 mM NaOH can be used to remove loosely bound structures and stabilize the baseline. This treatment may damage liposomes containing additives such as cholesterol, ceramides or proteins.

Perform a number of blank injections with buffer or regeneration solution at the beginning of the assay to stabilize the surface.

Depending on the liposome composition, buffer conditions and temperature, the liposomes may fuse on the dextran matrix to form a supported lipid bilayer that partially or wholly covers the sensor surface. In general, the adsorption capacity of the surface for unfused liposomes amounts to over 10,000 RU, while a fused lipid bilayer gives a response in the region of 5,000 RU. In some cases, the process of fusion with release of excess lipid may be seen as a downward drift in the response after liposome adsorption. It may be necessary to wait until the response has stabilized before injecting samples.

5.3   Liposome attachment on other sensor chips

Liposomes may be attached to Sensor Chip CM5 and other sensor surfaces using standard capturing approaches in detergent-free buffer. Techniques that have been described in the literature include inclusion of biotinylated lipids in liposomes for capture on Sensor Chip SA[1] and capture using antibodies directed against specific liposome constituents[2]. Proteoliposomes can also be covalently immobilized using standard coupling chemistry to attach the protein component to the sensor chip surface[3].

Publication of these approaches predated the development of Sensor Chip L1 as a surface specifically designed for liposome capture. There is essentially no inherent advantage in these alternative approaches compared with the use of Sensor Chip L1: on the contrary, streptavidin-based capture requires the preparation of liposomes incorporating suitable biotinylated lipids, and antibody-based capture is limited by the availability and affinity of antibodies against specific liposome constituents.

5.4   On-surface reconstitution

On-surface reconstitution (OSR) is a method for re-establishing a lipid environment around membrane proteins that have been immobilized on the sensor surface in a detergent-solubilized form. The major advantage of this approach compared with adsorption of liposomes to Sensor Chip L1 is that the protein ligand is immobilized or captured

---

[1] Cooper, M. A., Hansson, A., Löfås, S. and Williams, D. H. (2000) *Analyt. Biochem.* **277**, 196-205.

[2] MacKenzie, C. R., Hirama, T., Lee, K. K., Altman, E. and Young, N. M. (1997) *J. Biol. Chem.* **272**, 5533-5538.

[3] Hoffman, T. L., Canziani, G., Jia, L., Rucker, J. and Doms, R. (2000) *Proc. Natl. Acad. Sci. USA* (**97**) 11215-11220.

on the surface instead of being passively carried in the adsorbed liposomes, giving generally higher potential ligand capacity and allowing greater control over immobilization characteristics such as ligand density and orientation. The approach is suitable for reconstituting the membrane environment around transmembrane proteins such as G-protein coupled receptors.

To achieve OSR, the detergent-solubilized ligand is first immobilized or captured on the amphiphilic surface of Sensor Chip L1, and then exposed immediately to an injection of mixed detergent-lipid micelles. The micelles adsorb spontaneously to lipophilic residues on the sensor surface and to hydrophobic domains on the ligand. The detergent is subsequently eluted from the surface in the buffer flow, leaving reconstituted lipid bilayers with embedded ligand (see Figure 3-8).

The running buffer used in OSR is detergent-free, to allow elution of detergent from the surface and reconstitution of the lipid membrane. To avoid exposing the immobilized ligand to detergent-free buffer, the injection of mixed micelles should if possible follow the ligand injection directly, with no interruption by running buffer. This may be implemented using the COINJECT function in systems where this option is available or by a dedicated injection sequence designed specifically for OSR. It may not be possible to perform OSR successfully on systems which do not support either COINJECT or dedicated functionality, unless the immobilized membrane protein can withstand brief exposure to detergent-free conditions without loss of activity.

5.4.1   *Preparation of ligand and micelles*
Ligand should be solubilized using a detergent with a high critical micelle concentration (CMC) such as octyl glucoside that can be eluted with reasonable ease in running buffer. If the ligand concentration is sufficiently high, ligand can often be captured on the sensor surface directly from the supernatant of a solubilized cell extract, with no additional purification steps. Ligand that is to be immobilized covalently must first be enriched in a detergent-solubilized form.

Mixed micelles are prepared in running buffer, generally by dissolving dried lipid preparations in a buffer-detergent mixture. The proportion of detergent and lipid in the micelles is important to the success of OSR, and is determined by the CMC of the detergent and the solubility of the lipid at the given conditions of buffer and temperature. Optimal proportions can vary considerably, and must often be fine-tuned empirically. For micelles composed of octyl glucoside and POPC, the best mixture in HBS buffer has been found to be 27.5 mM octyl glucoside and 3.75 mM POPC. It is important that the mixed micelle preparation is not turbid (indicating the presence of vesicles), since this will lead to vesicle capture instead of membrane reconstitution. Excess lipid will generally result in the formation of

vesicles with resultant turbidity, while excess detergent will impair the adsorption of the mixed micelles to the surface.

A recommended procedure for preparing mixed micelles is as follows. For optimization of the lipid-detergent ratio, prepare five concentrations each of lipid and detergent within the concentration ranges suggested below, giving a total of 25 lipid-detergent ratios. Note that both the lipid and detergent concentrations need to be optimized.

1. Pipette lipids at 10 mM in chloroform into glass tubes that have been washed in chloroform, in amounts to give final concentrations of 0.1–10 mM.

2. Evaporate the chloroform under a stream of nitrogen, and remove final traces of solvent by evaporation under reduced pressure for at least 2 hours.

3. Prepare solutions of octyl glucoside at 20–40 mM in HBS buffer by dilution from a 0.5 M octyl glucoside stock solution and 10x concentrated HBS.

4. Add the detergent solution to the lipid and shake every 10 minutes for at least 45 minutes at room temperature. Make sure that lipid residues do not remain on the walls of the tube.

5. Preparations which remain turbid have too little detergent so that the lipid forms vesicles instead of mixed micelles. These preparations can be discarded.

6. Clear preparations can be tested for adsorption to Sensor Chip L1 using an unmodified chip that has been conditioned with two injections of 40–50 mM CHAPS. Inject each preparation for 1–8 minutes at 5–10 µl/min, regenerating the surface with two 1-minute injections of 50 mM octyl glucoside between each test. The optimal lipid-detergent ratio is the clear solution that gives highest response in the test injection (Figure 5-1). Responses up to about 3–6000 RU above baseline may be expected for POPC liposomes. Levels may vary with other liposome types: for example anionic lipids often give lower response levels.
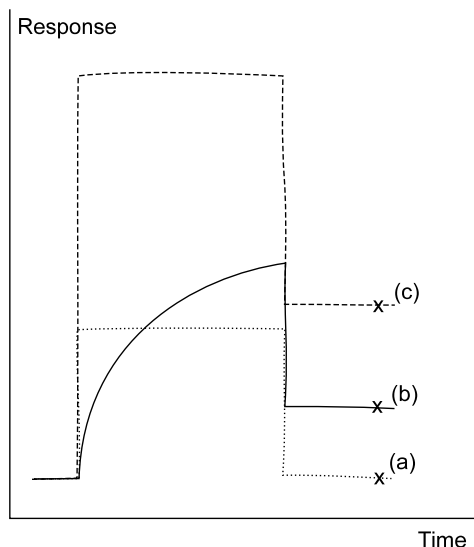
Figure 5-1. *Schematic sensorgrams illustrating preparations with different content of octyl glucoside and POPC injected over Sensor Chip L1. The response level at the marked report point indicates the amount of lipid stably adsorbed to the surface. (a) Lipid does not adsorb from a detergent-rich sample. (b) A lipid-rich sample gives a slower increase in response during the sample injection with a low final level of lipid deposition. (c) The optimal lipid-detergent ratio gives a high level of lipid deposition.*

5.4.2   Immobilizing the ligand

A capturing approach is generally preferable for attaching the ligand to the surface, since the ligand injection can then be followed immediately by the injection of mixed micelles. Covalent immobilization methods may work in some cases provided that the detergent in the ligand preparation does not interfere with the immobilization chemistry and that detergent is included in all washing and deactivation solutions to preserve the ligand activity.

It is generally easier to achieve higher ligand levels (analyte binding capacities) with OSR than with adsorption of ligand-containing liposomes.

5.4.3   Lipid deposition and membrane reconstitution

The mixed micelle preparation is passed over the surface in a short (typically 1–2 minutes) injection directly after the detergent-solubilized ligand has been attached to the surface, followed by detergent-free running buffer to elute the detergent and reconstitute the membrane.

6. Regeneration

Regeneration is the process of removing bound analyte from the sensor chip surface after analysis of a sample, in preparation for the next analysis cycle. The number of times a sensor surface can be regenerated depends on the nature of the attached ligand, but is frequently greater than 100 and may even be 1000 or more.

When the ligand is attached directly to the surface, regeneration removes analyte from the ligand without destroying the ligand activity. When a capturing approach is used (Section 3.2), regeneration generally removes both ligand and analyte from the capturing molecule. In this case the stability of the ligand under regeneration conditions is irrelevant.

Efficient regeneration is important for successful assays. Incomplete regeneration or loss of the binding activity from the surface will impair the performance of the assay and the useful lifetime of the sensor chip will be shortened. Time spent on establishing suitable regeneration conditions is therefore a valuable investment.

Regeneration of the surface with a regeneration solution is not always necessary: if the analyte dissociates fast enough, all analyte may be removed within a reasonable time simply by washing with buffer. This is immediately evident from the sensorgram, since the response returns to baseline after the sample injection. This chapter discusses the choice of conditions for regeneration in cases where spontaneous dissociation is not sufficient. Section 7.2.3 discusses how to deal with situations where regeneration fails.

Determining suitable regeneration conditions is basically a two-step process, involving scouting for possible conditions followed by verification of the suitability of chosen conditions. In some cases, conditions found in the initial scouting may need to be fine-tuned in additional experiments before verification. A set of reagents suitable for regeneration scouting with most protein ligands is available as Regeneration Scouting Kit from Biacore.

6.1   Regeneration scouting strategy

6.1.1   General considerations
Regeneration scouting is a procedure for finding potential conditions for regeneration, which can later be refined if necessary and tested more extensively. Scouting is performed by testing a few (suggested 5) repeated cycles of analyte binding and regeneration attempt with each

condition, and examining trends in the response levels within each condition. To avoid complicating the interpretation unnecessarily, scouting should start with a previously unused sensor surface immobilized with ligand.

General guidelines for regeneration scouting are:

Use a sensor surface which is representative for the surface you intend to use in your experiments. Optimal regeneration conditions for the same ligand may vary slightly with immobilization chemistry, immobilization level and sensor chip type (Section 6.1.3).

Where possible, use a high concentration of analyte so that at least half the maximum binding capacity of the surface is occupied. This will permit more confident interpretation of the scouting results. If only a small fraction of the analyte binding sites are occupied by analyte, loss of binding capacity will more easily go undetected. Regeneration conditions established at a low analyte binding level may not necessarily be optimal for higher levels (Section 6.1.3).

Test regeneration conditions using analyte that reflects the experimental samples if possible. This is particularly important if the samples are complex mixtures such as cell culture medium or body fluids, where binding of non-analyte components to the surface can complicate the regeneration behavior.

Use your knowledge and experience of the ligand and analyte to guide your initial choice of regeneration conditions. For molecules used in affinity chromatography contexts, conditions for elution from the chromatographic medium can provide a starting point for scouting for regeneration conditions.

Start scouting with the mildest conditions and progress towards more harsh treatment. This will reduce the risk that the surface is damaged in early scouting cycles and rendered useless for later tests. For scouting regeneration conditions of different types (e.g. pH and high ionic strength), run at least the initial scouting for each type on a new sensor surface to avoid complications that can arise from mixed treatments of the surface.

Assess the results in terms of trends in analyte response and baseline level within and between tested conditions. Overlay plots of the repeated injections for one condition can provide further help in assessing regeneration performance. Single test cycles seldom give sufficient information for establishing regeneration conditions.

6.1.2   Choice of regeneration solution

Conditions for suitable regeneration are determined by the nature of the ligand-analyte interaction and by the micro-environment on the surface of the sensor chip. Experience of a range of situations suggests that regeneration is often accomplished by denaturing or otherwise disturbing the structure of the analyte while the ligand may be stabilized against denaturation by immobilization on the surface. This is not however necessarily a guiding principle, and determination of optimal regeneration conditions can only be done through experimentation.

The most commonly successful regeneration conditions for protein surfaces are low pH (10 mM glycine-HCl at pH 1.5–3, available as ready-to use solutions from Biacore) and ethylene glycol at concentrations up to 100%. Other conditions which have proved useful include:

  high pH (1–100 mM NaOH)

  high ionic strength (e.g. up to 5 M NaCl or 4 M $MgCl_2$)

  low concentrations of SDS (up to 0.5%)

A more detailed list of recommended conditions for regenerating protein and other ligands is given in Section 6.4.

Always make sure that the regeneration solution is fully compatible with the running buffer, so that precipitation does not occur at interfaces between the two solutions. For example, $MgCl_2$ can cause precipitation with phosphate buffers, and SDS may precipitate if the potassium content of the running buffer is too high. Use a different running buffer if precipitation occurs on mixing with the regeneration solution.

6.1.3   Factors affecting regeneration

Even if the nature of the ligand-analyte interaction is probably the major factor determining suitable regeneration conditions, the optimum conditions are affected by the surface properties, ligand density and analyte binding levels.

Sensor chip type
Optimal regeneration conditions can differ slightly for the same ligand-analyte pair on different sensor chip types. In a test study using anti-myoglobin antibodies as ligand and myoglobin as analyte, optimal regeneration with glycine-HCl on Sensor Chip CM3 required pH values 0.2–0.4 units lower than on Sensor Chip CM5.

Coupling chemistry
Regeneration properties of the same ligand immobilized on the same sensor chip type can differ slightly with different coupling chemistries.

Ligand density
The amount of immobilized ligand can have a significant effect on the optimal regeneration conditions. Monoclonal antibodies regenerated with glycine-HCl, for example, have been found to require lower pH values at lower ligand densities. The effect can be as much as 0.5 pH units between 600 and 10,000 RU immobilized antibody.

Analyte binding level
The amount of analyte bound to the surface can affect the conditions required for optimal regeneration. In general, higher analyte levels require slightly harsher conditions. Regeneration scouting should thus be performed using high binding levels (at the top of or above the range that will be used in the application): the conditions determined will suffice for regeneration of lower levels. If the scouting is performed using low analyte binding levels, regeneration may be inadequate for higher levels encountered in the application.

In general, interpretation of regeneration scouting is easier if high analyte levels are used, since trends in analyte response values are more readily detected.

Temperature
Temperature can have a significant effect on regeneration performance, and it is important that regeneration is optimized and tested at the temperature at which the assay will be run. It is not practical to exploit a change in temperature during an assay to optimize regeneration, but in cases where the temperature is not critical for the ligand-analyte interaction, it may be possible to run the assay at a temperature chosen to favor regeneration.

6.1.4   Regeneration scouting procedure
The procedure outlined below is recommended for scouting for regeneration conditions. Scouting solutions suitable for a range of applications are available in the Regeneration Scouting Kit from Biacore.

1.   Using a new sensor chip, immobilize approximately the same amount of ligand that will be used in the analysis experiments, using the same immobilization chemistry.

2.   Inject analyte at a reasonably high concentration, so that at least 50% of the theoretical binding capacity of the surface is

occupied. The theoretical capacity is estimated from the
molecular weights of analyte and ligand as described in Section
3.6.1. (If molecular weight values are not available for estimation
of the theoretical binding capacity, use the highest analyte
concentration that is reasonable for your system and a contact
time that allows steady state binding to be attained if possible.
Practical considerations such as availability of analyte may limit
the amounts that can be used.)

Set a baseline report point just before the start of the analyte
injection and a report point to measure analyte response levels
just after the end of the injection.

3. Inject the mildest regeneration solution that you intend to test.
    Use a short contact time: 30–60 s is usually sufficient. Longer
    exposure to regeneration conditions involves greater risks for loss
    of ligand activity, and often does not lead to improved
    regeneration.

4. Repeat steps 2 and 3 for a suggested total of 5 cycles of analyte
    injection and attempted regeneration using the same regeneration
    solution.

5. Repeat steps 2–4 for the next regeneration solution to test.

    Progress from milder to harsher conditions: for example if
    regeneration with low pH is being tested, start at pH 3 or 2.5 and
    decrease the pH in steps of 0.5 or 0.25 units. Larger steps will
    involve fewer cycles to test a given range of pH values, but
    additional experiments may be needed to refine the conditions. A
    resolution of 0.25 pH units or less is recommended for fine-tuning
    of regeneration with acidic conditions.

Surfaces used for regeneration scouting can seldom be re-used for
analysis purposes, except in the fortunate circumstances where the last
regeneration conditions tested are optimal or too mild. In planning
regeneration scouting procedures, be aware that ligand used for a
scouting series will probably not be useful for any further experiments.

6.2   Interpreting regeneration scouting

6.2.1   *Interpretation principles*
The goal of regeneration is to remove all bound analyte while leaving
the ligand undamaged. Because of the way scouting experiments are
usually constructed, the response levels (baseline and analyte response)
in one cycle report on the efficiency of regeneration in the previous
cycle (Figure 6-1).
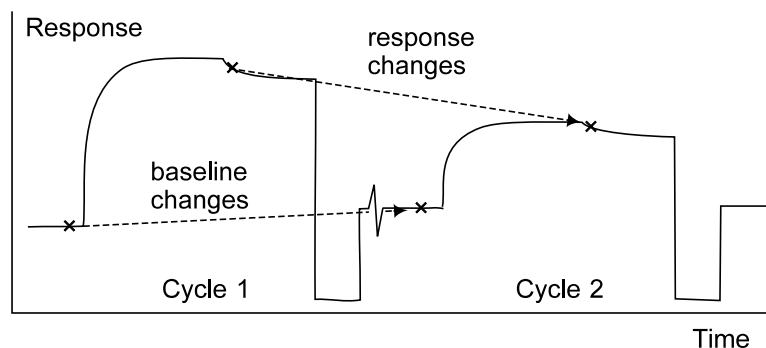
Figure 6-1. *Regeneration scouting is assessed by following the effects of regeneration attempts on the analyte binding capacity and baseline levels. The measurements from one cycle in the scouting procedure report on the efficiency of the regeneration attempt in the previous cycle.*

For the first cycle of regeneration scouting, the surface has not yet been exposed to any regeneration attempt. The analyte response correspondingly provides a starting reference for the level of binding that may be expected. Thereafter, the baseline report point indicates whether analyte has been removed from the surface in the previous regeneration cycle, while the analyte response indicates whether the ligand retains analyte-binding activity.

Note: Injection of regeneration solution often gives a considerable bulk response, since the refractive index is not matched with the running buffer. The relative bulk response may be either positive or negative depending on the solution used.

Regeneration conditions which do not remove analyte sufficiently lead to an increase in baseline between cycles with a corresponding decrease in analyte binding response measured relative to the baseline in the same cycle (Figure 6-2, top panel). Conditions which are too harsh may remove all bound analyte but result in a loss of analyte binding capacity as the ligand activity deteriorates (Figure 6-2, bottom panel).
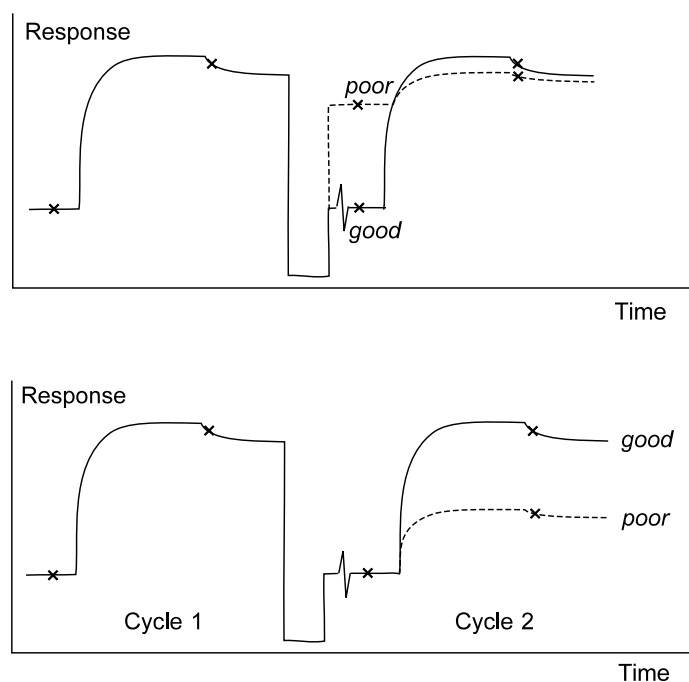
Figure 6-2. *Efficient regeneration removes all bound analyte. Inefficient regeneration leaves analyte on the surface (top panel), seen as an increase in the baseline. The analyte response in the second injection reveals whether the ligand is still fully active (bottom panel).*

Assess the regeneration performance in the first place from trends in the analyte response both within and between tested conditions. Trends in the baseline level are seldom conclusive in themselves, but may provide complementary information to aid in interpretation of the analyte response. The following general guidelines apply:

The sample binding response should be constant. A falling trend in sample response indicates either that the ligand is losing activity (regeneration is too harsh) or that material is accumulating on the surface (regeneration is too mild).

The baseline after regeneration should not increase. If it does, this indicates that material is accumulating on the surface (regeneration is too mild). Some increase in baseline may however be tolerated provided that the analyte binding response is not affected.

The baseline level after regeneration may fall, particularly during the first few cycles using a newly prepared chip. This is acceptable provided that the analyte response is not affected.

A schematic example of regeneration scouting using four conditions is illustrated in Figure 6-3. The first condition tested (A) is too mild: the sample response is consistently low and the baseline is consistently high, indicating that analyte bound in the first cycle is not removed. The second condition tested (B) shows some improvement, but the progressively increasing sample response indicates the analyte binding capacity is not completely restored by one regeneration injection of this solution. Condition C gives a stable level of both sample response and baseline, while condition D results in progressive deterioration of the analyte binding capacity. C is the best of these four conditions, while D is too harsh.
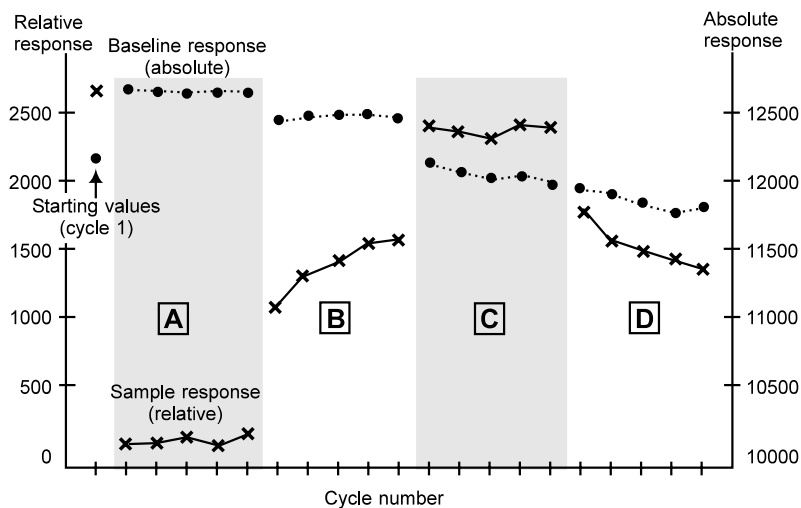
Figure 6-3. *Scouting for regeneration conditions. The report points from the first cycle give the starting values: thereafter the points are grouped according to the regeneration conditions tested. See text for interpretation. ● = baseline response; x = sample response.*

In practice, observed behavior in regeneration scouting may deviate from the ideal in a number of respects:

After efficient regeneration, the analyte response should in principle be the same as that in the first cycle, before exposure to any regeneration conditions. In practice, the response after regeneration often differs from the first value, and a consistent analyte response during repeated cycles of binding and regeneration is more important than achieving the same response as in the first cycle. The analyte binding capacity is often seen to deviate by 5–10% during the first few cycles on a newly prepared sensor chip, and start-up cycles should normally be included in assay protocols to compensate for this. Provided that only a relatively small fraction of the activity is lost in the first few cycles and that analyte response in subsequent cycles maintains a constant level, this effect may be ignored.

When regeneration conditions are too mild and analyte accumulates on the surface, progressive recovery of analyte binding capacity over a few cycles with harsher conditions may be observed. This is usually an indication that the conditions are on the borderline between too mild and acceptable.

Trends in the baseline level often do not follow the ideal pattern and are of secondary value in interpreting the results. As long as the analyte response relative to baseline is constant, trends in the absolute baseline level can usually be ignored if the changes are not excessive. A slight downward drift in baseline is common, even with fully optimized regeneration conditions.

While trend plots provide a convenient overview of the regeneration performance in a scouting experiment, the sensorgrams from each cycle contain more information about the effect of regeneration conditions. Preparing overlay plots of the sample injection and dissociation phase from the regeneration scouting cycles can help to reveal changes in the interaction behavior that might be missed in a trend plot (for example a change in the dissociation rate of the analyte). For critical experiments such as high resolution kinetic measurements, the sensorgram shape should not be affected by the regeneration treatment.

6.2.2   Case studies

The following case studies showing regeneration scouting for three different monoclonal antibody-antigen interactions with low pH illustrate interpretation of regeneration scouting in practice. Solutions used in each case were 10 mM glycine-HCl at pH 3.0, 2.5, 2.0 and 1.5. The antibody is immobilized on the surface as ligand.

Only trends in the analyte response level are shown: these are sufficient in all cases for interpreting the results.

Case 1
This antibody requires pH values below 3 for regeneration, but tolerates conditions down to pH 1.5 or below.
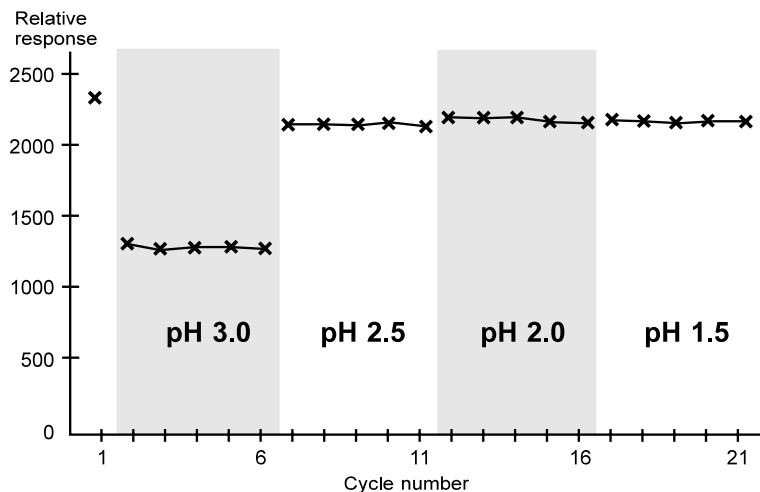
Figure 6-4. *Scouting for regeneration conditions, case 1. This ligand tolerates a range of acidic conditions and can be regenerated at pH between 1.5 and 2.5.*

The low analyte responses obtained with pH 3.0 suggest that these conditions are too mild. This is supported by the observations that longer injections give more efficient regeneration and that harsher conditions restore analyte binding capacity (not illustrated).

All the harsher conditions tested restore analyte responses to a constant level which is 90% or more of the response in the first cycle. Regeneration is thus achieved at pH 2.5 or below: regeneration can be performed confidently at pH 2.0, allowing a margin of variation in the behavior of different samples. There is no need to fine-tune the conditions further.

Case 2
This antibody can be regenerated at pH 2.0–3.0 (or possibly higher),
but begins to lose activity at pH 1.5.
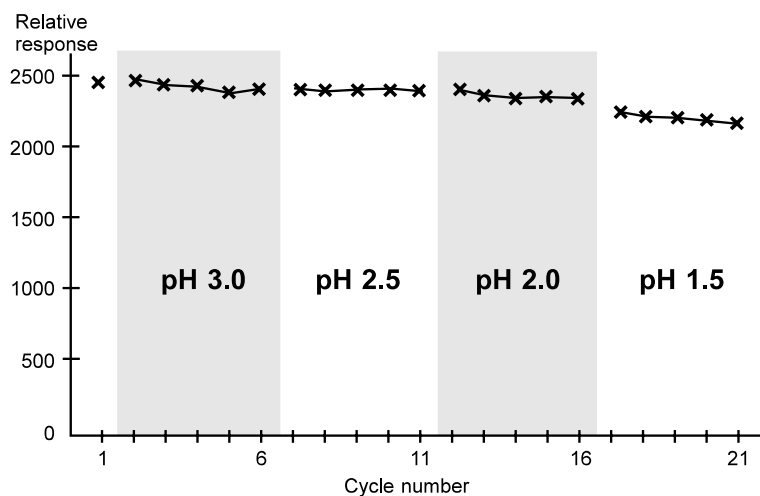
Figure 6-5. *Scouting for regeneration conditions, case 2. This ligand begins
to lose activity significantly at pH 1.5.*

Satisfactory regeneration is obtained at pH 3.0 and pH 2.5. There is a
slight loss of analyte response at pH 2.0, and progressive deterioration
in the response is pronounced at pH 1.5 suggesting that this antibody
surface does not tolerate conditions below pH 2.0. It may be possible
to use even milder conditions than pH 3.0.

Case 3
This antibody presents a difficult case: none of the regeneration
conditions tested is satisfactory, and scouting should be pursued
further either with closer pH intervals around 2.5 and varying contact
times or with a different regeneration approach.
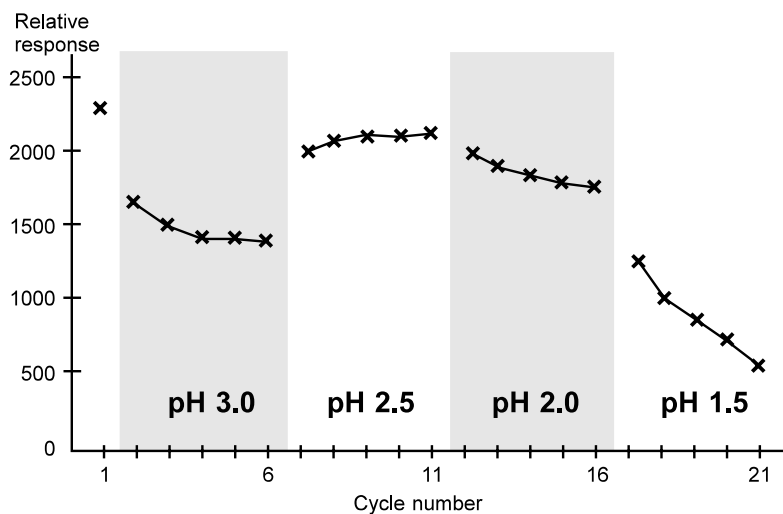
Figure 6-6. *Scouting for regeneration conditions, case 3. For this ligand, it
may be possible to find suitable regeneration conditions by further scouting
around pH 2.5.*

In this case, pH 3.0 is too mild since the analyte response falls
progressively. The response is largely restored at pH 2.5, although a
stable level is not reached until the third cycle at this pH. Lower pH
values cause progressive deterioration in the response.

This antibody shows considerably less tolerance in regeneration
conditions than the previous two cases, and for optimal performance
the conditions should be refined further around pH 2.5.

6.3   Verifying regeneration conditions

Once regeneration scouting has given an indication of suitable
conditions, it is important to verify the performance of regeneration
over a larger number of repeated cycles of analyte injection and
regeneration (recommended minimum 20 cycles).

The criterion of acceptance for the regeneration conditions will depend
to some extent on the demands of the application: for example, careful
kinetic analyses demand more rigorous regeneration than qualitative
screening for binders and non-binders. As a general recommendation,
good regeneration conditions should give an analyte response that does

not vary by more than 5–10% over 20 cycles. Because there is often a slight reduction in activity in the first few cycles, the assessment should be made over for example cycles 6–25 rather than cycles 1–20.

6.4   Suggested regeneration solutions

This section offers some recommendations for regeneration solutions based on experience at Biacore and in the literature. For molecule types that are not specifically mentioned here, an empirical approach is the best route to success.

6.4.1   *Protein surfaces*

Antibodies
Antibody ligands can usually be regenerated using acidic conditions (10 mM glycine-HCl, pH 3.0–1.5).

Other proteins
Although the range of protein-protein interactions studied with Biacore is very broad, experience has shown that a relatively small set of conditions provides for regeneration. Many surfaces are regenerated satisfactorily by one of the following solutions:

   10 mM glycine-HCl pH 3.0–1.5 (available as ready-to-use solutions from Biacore)

   50–100% ethylene glycol

   1–100 mM NaOH

   1–4 M MgCl2

   0.5–5 M NaCl

   0.02–0.5% SDS

Special cases
For some studies, the nature of the interaction points the way to a suitable regeneration approach which is specific for that interaction. Interactions that require metal ions, for example, can often be regenerated with chelating agents such as EDTA or EGTA, provided that the ligand survives removal of the metal ion. A specific example of this approach is Sensor Chip NTA, which uses Ni^{2+} ions chelated by nitrilotriacetic acid to capture his-tagged ligands, and which can be regenerated with EDTA that strips the Ni^{2+} ions from the surface (see Section 3.2.3).

6.4.2 **Nucleic acids**

Hybridized oligonucleotides can usually be regenerated with freshly prepared 1 mM HCl.

Proteins can be removed from RNA or DNA surfaces with 50 mM NaOH containing 1 M NaCl, or with 0.2–0.5% SDS.

6.4.3 **Membrane-associated ligands**

Lipid monolayers on Sensor Chip HPA
Regeneration of the Sensor Chip HPA surface is directed towards removing bound analyte from the ligand, and uses the same approach as for ligands covalently attached to CM-series and other sensor chips (with the exception that regeneration solutions should not contain detergents or organic solvents). Lipid monolayers composed of synthetic lipids such as DMPC and POPC are generally quite robust in this respect and can withstand exposure to e.g. 100 mM HCl and 100 mM NaOH.

Although the lipid monolayer with associated ligand and bound analyte can in principle be removed from Sensor Chip HPA by washing with detergents or organic solvents, this approach to regenerating the hydrophobic surface is not recommended and Biacore does not guarantee the performance of Sensor Chip HPA treated in this way.

Supported lipid bilayers
Proteoliposomer and membrane vesicles are often difficult to remove completely from Sensor Chip L1, and regeneration of this kind of surface should be directed towards removing bound analyte from the ligand (using the same approach as for ligands covalently attached to CM-series and other sensor chips, with the exception that regeneration solutions should not contain detergents or organic solvents).

For surfaces prepared with pure liposomes, the lipid can be removed with a 10–30 second injection of 2:3 isopropanol:50 mM NaOH is sufficient to strip the lipids from the surface. Removal of the lipid is generally recommended for applications that address interaction of the analyte with the lipid bilayer itself, such as studies of absorption of small molecules into membranes.

OSR surfaces
An OSR surface is regenerated with the aim of removing analyte and other bound sample components while leaving the reconstituted membrane intact. Detergents and organic solvents that may destabilize the membrane should be avoided.

6.4.4   *Small molecules*

Small molecules are generally more tolerant of harsh conditions than macromolecules, so that a wider range of regeneration conditions can often be considered. Solutions that have proved successful in regenerating low molecular weight ligands with protein analytes are (in approximate order of convenience):

20–100 mM NaOH in 30% acetonitrile. This solution is not stable and must be prepared fresh each day.

20–100 mM NaOH containing 0.5% Surfactant P20 or 0.05% SDS

10 mM glycine-HCl pH 3.0–1.5 (available as ready-to-use solutions from Biacore)

1–4 M $MgCl_2$

6.4.5   *Other regeneration strategies*

Some ligand-analyte interactions may be difficult to regenerate with the conditions suggested above. Other conditions that have been found useful include:

10–100 mM HCl

~0.1 % trifluoroacetic acid

~1 M formic acid

~1 M ethanolamine-HCl at pH 9 or higher

In particularly stubborn cases, or when several components from the sample may bind to the surface, regeneration cocktails using mixtures of different types of solution (e.g. acidic and chaotropic conditions) may prove fruitful. The control software for most Biacore systems supports regeneration with two consecutive injections, and this approach can in some cases be an alternative to mixed reagents.

7. Sensor surface performance

This chapter considers aspects of sensor surface performance including testing the surface activity and troubleshooting surface preparation and performance problems.

7.1   Testing the surface

Once the surface has been prepared, the analyte binding activity should be tested before proceeding to further stages in assay development. The same protocol can also be used to test the activity of sensor chips that have been stored, to ensure that activity is retained throughout the storage.

To test the binding activity of the surface, inject analyte that is known to bind to the ligand. Use successively higher analyte concentrations (e.g. 1, 10, 100, 1000 nM) with a moderate flow rate (20–40 µl/min) and a contact time that is sufficient if possible for the binding curve to flatten out. The response reached in a single injection can be used to check the consistency of surface activity between different surfaces and during storage of surfaces.

To estimate the maximum analyte binding capacity of the surface, perform repeated injections of analyte without regenerating the surface between injections. The sensorgram plot will show a steadily increasing response over the initial baseline. Eventually, injection of analyte will give no further increase in response when the maximum binding capacity is reached. The maximum analyte binding capacity compared with the theoretical value (Section 3.6.1) gives a valuable indication of the activity of the ligand on the surface. If the experimental capacity is low in comparison with the theoretical value (e.g. 30% or lower) and the starting material is known to be active, consider revising the immobilization method to maintain ligand activity.
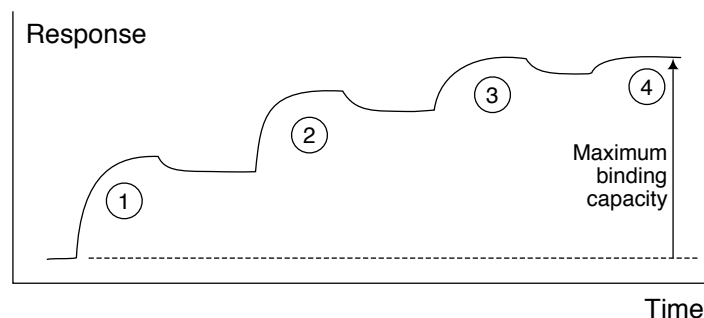
Figure 7-1. *Repeated injections of analyte without regeneration can be used to estimate the maximum analyte binding capacity of the surface.*

7.2   Troubleshooting surface preparation

This section deals with the most common problems associated with ligand immobilization.

7.2.1   *Low immobilization levels*

If the level of immobilized ligand is too low, examine the immobilization results to identify the cause of low immobilization levels:

If pre-concentration of ligand on the surface is not satisfactory (Figure 7-2):

Test pre-concentration at different pH values. As a general rule, pH values down to about 3.5 can be used (use citrate buffers for pH 3.5–4.0). If pre-concentration is inadequate even at pH 3.5, the ligand may be too acidic, and you should consider using a different immobilization approach. Some ligands can be immobilized satisfactorily at pH values above 5.5. Maleate buffers are suitable for immobilization at pH values in the range 5–6.

Make sure you are using low ionic strength buffer and that the ligand is sufficiently diluted or desalted from salt-containing stock solutions. The total ion concentration should ideally be 10 mM or less. Only use higher salt concentrations if this is necessary to maintain ligand stability.

Increase the contact time if the immobilization sensorgram indicates that more ligand can bind.

Increase the ligand concentration.
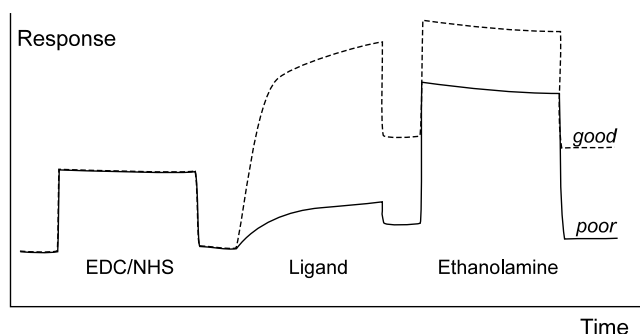
Figure 7-2. *Inadequate binding of ligand to the surface is seen as a poor increase in response during and after the ligand injection (the illustration shows a sensorgram for amine coupling).*

If ligand is pre-concentrated on the surface but is not immobilized (Figure 7-3):

Make sure you are using fresh EDC and NHS solutions.

Make sure that the immobilization buffer and running buffer do not contain substances that compete with the ligand for reactive groups on the surface (e.g. Tris or sodium azide for amine coupling).

Consider an alternative immobilization chemistry.
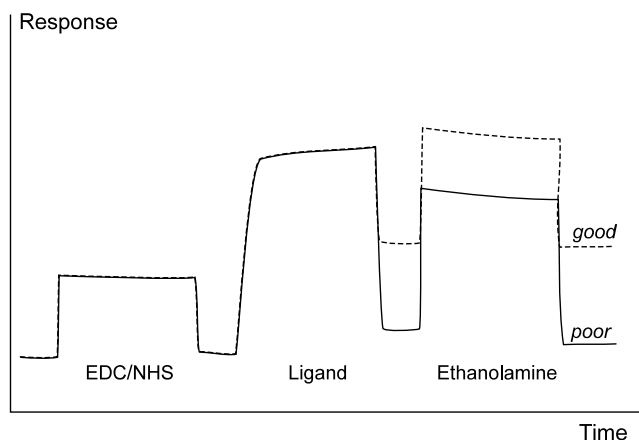
Figure 7-3. *Inadequate immobilization of ligand is seen as an increase in response during ligand injection but a low level over baseline after the injection.*

7.2.2   Low analyte responses

If the amount of ligand immobilized appears to be sufficient but the analyte response or maximum binding capacity is too low, the ligand may have lost activity either during ligand preparation or by the immobilization procedure. Check analyte binding activity of the ligand preparation by independent techniques if possible. If it seems that the ligand loses activity during immobilization, try alternative immobilization methods or use a capturing approach. Review the composition of the immobilization buffer (e.g. avoid chelating agents for proteins that require metal ions for activity).

If both the analyte and ligand are large molecules and the amount of ligand on the surface is high, steric hindrance may reduce the maximum binding capacity of the surface.

7.2.3 When regeneration fails

It is not always possible to find satisfactory regeneration conditions for a given interaction. It might be expected that systems involving heterogeneous ligand or analyte could require regeneration by sequential exposure to different solutions (e.g. acidic conditions followed by high ionic strength).

For particularly difficult systems, two alternative approaches may be available:

Reverse the roles of ligand and analyte. Regeneration is only required to preserve the activity of the ligand attached to the surface: damage to the analyte does not matter.

Use a capturing approach instead of attaching the ligand directly to the sensor surface. Regeneration is then directed to removing the ligand from the capturing molecule, and any damage to the ligand does not matter.

7.2.4 Problems with non-specific binding

For some applications, particularly those where samples are complex mixtures such as serum or crude cell extract, responses arising from other sources than binding of analyte to ligand can complicate the interpretation of the results. Such responses may arise from binding of non-analyte molecules in the sample to the ligand or from non-specific binding of analyte or non-analyte molecules to the sensor chip surface. The contribution of this kind of binding to the observed response varies with the kind of ligand and sample used, but also with the amount of ligand on the sensor surface. Moderate to high levels of immobilized ligand have been observed to suppress non-specific binding of sample components to the sensor surface, but can incur higher levels of unwanted binding to the ligand.

Problems with non-specific binding can in general be addressed in three ways: design of the experiment, choice of sensor surface and the use of additives in the sample.

Experimental design
Apart from the obvious approach of partially purifying the sample to remove components that interfere with the assay, non-specific binding may in some cases be reduced by optimizing the composition of the running buffer. In general, physiological (150 mM) or higher salt concentrations will help to suppress non-specific electrostatic interactions, and inclusion of salt in the running buffer is always recommended. Other buffer components may have a significant effect on non-specific binding in individual cases.

A powerful technique for dealing with non-specific binding in applications that measure binding levels (as opposed to kinetic interaction profiles) is to use enhancement reagents to specifically amplify the signal from the analyte. An enhancement reagent binds to the analyte independently of the ligand, so that the enhancement response is a direct indication of the amount of analyte on the surface. In this way the analyte is detected and identified with a double specificity, once by interaction with the immobilized ligand and once by interaction with the injected enhancement reagent.

Choice of sensor surface

Different sensor chip types have different characteristics with respect to non-specific binding. It is difficult to provide general recommendations in this respect, since the effects vary according to the nature of the sample: for example, Sensor Chip CM4 tends to show lower non-specific binding than Sensor Chip CM5 with cell culture medium and crude cell extracts, but the reverse is often true with serum and plasma. As a general guideline, if non-specific binding is a problem, it can be worth testing the application on a different sensor chip type.

Similarly, the ligand immobilization chemistry can influence the level of non-specific binding in an unpredictable manner. When the ligand is amenable to alternative immobilization chemistries, testing a range of different immobilization methods can help to identify conditions that reduce non-specific binding.

In some applications using serum samples, immobilization of aminomethyl-polyethyleneglycol (aminomethyl-PEG) to the surface prior to ligand immobilization has been found to reduce the levels of non-specific binding. To use this approach, activate the surface with a 10-minute injection of 0.05 M EDC/0.2 M NHS. Follow this activation with an injection of 1–5 mM aminomethyl-PEG in 10 mM sodium borate pH 8.5 for 5–10 minutes. Immobilize the ligand using the chosen chemistry directly after the aminomethyl-PEG injection.

The immobilization capacity of the surface for ligand is reduced after immobilization of aminomethyl-PEG, and a balance has to be struck between the level of aminomethyl-PEG substitution and the remaining capacity of the surface. Acceptable results have been observed with levels of aminomethyl-PEG around 500 RU: higher levels can reduce non-specific binding further but at the expense of a reduction in ligand immobilization capacity.

Where alternative ligands are available (for example with monoclonal antibody ligands), the choice of ligand may have a decisive effect on the extent of non-specific binding. In many cases, immobilization of Fab'$_2$ fragments can be preferable to the use of intact antibodies, eliminating binding of sample components to the Fc portion of the antibody.

Sample additives

Soluble carboxymethyl-dextran added to the sample at 0.5–1 mg/ml can compete for molecules that bind to the dextran on the sensor surface without interfering with the analyte-ligand interaction.

In some cases, addition of "ligand mimics" can help to reduce unwanted binding to the immobilized ligand. An example of this approach is the addition of polyclonal antibody preparations (at 100–200 µg/ml) from non-immune animals to counteract non-specific binding to immobilized monoclonal antibodies.

8. Storing and re-using sensor chips

Sensor chips are supplied in individually sealed packages in a nitrogen atmosphere. Unopened packages have a shelf life of at least 3 months at 4°C (each package is marked with a "Use before" date). Sensor chips should be used as soon as possible after opening the package.

With ligand immobilized on the sensor chip surface, the sensor chip should preferably be used within a few days, and is best kept docked in the instrument under conditions of standby flow. Sensor chips with immobilized ligand can under some circumstances be stored outside the instrument and re-used at a later date. This chapter describes the procedures for storage of used chips.

> *Important note*
> Storage of used chips outside the instrument is not recommended for systems with multiple detection spots in each flow cell (e.g. Biacore S51). Precisely reproducible positioning of the sensor chip in relation to the IFC cannot be guaranteed, so that previously immobilized spots on the surface may not line up exactly with the detection spots when the chip is re-docked.

Storage lifetime will depend on the nature of the attached ligand, but for many ligands the prepared chip can be stored wet or dry for periods of several weeks with only small losses in binding activity. This section describes storage procedures for prepared sensor chips, based on tests performed on Sensor Chip CM5 prepared with a range of different proteins.

For most ligands, wet storage is preferable to dry.

8.1 Recommended procedures – wet storage

Materials:
HBS-EP buffer (Biacore)
A 50 ml polypropylene test tube

Storage
1. Undock the sensor chip and take it out of the instrument.

2. Take the sensor chip support out of the sheath with a pair of tweezers. Avoid touching the support with your fingers.

3. Place the support in a 50 ml tube containing buffer so that the support is completely covered, cap the tube securely and place it in a refrigerator.

Re-use
1. Take the support out of the tube using a pair of tweezers. Avoid touching it with your fingers.

2. Rinse the support with distilled water, and shake it gently to remove most of the water.

3. Wipe the support and the glass side of the sensor chip dry with a lint-free tissue or dry with pressurized oil-free air or nitrogen. Do not wipe the sensor surface itself. The glass side is flush with the support: the sensor surface is on the side that is recessed into the support.

4. Re-insert the support into the sheath. The glass side should be towards the label on the cassette, and the white tab on the support should be at the outer end (Figure 8-1).

5. Insert the sensor chip in the instrument and dock it.

8.2   Recommended procedures – dry storage

Materials
Blue indicating silica gel
A 50 ml polypropylene test tube

Storage
1. Undock the sensor chip and take it out of the instrument.

2. Take the sensor chip support out of the sheath with a pair of tweezers. Avoid touching the support with your fingers.

3. Shake excess liquid from the sensor chip or dry with oil-free pressurized air or nitrogen.

4. Place the support in a test tube containing about 5 g blue indicating silica gel, cap the tube securely and place it in a refrigerator.

Re-use
1. Allow the sensor chip to equilibrate to room temperature before opening the test tube. Take the support out of the tube using a pair of tweezers.

2. Re-insert the support into the cassette. The glass side of the chip (the side that is flush with the support) should be towards the label on the cassette, and the white tab on the support should be at the outer end (Figure 8-1).

3. Insert the sensor chip in the instrument and dock it.
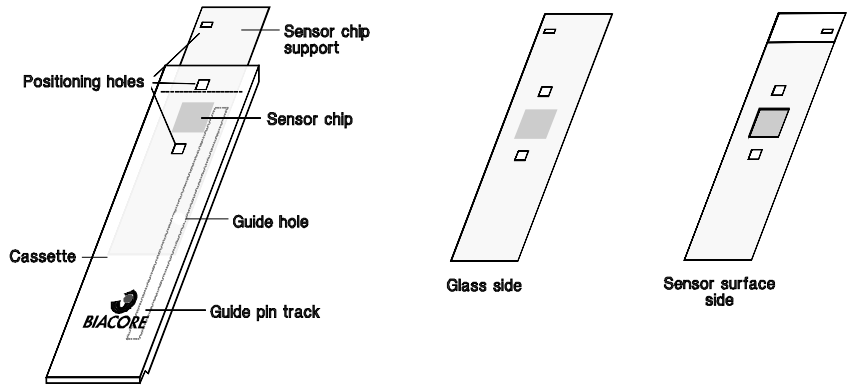
Figure 8-1. *Sensor chip cassette and support, showing the orientation of the chip support in the cassette.*

A. Immobilizing small molecules

Small molecules (typically organic compounds such as hormones, effectors, drug candidates etc, with molecular weight less than about 500 daltons) present some special problems with respect to immobilization on the sensor chip surface:

> The number and kind of functional groups available for coupling to the sensor surface is usually limited.

> There is a significant risk that immobilization will interfere with the binding site for the analyte, either sterically or through alteration of the chemical properties of the molecule.

> Electrostatic pre-concentration is generally not useful for enhancing the efficiency of immobilization. Coupling is instead performed at relatively high ligand concentrations (typically 1–50 mM) at pH 7–8.5.

> The solubility of many organic compounds is limited in aqueous buffers, so that it may be difficult to achieve sufficiently high concentrations in coupling buffer. Solutions for immobilization are often best prepared by dissolving the ligand in a water-miscible solvent (e.g. dimethyl sulfoxide DMSO or dimethyl formamide DMF) and diluting the solution in immobilization buffer. Amine coupling has been performed successfully in the presence of up to 50% DMSO, DMF or acetonitrile. Check the respective Instrument Handbook for compatibility of the Biacore flow system with organic solvents.

> The amount of ligand immobilized cannot usually be accurately assessed from the response levels since small molecules give inherently low responses.

This appendix provides some examples of small molecule immobilization procedures. Except in cases where the native molecule contains a functional group amenable to standard coupling chemistry as described in Chapter 4, organic chemical expertise will generally be required for modification of the ligand, or for devising alternative coupling chemistries specific to the individual ligands.

Some of the conditions required for small molecule immobilization (in particular the use of organic solvents to maintain solubility) are not compatible with the IFC and flow system in Biacore instruments, and immobilization must be performed outside the instrument, either by pipetting solutions directly on to the sensor chip or in the special Surface Prep unit that is supplied with some Biacore systems. Immobilization outside the instrument does not provide any monitoring of the immobilization process, and the success or failure of immobilization can only be determined by docking the chip in the instrument and testing analyte binding capacity.

In many cases where the interaction being studied involves a small molecule and a macromolecular partner, immobilization of the macromolecule binding partner as ligand and measurement of interaction with the small molecule either by direct binding assays (despite the low response levels) or surface competition assays may be a more workable alternative to immobilizing the small molecule.

A.1   Direct immobilization

Many small molecules carry a functional group that can be used to immobilize the molecule to the sensor surface using the methods described in Chapter 4. Amine, thiol and aldehyde groups can be exploited directly: carboxyl and hydroxyl groups can be used after modification to introduce for example an amine or thiol function.

Two examples of direct immobilization using amine coupling are given below. Concentrations and contact times may vary for other compounds.

Amine coupling – ampicillin
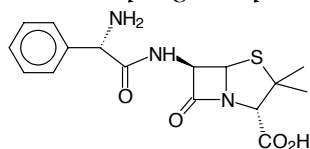
Ampicillin

Ampicillin contains a native amine group that is sufficiently reactive to allow direct immobilization by amine coupling. The ligand is dissolved at 10 mM in 50 mM sodium borate pH 8.5 (coupling is efficient at high pH, and there is no electrostatic pre-concentration effect to motivate the use of low pH buffers).

Sensor Chip	CM5	
Location	In Biacore instrument	
Activation	EDC/NHS	7 min
Coupling	10 mM ampicillin in 50 mM sodium borate pH 8.5	7 min
Deactivation	1 M ethanolamine HCl pH 8.5	1 min

Amine coupling – sulfamethazine
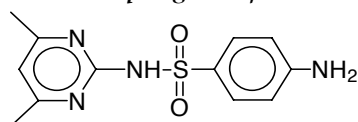
Sulfamethazine

The benzylamine group in sulfamethazine has relatively low reactivity and the compound is only sparingly soluble in aqueous buffers. Successful immobilization has been achieved with 7 mM sulfamethazine in 10 mM HCl pH 3.0 containing 10% DMF[1]. Immobilization was performed outside the instrument.

Sensor Chip	CM5	
Location	Outside instrument	
Activation	EDC/NHS	18 min
Coupling	7 mM sulfamethazine in 10 mM HCl, 10% DMF, pH 3.0	3 h
Deactivation	1 M ethanolamine HCl pH 8.5	18 min

A.2 Modifying the sensor surface

Modification of the sensor surface to introduce new functional groups extends the range of chemistries that can be used for immobilization. Introduction of thiol or reactive disulfide groups is described in Section 4.5. Another alternative is to immobilize a diamine compound using amine coupling to create a surface carrying free amine groups. This allows for example immobilization via carboxyl groups on the ligand, by an "inverted" amine coupling procedure where the ligand is activated with EDC/NHS and reacts with amine groups on the surface. Suggested conditions for creating an amine surface by immobilization of ethylenediamine is described below. Note that the amine surface is not stable and should be used directly after preparation.
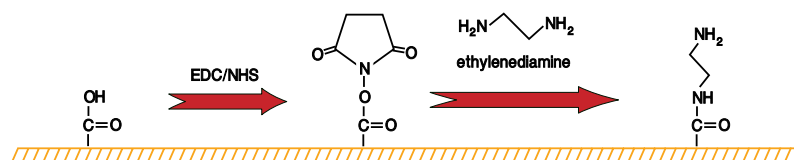
Preparing an amine surface by immobilizing ethylenediamine.

---

[1] Sternesjö, Å., Mellgren, C. and Björck, L. (1995) *Analyt. Biochem.* **226**, 175-181.

Sensor Chip	CM5	
Location	In Biacore instrument	
Activation	EDC/NHS	7 min
Coupling	0.1 M ethylenediamine in 50 mM sodium borate pH 8.5	7 min
Deactivation	1 M ethanolamine HCl pH 8.5	3 min

Other functional groups can be introduced using similar principles.

A.3   Ligand activation

In some cases, the ligand may need to be activated to enable immobilization. The example below illustrates activation of hydroxyl groups for immobilization on an amine surface (prepared as described above).

Ligand activation – trenbolone
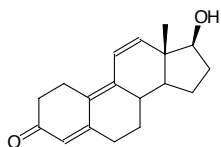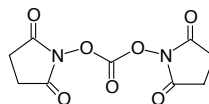
Trenbolone   Disuccinimidyl carbonate

Modification of the hydroxyl group on trenbolone with disuccinimidyl carbonate (DSC) introduces a succinimidyl group on to the molecule which can react with amine groups on an amine-modified sensor chip surface. Modification of trenbolone is performed by incubation with DSC for 30 minutes in pyridine containing 4-dimethylaminopyridine. The product is diluted into buffer at pH 7 before injection over the amine surface.

Sensor Chip	CM5	
Location	Outside instrument	
Activation of trenbolone	10 mM trenbolone, 40 mM DSC and 40 mM dimethylaminopyridine in pyridine	30 min
Coupling	1:1 activated trenbolone:50 mM sodium borate pH 7.0	7 min

A.4 Ligand modification

Ligands which do not contain a native group amenable to immobilization can be modified to introduce such a group. While ligand modification may require expertise in organic chemical synthesis, this approach has some inherent advantages:

The functional group used for immobilization can be chosen to suit a selected immobilization chemistry. Side reactions with other functional groups on the ligand can in this way be reduced or eliminated.

A spacer of chosen length can be introduced between the ligand and immobilization site, which can often help to reduce steric interference in the interaction with the analyte.

The position of the ligand modification can be chosen to minimize adverse effects on the interaction with analyte.

An example of modification to introduce amine groups is given below. Procedural details may be found in the literature[1].

Ligand modification – biotin
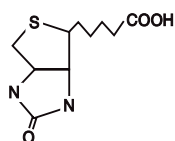
Biotin   Jeffamine

A primary amine can be introduced on to the carboxyl group of biotin by activation with EDC/NHS followed by reaction with a diamine. In this example jeffamine (1,8-diamino-3,6-dioxaoctane, JA) was used as the diamine rather than a simple aliphatic diamine, to increase solubility of the resulting derivative. The product can be immobilized using standard amine coupling procedures.

---

[1] Wilbur, D. S., Pathare, P. M, Hamlin, D. K. and Weerawarna, S. A (1997) *Bioconjugate Chem*. **8**, 819.832.

Biacore Sensor Surface Handbook
version AA

Index

Biacore Sensor Surface Handbook
version AA

BIACORE

BR-1005-71