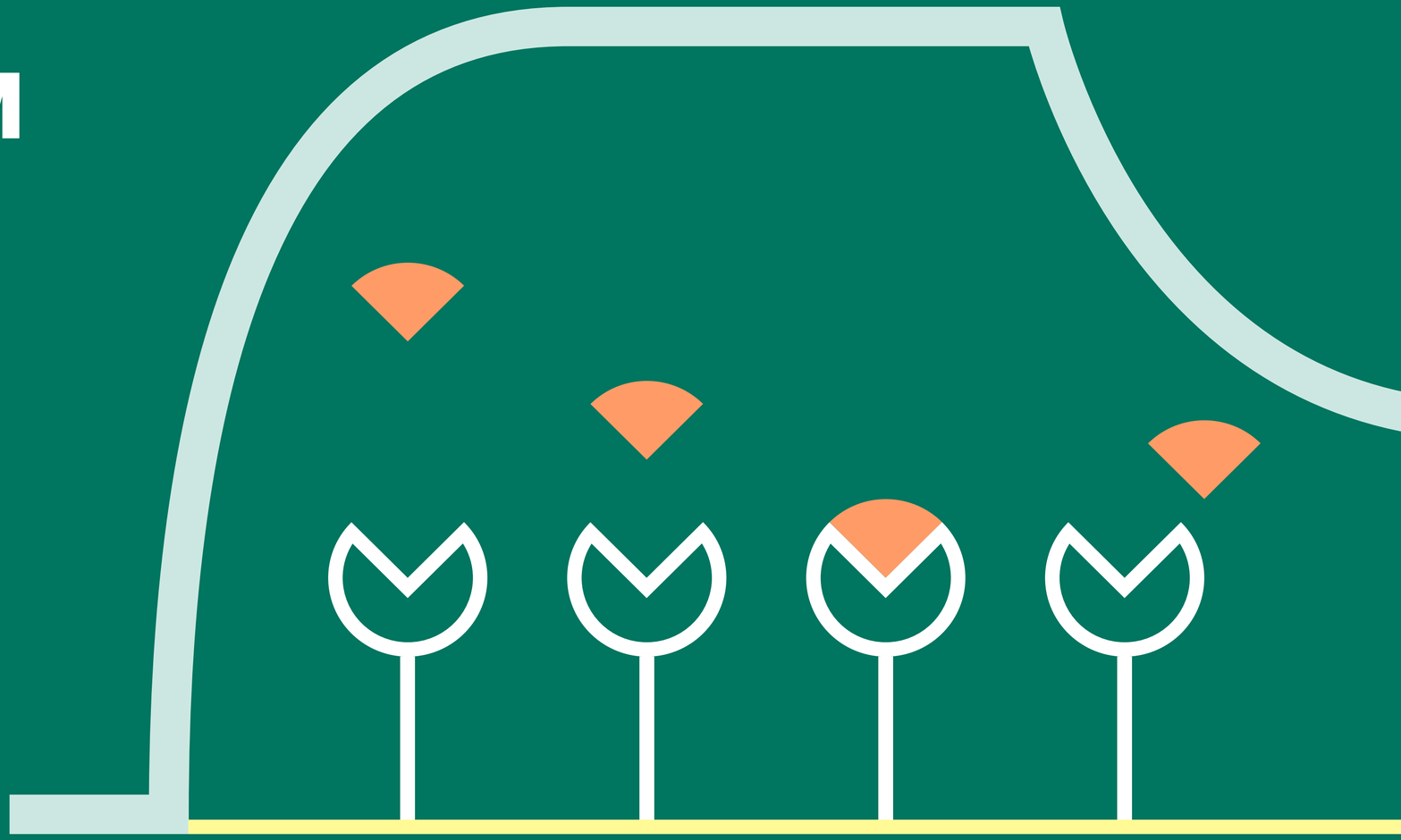
Handbook

Biacore™ sensor surface



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01
Introduction

1.1 Principles of Biacore™ SPR systems

Biacore™ systems allow you to monitor the interaction between molecules in real time using the phenomenon of surface plasmon resonance (SPR). This involves attaching one interacting partner to the surface of a sensor chip, and then passing a sample containing the second interaction partner over the surface. Binding of molecules to the sensor surface generates a response which is proportional to the bound mass. These changes 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. You can follow the binding events in real time and a range of interaction characteristics can be determined. Some of the questions that can be answered using Biacore™ systems include:

- Investigating the specificity of an interaction by testing the extent of binding between different pairs of molecules.
- Investigating the kinetics and affinity of an interaction by analyzing the binding behavior in terms of molecular interaction models.
- Investigating the concentration of specific molecules present in a sample by measuring the response obtained from the sample.
- Investigating the similarity between samples, for example, different batches of a biotherapeutic drug, by comparing curve shapes between batches.

1.2 Biacore™ SPR terminology

Biacore™ systems monitor the interaction between two molecules, one of which is attached to the sensor surface and the other is free in solution. The following terms are used in the context of work with Biacore™ systems (Fig 1.1):

- The partner attached to the surface is called the **ligand**. Attachment may be covalent or through high affinity binding to another molecule which is in turn covalently attached to the surface. In the latter case the molecule attached to the surface is referred to as the **capturing molecule.**

Note: *The term ligand used here is analogous with the terminology used in affinity chromatography contexts but does not imply that the surface-attached molecule is a ligand for a cellular receptor.*

- The **analyte** is the interacting partner that you wish to study, which is injected in solution over the sensor surface. Commonly, the analyte binds directly to the ligand. Assays can also be set up to assess analyte properties from the effect of binding of another molecule as in an inhibition assay format. Please refer to Biacore™ application guides available at cytiva.com/biacore for more information.

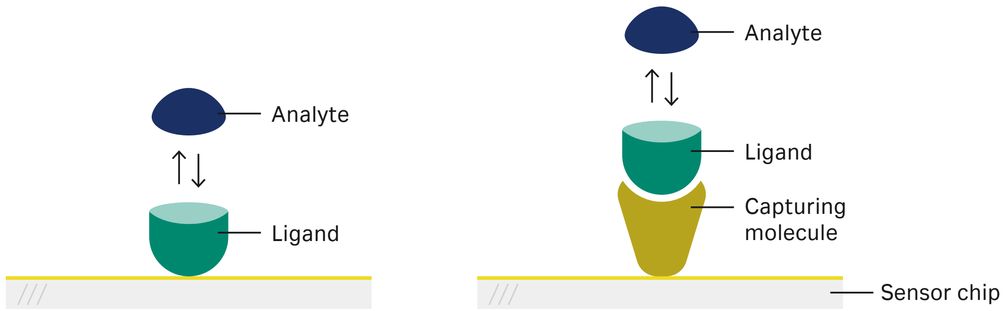

Fig 1.1. Ligand, analyte, and capturing molecule in relation to the sensor surface.

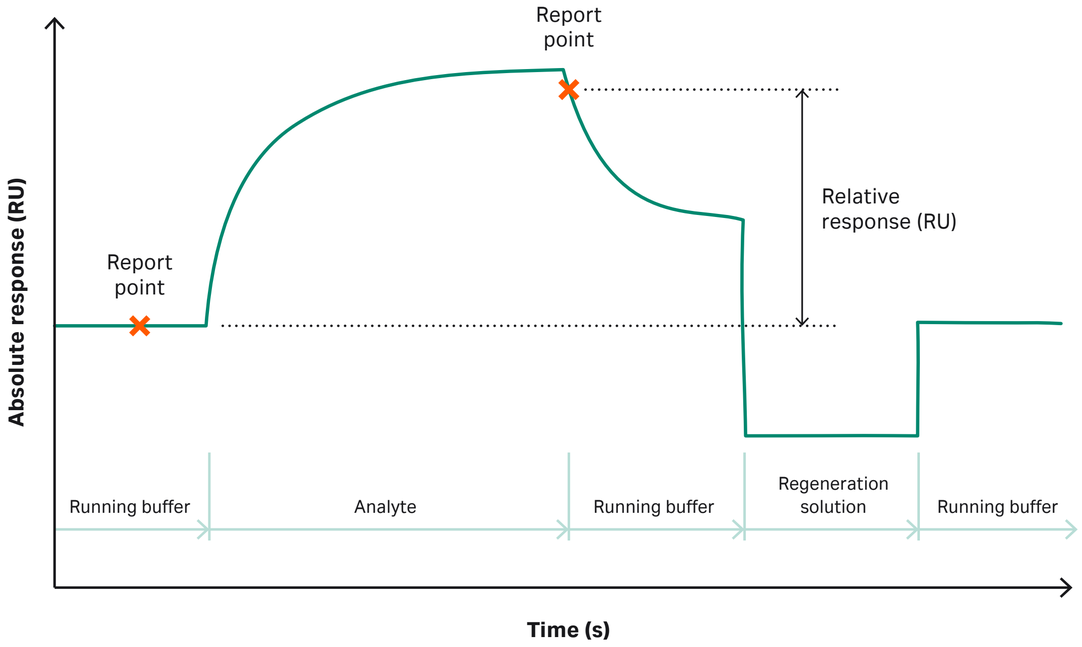

Fig 1.2. Schematic illustration of a sensorgram. The bars below the sensorgram curve indicate the solutions that pass over the sensor surface.

- **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 in RU against time (Fig 1.2), showing the progress of the interaction. This curve is displayed directly on the computer screen during an analysis. Sensorgrams may be analyzed to provide you with information on the rates of the interaction.
- In many assay situations, sample passes over two or more flow cells in series. The first flow cell in the series is usually blank and serves as a reference, while ligand is attached in the other flow cell(s). Surfaces with ligand are referred to as **active**. Blank surfaces used for reference purposes are referred to as **reference.**
- A particular sensorgram is referred to as a **curve** in several contexts in the software. This terminology is used to distinguish between different classes of sensorgram that recur within a run. For example, measurements on one active and one reference surface can generate separate curves for each of the two flow cells and a third **reference-subtracted curve** (active minus reference).
- 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 of your choice.

1.3 Components of Biacore™ SPR 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 indices. In Biacore™ SPR systems the conducting film is a thin layer of gold on the sensor chip surface, while the media are the glass of the sensor chip and the sample solution.

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 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 150 nm in Biacore™ SPR systems.

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 (Fig 1.3).

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 an SPR response. Only refractive index changes within the effective penetration depth of the evanescent wave (about 150 nm from 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. Light from a light-emitting diode (LED) is focused in a wedge onto the sensor surface, covering a fixed range of incident and reflected angles.

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™ SPR system sensor chips because it combines favorable SPR characteristics with stability and a high level of inertness in biomolecular interaction contexts.

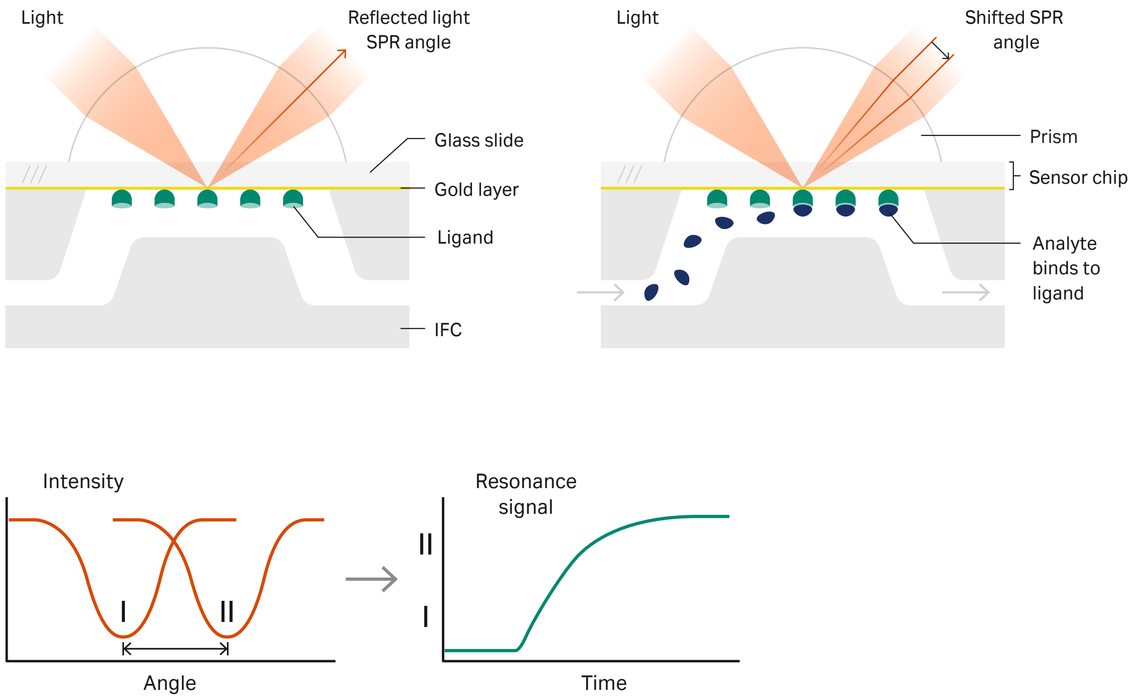

Fig 1.3. The SPR detection principle.

1.3.2 The sensor surface

The sensor chip is at the heart of Biacore™ SPR systems providing the physical conditions necessary to generate the SPR signal. The interaction that you want to study occurs on the surface of the sensor chip (Fig 1.4).

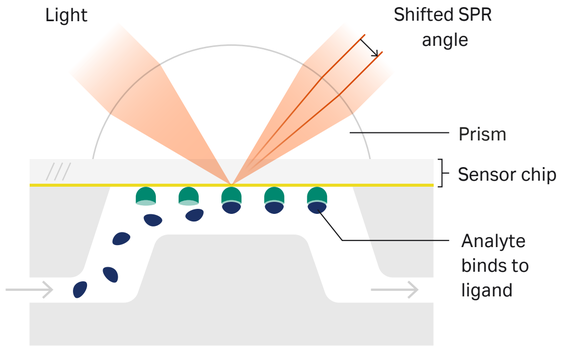

Fig 1.4. Illustration of a solution containing the analyte flowing over the sensor chip and interacting with the ligand.

The specificity of a Biacore™ analysis is determined through the nature and properties of the molecule attached to the sensor surface. You may attach biomolecules to the sensor chip surface using three different approaches (Fig 1.5):

- Covalent immobilization, where the molecule is attached to the surface through a covalent bond linkage.
- High affinity capture, where the molecule of interest is attached by noncovalent interaction with another molecule (which in turn is usually attached using covalent immobilization).
- Hydrophobic adsorption, which exploits 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.

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

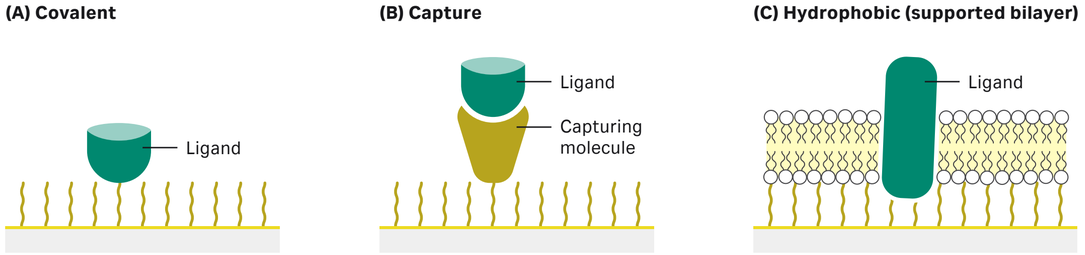

Fig 1.5. Three approaches for attaching biomolecules to the sensor chip surface.

1.3.3 The flow system

During your analysis, a continuous nonturbulent flow of liquid is maintained over the sensor surface at a controlled flow rate, through the integrated microfluidic cartridge (IFC). The sensor chip is docked against the IFC to form a flow cell, and the flow is switched between buffer, sample, and reagent as required by a set of valves in the IFC (Fig 1.6). Switching valve positions is performed with high precision so that the transition time between different liquids is minimized.

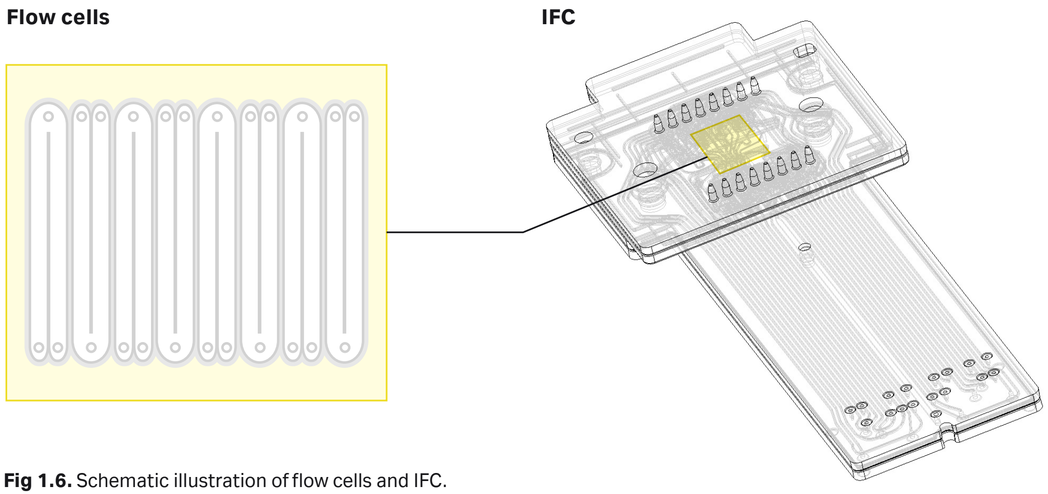

Fig 1.6. Schematic illustration of flow cells and IFC.

1.4 Scope of this handbook

This handbook provides you with a general guide to the design and use of sensor surfaces for Biacore™ system 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 reuse

Aspects of sensor chip usage that are specific to individual instruments are described in application guides and user manuals of the respective instruments.

1.5 Additional information

More information about Biacore™ systems, sensor chips, and applications may be found at cytiva.com/biacore.

02

Sensor chip overview

2.1 The sensor surface

The sensor chips used in Biacore™ systems have two essential features:

- A glass surface covered with a thin gold layer, creating the physical conditions required for the surface plasmon resonance (SPR) signal. This feature is common to all Biacore™ sensor chips.
- A coating on the gold layer which provides the means for attaching molecules and an environment where the interaction being studied will occur. The coating varies between different types of chip. Uncoated chips are also available for users wishing to prepare their own customized surfaces.

The sensor chip itself is mounted on a plastic support to facilitate handling. The support is in turn held in a protective plastic sheath (Fig 2.1). Two different sheath and support formats are used in the current instrument platforms, but the properties of the sensor surface itself are unchanged.

The gold layer and surface coating on the sensor surface are stable under a wide range of conditions, including pH and moderate concentrations of many organic solvents. Please refer to instructions for the specific sensor chip for more information. 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

SPR arises under conditions of total internal reflection at a thin film of a conducting material. The characteristics of SPR 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. To provide the correct conditions for SPR, the glass surface of the sensor chip is coated with a uniform, approximately 50 nm thick layer of gold. Gold gives a well-defined reflectance minimum at visible light wavelengths and is also suitable for attachment of surface layers.

Fig 2.1. Sensor chip and support construction (top: classic format, bottom: Series S format).

2.1.2 The surface coating

Unmodified gold is in general not a suitable surface environment for biomolecular interactions, so the gold on almost all Biacore™ sensor chips is covered with a covalently bonded monolayer of alkanethiol molecules. This serves to protect the samples from contact with the gold and at the same time allows for attachment of a surface matrix. Modification of the gold by attachment of the alkanethiol layer does not affect the SPR properties of the surface.

On most sensor chips, the surface is then covered with a matrix of carboxymethylated dextran, a flexible unbranched carbohydrate polymer forming a thin surface layer typically about 100 nm thick. The dextran matrix close to the surface is equivalent in concentration to an aqueous solution of about 2% dextran (1). It imparts several important properties to the sensor surface including:

- 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 surface without the matrix.
- It extends the region where interactions occur to encompass a surface layer with a thickness of the same order of magnitude as the penetration depth of the SPR detection.
- Dextran is a relatively inert molecule in the context of most biomolecular interactions and consists of flexible unbranched polymer chains that allows immoblized and captured 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 Sensor chips and reagent kits

In this section we give an overview of the different sensor chip types and reagent kits available from Cytiva. For more detailed information visit cytiva.com/biacore.

Table 1. Biacore sensor chips

Sensor chip purpose	Characteristics	Products
General	Sensor chips with carboxymethyl groups on the chip surface for ligand attachment.	Sensor Chip CM3, CM4, CM5, CM7, and C1
General	Sensor chip based on a polyethylene glycol (PEG) matrix with carboxyl groups on the chip surface for ligand attachment.	Sensor Chip PEG
Capture of tagged ligands	Sensor chips preimmobilized with capturing molecules or reagent kits containing reagents for coupling of capture molecules specific for tagged ligands.	Sensor Chip SA , NA , and NTA NTA Reagent Kit, Biotin CAPture Kit, GST Capture Kit, His Capture Kit
Antibody capture	Sensor chips preimmobilized with capturing molecules or reagent kits containing reagents for coupling of capture molecules specific for antibodies.	Sensor Chip Protein A, Protein G, Protein L, and PrismA Human Antibody Capture Kit, Mouse Antibody Capture Kit, Human Fab Capture Kit
Lipid attachment	Hydrophobic surfaces for attachment of lipid bilayers or liposomes.	Sensor Chip HPA and L1
Customizable	Unmodified gold layer for preparation of customized surfaces.*	Sensor Chip Au SIA Kit

*Customization of unmodified sensor chip surfaces is the responsibility of the user. Cytiva does not provide customization services or support for customized surfaces.

2.2.1 General purpose sensor chips

General purpose sensor chips (often referred to as CM-series sensor chips) carry carboxymethyl groups attached to the surface to allow covalent attachment of ligands using established chemistries (*see* Chapter 4). For most of these sensor chips, the carboxymethyl groups are attached to a dextran matrix. The dextran matrix is flexible, allowing relatively free movement of attached ligands within the surface layer.

Dextran-based general purpose sensor chips include:

- Sensor Chip CM5, a general-purpose sensor chip.
- Sensor Chip CM4 with a lower carboxymethylation level, use to reduce background binding.
- Sensor Chip CM3 with a shorter dextran matrix, for coupling of large molecules.
- Sensor Chip CM7 with a higher immobilization capacity, for analysis of small molecules.

General purpose sensor chips without dextran matrix

Sensor Chip C1 carries carboxymethyl groups attached to the alkanethiol layer and can be used for multivalent and very large analytes. Sensor Chip PEG is based on a polyethylene glycol (PEG) matrix instead of dextran. Both surfaces are flat 2D surfaces that can be used as alternatives in applications where the use of dextran is not applicable.

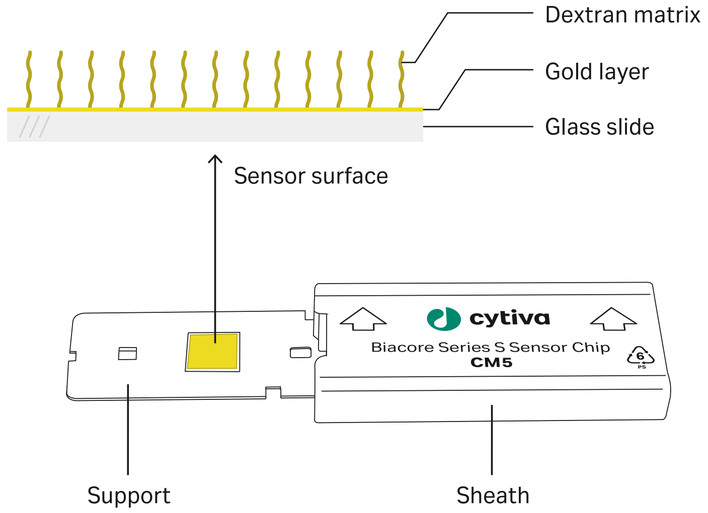

Fig 2.2. Schematic illustration of the structure of the sensor chip surface on CM-series chips.

2.2.2 Sensor chips and reagent kits for antibody capture

Sensor chips for antibody capture are preimmobilized with capturing molecules specific for different antibodies. Preimmobilized sensor chips for antibody capture include:

- Sensor Chip Protein A for capture of human antibodies.
- Sensor Chip Protein G for capture of antibodies from multiple species.
- Sensor Chip Protein L for capture of antibody fragments (kappa light chain).
- Sensor Chip PrismA for quantitation of human antibodies.

Reagent kits for preparing sensor surfaces for antibody capture are also available. Reagent kits for antibody capture include:

- Human Antibody Capture Kit for capture of human antibodies.
- Mouse Antibody Capture Kit for capture of mouse antibodies.
- Human Fab Capture Kit for capture of human Fab fragments (kappa and lambda light chain).

For more information visit cytiva.com/biacore.

2.2.3 Sensor chips for tagged ligand capture

Noncovalent capture provides a convenient way of attaching tagged ligands to the sensor surface and at the same time selecting the ligand from complex sample matrices such as cell extracts or partially purified material.

Preimmobilized sensor chips for capture of tagged ligands include:

- Sensor Chip SA with preimmobilized with streptavidin for capture of biotinylated ligands.
- Sensor Chip NA with preimmobilized with NeutrAvidin for capture of biotinylated ligands.
- Sensor Chip NTA for reversible capture of histidine-tagged (his-tagged) ligands through nickel ion (Ni^{2+}) chelation.

Reagent kits are available to support preparation of sensor surfaces for capture of tagged ligands. Reagent kits for tagged ligands include:

- Biotin CAPture Kit for reversible capture of biotinylated ligands.
- GST Capture Kit for capture of GST (glutathione S transferase) fusion proteins.
- His Capture Kit for capture of his-tagged ligands.

For more information, visit cytiva.com/biacore.

2.2.4 Sensor chips for lipid attachment

Lipids can be attached to the sensor chip by direct hydrophobic adsorption on Sensor Chip HPA, forming a lipid monolayer on the surface, or by attachment of liposomes to hydrophobic side chains on the dextran matrix of Sensor Chip L1.

Under suitable conditions, liposomes attached to Sensor Chip L1 may form a supported lipid bilayer.

2.2.5 Customizable sensor chips

Sensor Chip Au offers a plain gold surface mounted on the chip support for creation of customized surfaces. Unmounted gold surfaces are also available with a separate chip support in SIA Kit Au, for preparation of surfaces using procedures that the plastic chip support cannot withstand.

Spin-coating and other techniques for creation of self-assembled monolayers can be used in preparation of customized surfaces. For more information, refer to literature on surface coating and surface interaction studies as these techniques are not discussed further in this handbook.

2.3 Reference

1. Stenberg E, Persson B, Roos H, Urbanicksy C. Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. *J Coll Interf Sci.* May 1991;143(2):513-526. doi.org/10.1016/0021-9797(91)90284-F

03

Surface preparation strategies

Molecules can be attached to the sensor chip surface using several techniques, including covalent immobilization using a variety of chemical methods, high affinity binding to a specific capturing molecule, and adsorption of lipid mono- or bilayers.

This chapter provides 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 fixes the molecule permanently on the surface, often with a heterogeneous orientation. Functional groups on the ligands can be utilized for covalent coupling to a suitable sensor chip. The most common approach, and often the first method of choice, is amine coupling to Sensor Chip CM5 (Fig 3.1).

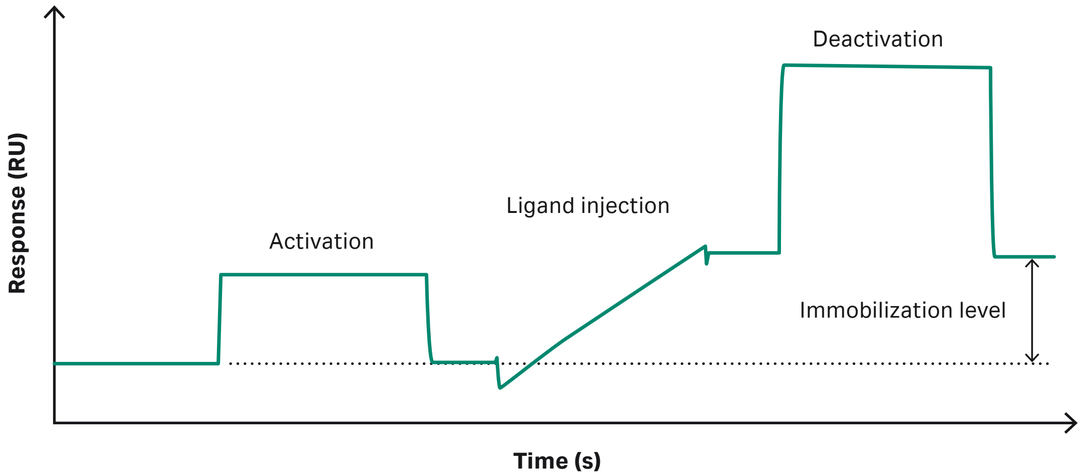

Fig 3.1. Covalently immobilized ligands are linked to the surface through different types of coupling chemistries. The figure shows a typical sensorgram for an amine coupling.

The carboxymethyl dextran on Sensor Chip CM5, other CM-series chips, and Sensor Chip L1 provide a foundation for a range of covalent immobilization chemistries. The most common covalent immobilization methods are:

- **Amine coupling** exploits 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** exploits thiol-disulfide exchange between thiol groups and active disulfides introduced on either the ligand or the surface matrix.
- **Maleimide coupling** is an alternative to thiol coupling when a surface is exposed to reducing agents or high pH.
- **Aldehyde coupling** uses the reaction between carbohydrazide groups introduced on the surface and aldehyde groups obtained by oxidation of carbohydrates in the ligand.

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 (Fig 3.2). 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 and/or at high pH.

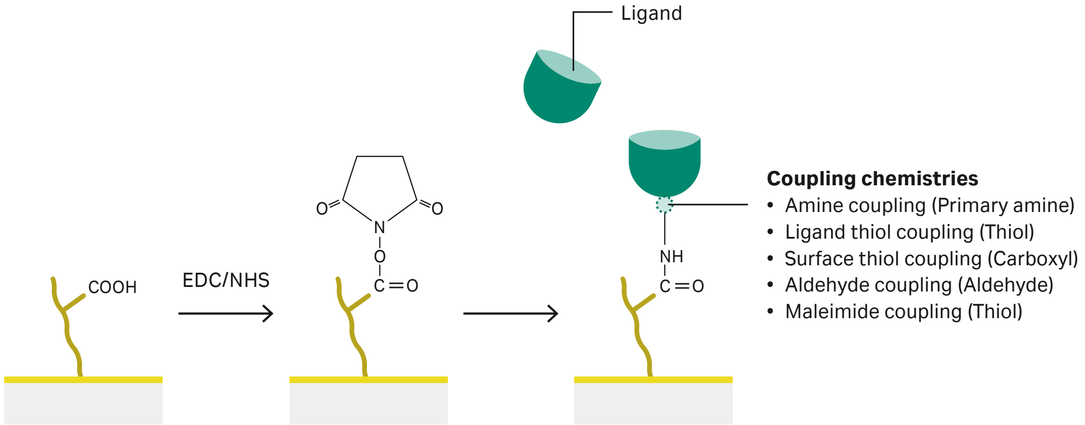

Fig 3.2. General purpose sensor chips (often referred to as CM-series sensor chips) carry carboxymethyl groups attached to the surface to allow covalent attachment of ligands using established chemistries**.**

Although covalent immobilization involves chemical modification of the ligand, most ligands can be immobilized using 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.

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 on 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. Capture approaches can provide an alternative if homogeneous orientation of the ligand is important for the experimental design.

The advantages of covalent direct attachment include:

- Less ligand consumption
- Higher binding capacity
- Very stable immobilization with no leakage
- Shorter cycle times

3.2 Capture approaches

Capture based attachment of ligand is another common approach and is used when the ligand is unstable or difficult to covalently couple or regenerate. Capture relies on binding of the ligand to a capturing molecule already attached to the sensor surface. Capture is usually reversible and ligand orientation specific. The capturing molecule is itself normally attached to the surface using covalent chemistry. The interaction between ligand and capturing molecule should have a high affinity (slow dissociation rate) so that ligand does not bleed off the surface significantly during the course of an analysis cycle. Normally, 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 (Fig 3.3).

Capture approaches may be preferable to covalent immobilization in several respects:

- The ligand is usually not modified in any way. It is captured under physiological conditions via a high-affinity interaction, either with an affinity tag or an antibody domain. The approach can be valuable for unstable ligands and/or 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.
- There is a lower requirement on ligand purity enabling selective capture of ligand from crude samples. Capturing provides a microscale affinity purification of the ligand, allowing immobilization of specific ligands from complex mixtures such as cell extracts or cell culture media.
- Capture 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.
- Capture is preferred for ligands that are difficult to immobilize or regenerate.
- Regeneration is preoptimized and thus there is no need to optimize regeneration for each individual ligand/analyte interaction.

On the other hand, capture approaches consume more ligand since fresh material is 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 capture interaction for applications like kinetic determination where dissociation of captured ligand during the analysis cycle complicates evaluation of the results.

Some common capture approaches (described in the following sections) are biotin-based capture, antibody-based capture, and capture of his-tagged proteins. Sensor chips and reagent kits for common capture approaches are available from Cytiva. Capture may in principle be applied to any ligand for which a suitable high affinity interactant is available.

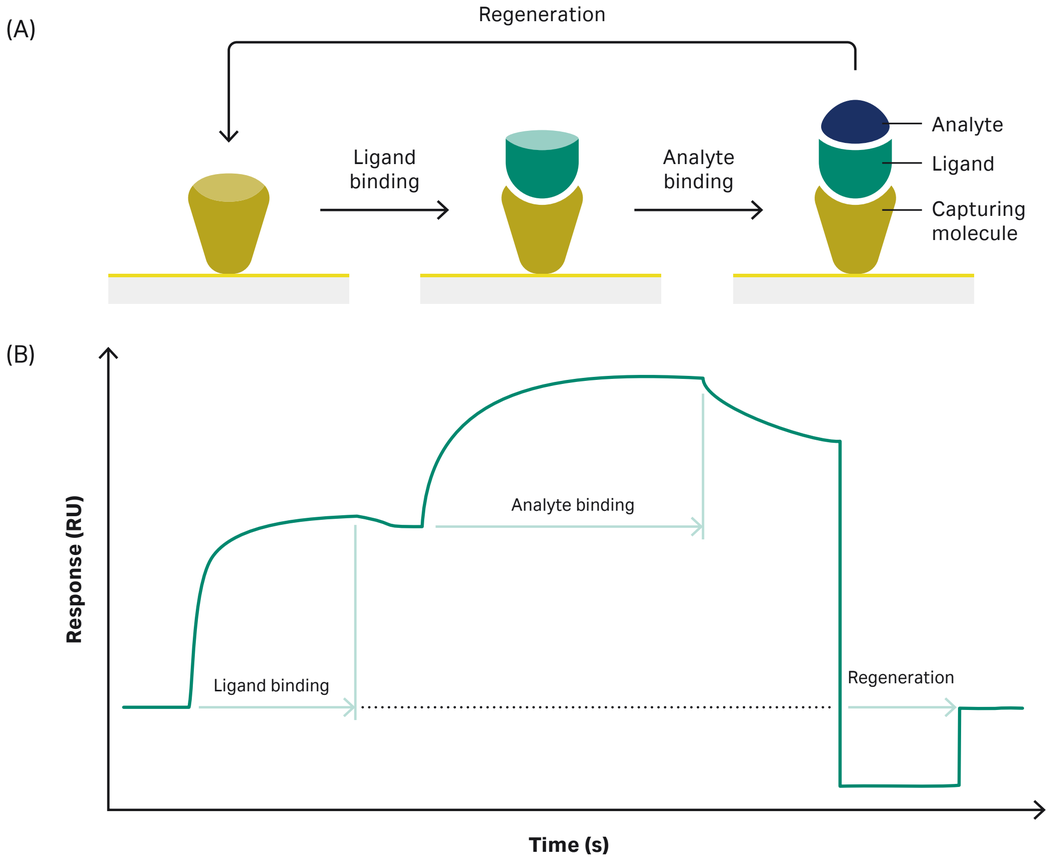

Fig 3.3. Typical analysis cycle using the capture approach, illustrated as molecules on the sensor chip surface (A) and as a schematic sensorgram (B).

3.2.1 Streptavidin-biotin capture

Sensor Chip SA

Sensor Chip SA provides a sensor surface with covalently attached streptavidin, designed for capture of biotinylated compounds (Fig 3.4). The affinity of streptavidin for biotin is extremely high, with an equilibrium dissociation constant of about 10^{-15} M dissociation of biotinylated ligands from the surface of Sensor Chip SA is generally negligible during 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.

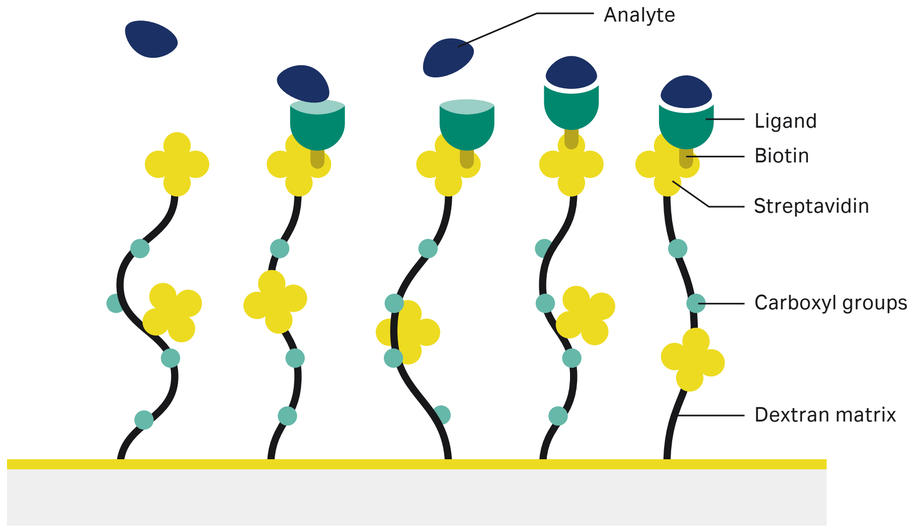

Fig 3.4. Biotinylated ligands can be captured by high affinity binding to streptavidin on the surface of the sensor chip.

Capture of biotinylated ligands on a streptavidin surface is a special case of the capture approach since the streptavidin-biotin interaction is so strong that capture 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 capture approaches.

3.2.2 NeutrAvidin-biotin capture

Sensor Chip NA

Sensor Chip NA consists of a carboxymethylated dextran matrix preimmobilized with NeutrAvidin for high-affinity capture of biotinylated ligands, such as peptides, proteins, and nucleic acids. Sensor Chip NA is an alternative to Sensor Chip SA. The affinity of the NeutrAvidin-biotin interaction is in the same range as for the streptavidin-biotin interaction.

3.2.3 Reversible capture of biotinylated ligands

Biotin CAPture Kit

Capture of biotinylated ligands on Sensor Chip CAP (included in Biotin CAPture Kit) uses hybridization of single-stranded (ss) DNA to attach streptavidin reversibly to the surface (Fig 3.5). Sensor Chip CAP carries covalently attached ss-DNA, while Biotin CAPture reagent consists of streptavidin attached to the ss-DNA containing the complementary nucleotide sequence. Hybridization of the two DNA strands attaches streptavidin to the surface, allowing capture of biotinylated ligands. Regeneration is performed by denaturing the double-stranded (ds) DNA using a mixture of guanidine hydrochloride and NaOH.

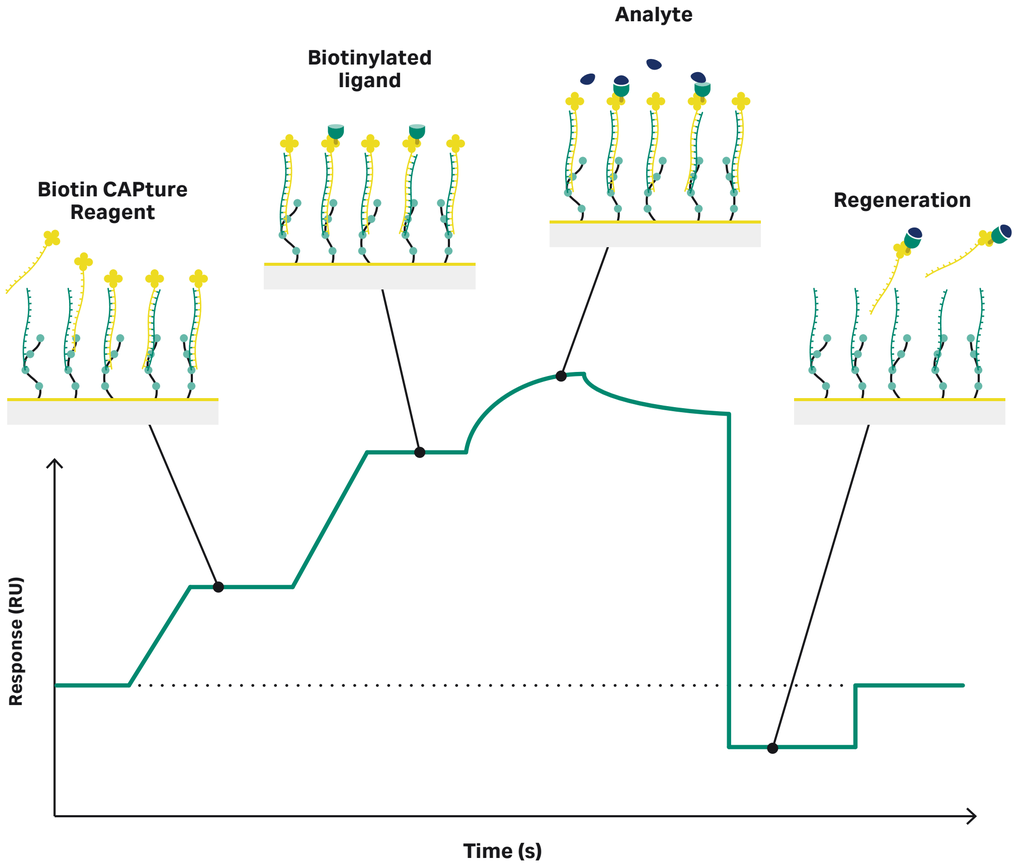

Fig 3.5. Illustration and sensorgram of reversible capture of biotinylated ligands using Biotin CAPture Kit. The surface is regenerated by denaturing the hybridized DNA, leaving ss-DNA on the surface.

Note: *This approach is not suitable for studies involving DNA-binding proteins or enzymes that degrade DNA.*

3.2.4 Antibody-based capture

Antibodies offer several advantages as capturing molecules. Monoclonal or polyclonal antibodies are widely used tools in biotechnology contexts, and specifically tailored high affinity antibodies are commercially available for many antigens. 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 antigen 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 (Fig 3.6).

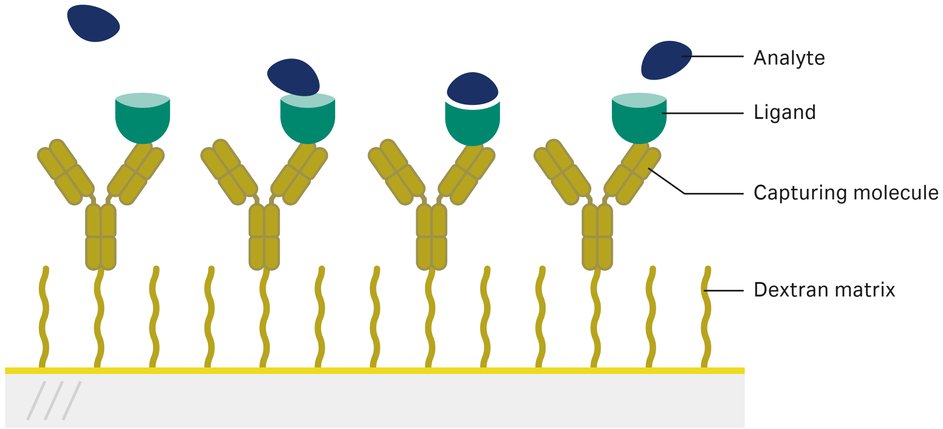

Fig 3.6. Antibody-based capture relies on the specific binding between an immobilized antibody and the antigenic epitope on the ligand.

3.2.5 Sensor chips and kits for antibody capture

If your ligand is an antibody-like molecule, Sensor Chip PrismA, Protein A, Protein L, Protein G, or an antibody capture kit can be used for antibody capture.

If your ligand is a human antibody or a molecule tagged with an Fc fusion protein, capture via the Fc region on the antibody to prefunctionalized Sensor Chip PrismA, Protein A, Protein G, or use of Human Antibody Capture Kit is recommended. Sensor Chip PrismA and Sensor Chip Protein A have high capacity and bind all human IgG subclasses except IgG3. Sensor Chip Protein G binds IgG_3 as well. Human Antibody Capture Kit can be used with various sensor chips such as Sensor Chip CM5 or Sensor Chip CM3, depending on assay requirements. The kit binds all human IgG subclasses.

You can also capture using the antigen-binding fragment (Fab) region. If your ligand is a human Fab-fragment, capture can be achieved via the Fab-region to prefunctionalized Sensor Chip Protein L or by using Human Fab Capture Kit. Sensor Chip Protein L binds to kappa light chains, specifically to human kappa I, III, and IV and mouse kappa I. Human Fab Capture Kit binds both human kappa and lambda chains. This kit is only recommended for use with Sensor Chip CM5.

If your ligand is a mouse antibody, use capture via the Fc-region to prefunctionalized Sensor Chip Protein A, Sensor Chip Protein G, or use Mouse Antibody Capture Kit. The kit can be used for all mouse antibody IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3), IgA, and IgM. It can also be combined with various sensor chips such as Sensor Chip CM5 or Sensor Chip CM3, depending on assay requirements.

3.2.6 Capturing tagged recombinant proteins

Recombinant proteins are often tagged for purposes of identification and affinity purification. The same principle can be exploited for capture of recombinant proteins on the sensor surface. 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 dual 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.

Polyhistidine 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 (Fig 3.7). His Capture Kit is an alternative to Sensor Chip NTA and contains an antibody specific to consecutive histidine regions for capture of his-tagged ligands.

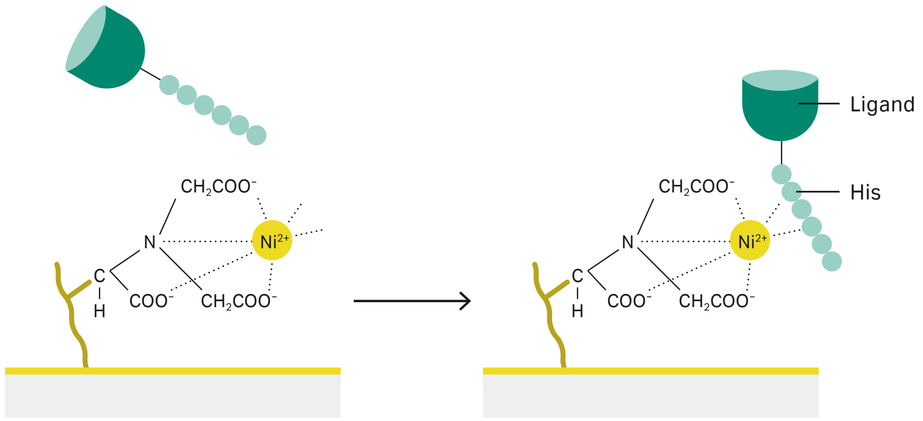

Fig 3.7. Nickel ions form a chelation bond between NTA immobilized on the surface and polyhistidine tags on the ligand.

Another commonly used recombinant tag is glutathione-S-transferase (GST), which can be captured on an anti-GST antibody surface. GST Capture Kit contains an anti-GST antibody for capture of GST-tagged ligands.

If you have a protein that has a different tag to those mentioned previously, such as FLAG and c-myc, covalent coupling of the respective capturing molecule to a suitable sensor chip is recommended.

3.3 Hydrophobic attachment

Hydrophobic attachment is used for work with lipid monolayers and bilayers or liposomes. 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 ligand to attach to the sensor chip surface.

3.3.1 Monolayer attachment

Sensor Chip HPA consists of a hydrophobic alkanethiol layer that covers the gold film, with no dextran layer or further modification of the sensor chip. Adsorption of lipids from micelles or liposomes on this surface creates a lipid monolayer with their 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 (Fig 3.8).

While this approach provides a stable environment for ligands associated with a membrane surface or partially inserted into the membrane, it is often 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 as discussed in the next section.

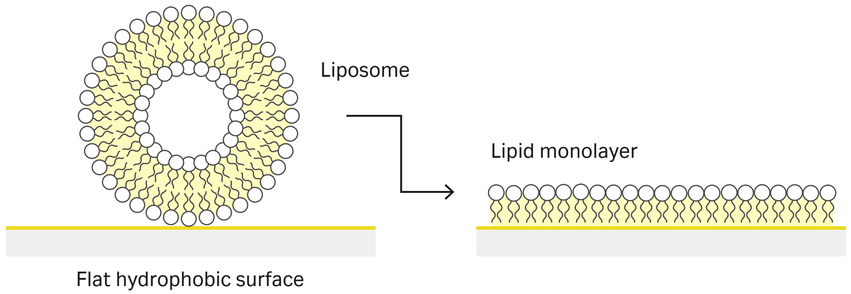

Fig 3.8. Adsorption of liposomes on Sensor Chip HPA results in the formation of a lipid monolayer covering the sensor chip surface.

3.3.2 Bilayer attachment

There are several approaches 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 via side chains that are exposed to the aqueous phase.
- Liposomes may be prepared with a specific antigenic component or with biotinylated lipids, allowing capture of the liposomes with immobilized antibody or streptavidin, respectively.
- Hydrophobic residues attached to the dextran matrix on Sensor Chip L1 can serve to attach membranes to the dextran matrix by insertion into liposomes (Fig 3.9).

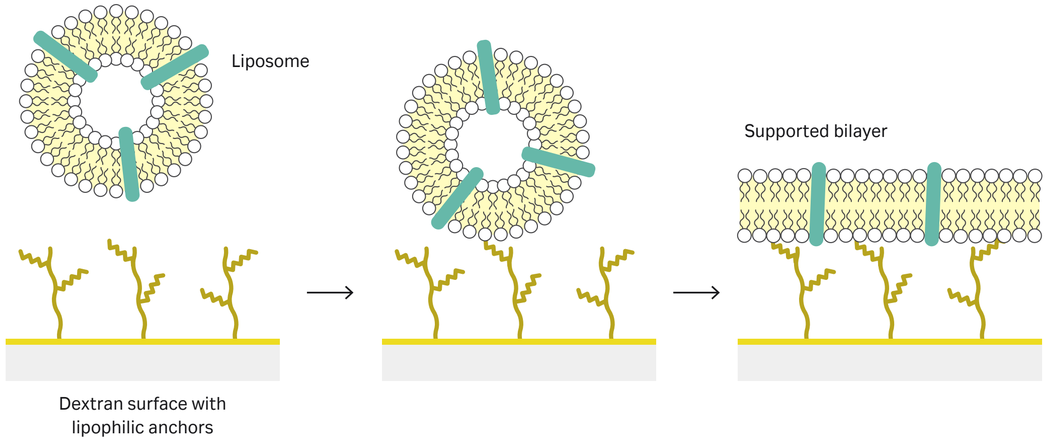

Fig 3.9. Liposomes attach to the hydrophobic anchors in the dextran matrix on Sensor Chip L1 and can fuse to form a supported lipid bilayer.

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, thiol, or carboxyl group may be present in the native molecule. In other molecules it may be necessary to introduce such a group through modification.
- The chemical procedure of immobilizing a small molecule involves a greater risk of interfering with binding to the interaction partner.
- 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.
- Many organic compounds have limited solubility. 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 10% DMSO, DMF, or acetonitrile.
- Electrostatic preconcentration (*see* Section 4.1.2) is generally not useful for small molecules. High concentrations (typically 5 to 10 mM), and for amine coupling basic pH, 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 has independent binding sites for the capturing molecule and the analyte. In some cases, it may 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.
- The amount of ligand immobilized cannot usually be accurately assessed from the immobilization levels since small molecules give inherently low responses.

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 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 and a high pH is avoided).

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. Short cross-linking treatment is usually sufficient (less than 30 s and in some cases as short as 5 s). The cross-linking step should be followed by an injection of ethanolamine to inactivate any remaining succinimide esters.

Noncovalently captured ligand will always dissociate from the surface to some degree during 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 or corrected by subtraction of an appropriate reference, since fresh ligand is captured for each analysis cycle (a dissociation rate constant of 1.5×10^{-4} s^{-1} results in loss of about 5% of the ligand during a 5 min 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 capture approach with immobilization of the captured ligand on the surface. Ligand that is captured and then cross-linked cannot however be removed by regeneration.

3.6 Strategies for surface preparation

3.6.1 How much ligand should be immobilized?

The amount of ligand to immobilize for any application depends on the relative molecular weights of the ligand and analyte, and on the sensitivity of the Biacore™ SPR system. Since the SPR response is directly proportional to the mass concentration of material at the surface, the theoretical analyte binding capacity of a given surface is related to the amount of ligand immobilized. The following expression can be used to calculate a suitable attachment (immobilization) level of captured or covalently coupled ligands.

$$R_{ligand} = \frac{R_{max}}{n} \times \frac{MW_{ligand}}{MW_{analyte}}$$

Where R_{ligand} is the ligand attachment level in RU, R_{max} is the theoretical maximum analyte binding capacity in RU, MW is the molecular weight in g/mol, and n is the interaction stoichiometry (analyte/ligand). Be aware that this estimation assumes that the ligand on the surface is 100% active. In practice, the activity is usually lower. As a result, the amount of attached ligand should be adjusted accordingly.

For example, if the ligand molecular weight is 100 000 g/mol and the analyte molecular weight is 50 000 g/mol, immobilizing 500 RU of ligand will give a theoretical analyte binding capacity of 250 RU assuming that the ligand is fully active. Note that this is the theoretical binding capacity, which can strictly be reached only at infinite analyte concentrations and infinite contact time and without crowding limitations.

In practice, the maximum observed response (the effective binding capacity) is affected by several factors including, the activity of the ligand, binding kinetics, available analyte concentration, and instrument limitations on the maximum analyte injection time. The theoretical binding capacity can however be useful as a guide to how much ligand to immobilize and 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. Because of the mass dependence of the SPR response, more ligands 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.

For concentration assays with macromolecular ligands and analytes, immobilize as much ligand as is reasonably possible. For protein ligands of average molecular size (MW on the order of 50 000 to 150 000), immobilization levels of 5 000 to 10 000 RU are typical. A high immobilization level will enable measurements at lower analyte concentrations. In addition, high levels of immobilized ligand ensure rapid binding of analyte which can be desirable for concentration measurement.

If the ligand is large and the analyte is small, responses will generally be low. Concentration assays for small molecules may be better designed as inhibition assays to overcome this limitation.

If the ligand is small and the analyte is large, it may be necessary to keep the amount of immobilized ligand very low to avoid steric crowding effects at high analyte concentrations. This is also true for assays that use capturing or enhancement reagents, where a multimolecular complex is built up on the sensor surface during 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. For the highest data quality, analyte R_{max} should preferably be 10 RU or lower.

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:

- Sensor Chip CM5 is the most common surface and works for a wide range of applications and molecules. 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 nonspecific binding of positively charged molecules may benefit from using Sensor Chip CM4, with a lower degree of carboxylation and thus a reduced net charge. With plasma or serum samples, however, Sensor Chip CM5 has been observed to give lower nonspecific binding than Sensor Chip CM4. Keep in mind that the immobilization capacity of Sensor Chip CM4 is lower than that of Sensor Chip 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.
- Sensor Chip CM7 is an alternative to Sensor Chip CM5 if there is a need for high capacity in LMW analysis.
- Sensor Chip C1 and Sensor Chip PEG are low-capacity sensor surfaces without dextran matrix that can be used as alternative surfaces when there is interference from the dextran matrix.
- Preimmobilized sensor chips and reagent kits are available for specific applications.
- 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 ligand attachment and capture 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. Relevant factors include the availability of suitable chemical groups on the ligand and the possibility that covalent attachment may inactivate analyte binding. Acidic proteins which are not efficiently preconcentrated in their native state (*see* Section 4.1.2) can be modified with PDEA for surface thiol coupling. This reaction raises the isoelectric point of the protein by modifying carboxyl groups on the protein.

Some biomolecules, notably carbohydrates and glycoconjugates, may be successfully immobilized using aldehyde chemistry after oxidation of *cis*-diols in the ligand to aldehydes. In some cases, the carbohydrate chains can provide a spacer for glycoprotein immobilization, improving the accessibility of protein sites.

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. Capture on Sensor Chip SA or Sensor Chip NA usually gives stable attachment, and regeneration is aimed at removing the bound analyte while leaving the ligand on the surface. Reversible capture using Biotin CAPture Kit provides an alternative that is amenable to regeneration of the capturing surface.

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 or on Sensor Chip NA after biotinylation.

Capture through streptavidin/NeutrAvidin-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 preconcentration, which is inefficient for nucleic acids.

Refer to the Biacore™ consumables selection tool for more information.

04

Covalent immobilization chemistry

This chapter gives a more detailed description of the conditions and methods used for covalent attachment of ligands or capturing molecules to the sensor surface. Corresponding aspects of hydrophobic adsorption are discussed 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 longer contact times for surface activation and ligand immobilization. Using higher temperatures may in some cases help to increase the immobilization level.

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 preconcentration

Immobilization is usually performed from reasonably dilute ligand solutions (10 to 50 µg/mL or less) and is achieved through electrostatic preconcentration 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 ligands on the surface. This mechanism works well for most proteins and for several other kinds of biomolecules.

The primary requirement for this electrostatic preconcentration on the surface is that the pH of the ligand solution should be between 3.5 and the isoelectric point of the ligand, so that the surface and the ligand carry opposite net charges (Fig 4.1). In addition, the electrostatic interactions involved in preconcentration are favored by low ionic strength in the coupling buffer. In general, covalent immobilization of proteins is best performed by diluting the ligand in 10 mM buffer at pH 4.0 to 5.5 (e.g., sodium acetate buffer). A range of ready-to-use buffers for protein immobilization are available from Cytiva. The extent of preconcentration 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 (*see* Section 4.1.5).

Some ligands (for example highly acidic proteins and nucleic acids) are negatively charged even at low pH values and cannot be efficiently preconcentrated on carboxymethyl dextran surfaces. Modification of the ligand can in some cases help to reduce the acidity, for example, introduction of PDEA for thiol coupling may raise the isoelectric point of proteins sufficiently to allow preconcentration (*see* Section 4.7). Another option is to use positively charged CTAB micelles as carriers to preconcentrate negatively charged ligands to the surface (*see* Section 4.6).

High affinity capture is an alternative immobilization approach that does not depend on electrostatic preconcentration (*see* Section 3.2). One example is attachment of biotinylated nucleic acids to streptavidin or NeutrAvidin. Capturing interactions can be exploited in buffers of physiological ionic strength or higher to reduce the effects of electrostatic repulsion of acidic ligands.

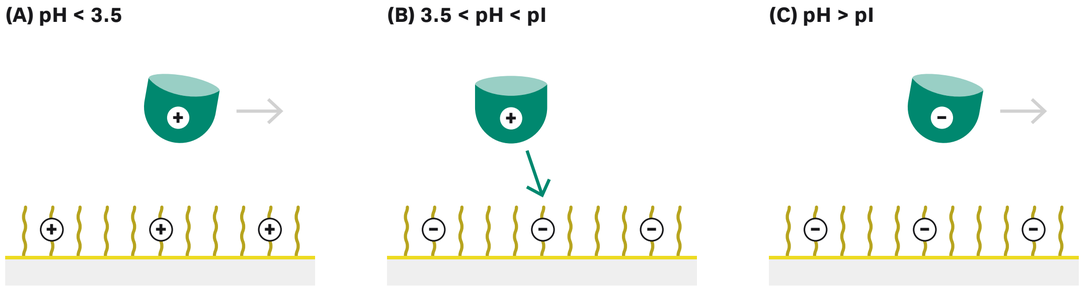

Fig 4.1. Ligand is concentrated on the surface through electrostatic attraction when the pH lies between the isoelectric point of the ligand and the pK_a of the surface. If the pH is too low or too high, ligand will not be concentrated on the surface.

4.1.3 Other buffer conditions

For many proteins, coupling in 10 mM acetate buffer, pH 5.0 works well. If you need to use other conditions, keep the following considerations in mind:

- The buffer pH should be at least 0.5 to 1.0 unit below the isoelectric point of the ligand. For ligands with isoelectric point (pI) above 8.0, the buffer pH may be increased to 7.0 or 7.5. The optimum pH can be determined experimentally (*see* Section 4.1.5).
- The ionic strength should be low (10 mM of monovalent cations is recommended).
- Buffer components containing primary amine groups and other strong nucleophilic groups (e.g., Tris or 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 (*see* Section 4.7).

4.1.4 Ligand concentration

Ligand solutions for immobilization are usually quite dilute (typically 10 to 50 µg/mL for most proteins) provided that efficient preconcentration 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 Immobilization pH scouting

While the general recommendation to immobilize proteins at pH 5.0 works adequately in many cases, you may need to optimize the coupling conditions for best results. To determine suitable coupling conditions without permanently modifying the sensor chip surface, inject your ligand in coupling buffer over a surface that has not been activated with EDC/NHS. Preconcentration 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. The electrostatically bound ligand can be washed off the surface with 50 mM NaOH, and the same surface can be used for ligand immobilization.

A method or wizard for immobilization pH scouting is provided with current Biacore™ systems.

1. Prepare ligand solutions in the different immobilization buffers to be tested. As a general rule, the pH range from 4.0 to 5.5 should be covered in steps of 0.5 pH units for initial scouting. For ligands with higher pI, a higher range should be used. If the ligand solutions are prepared by dilution from a stock solution, make sure that the ionic strength of the final diluted solution is sufficiently low. For ligand solutions at physiological ionic strength a 20-fold dilution is recommended. In some cases, it may be necessary to desalt the ligand solution by dialysis or size exclusion chromatography.
2. Set up the scouting procedure according to the predefined method in the Biacore™ system. Use a higher ionic strength buffer at pH slightly above neutral (e.g., HBS-EP+) as running buffer. Start at the highest pH to reduce the risk of aggregation or precipitation of the ligand on the surface.
3. Evaluate the results as described below.

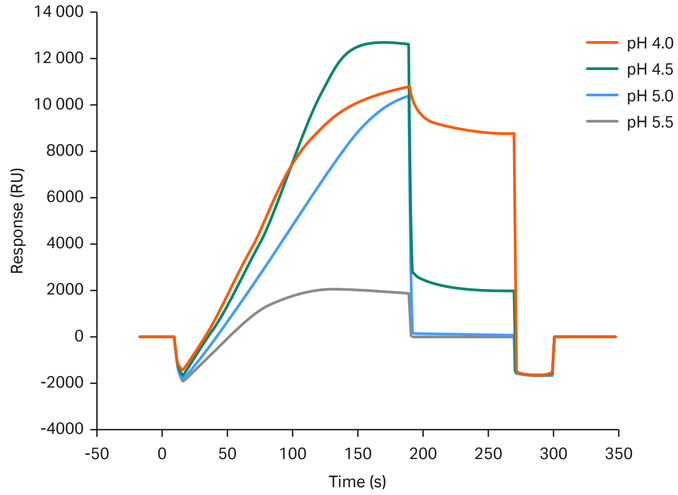

Fig 4.2. An example of preconcentration scouting results. Binding increases as pH is reduced from 5.5 to 4.5. At pH 4.0, bound material does not dissociate from the surface at the end of the injection, indicating that the protein is aggregating or denaturing. The best immobiliztion pH for this protein is between 4.5 and 5.0.

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

Many protein ligands tend to aggregate or precipitate at low pH. This is often visible as irregularities in the sensorgram or an increased baseline after the ligand injection (Fig 4.2). Avoid using buffers in which this behavior is observed.

For proteins with high pI, pH scouting should be performed using coupling buffers with pH greater than 5.5. For example, if you want to couple a protein with pI of 8.0, perform the scouting using 10 mM phosphate buffer with pH 7.5, 7.0, and 6.5.

If you do not obtain satisfactory electrostatic preconcentration at any pH, try increasing the ligand concentration and, if possible, reducing the ionic strength of the coupling buffer. Higher immobilization levels can also be achieved by extending the ligand contact time (10 to 20 min).

If this does not help, you may need to use a different coupling approach.

4.2 General immobilization procedures

The general steps involved in immobilizing ligand are essentially the same for 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 noncovalently 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. Predefined methods or wizards for common immobilization chemistries are supplied with Biacore™ SPR systems or can be obtained from Cytiva.

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 the immobilization will be reduced if solutions are not fresh.

Note: *Mixing of EDC and NHS should not be performed manually. It is performed by the instrument and is included in the immobilization method.*

Many protein ligands exhibit limited stability in low ionic strength, low pH buffers typically used for covalent immobilization. For this reason, ligand solutions should be prepared in immobilization buffer with as minimal time before use as possible. 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. If prepared from physiological ion strength solution, a dilution factor of 20 is a good “rule of thumb.” Rapid buffer exchange using desalting columns or microspin techniques are often a preferable alternative to dilution from stock solutions. Suitable desalting columns are available from Cytiva. Keep in mind that any components in the ligand solution containing primary amines, such as Tris or azide, will be immobilized alongside the ligand and will thus disturb the immobilization. A buffer exchange will help in those situations.

4.2.2 Evaluating covalent immobilization

You can usually get an indication of the ligand attachment level from the response at the end of the immobilization procedure, relative to the baseline before surface activation (Fig 4.3). To achieve this, measure the amount of ligand attached after final deactivation of the surface, which also serves to remove noncovalently bound ligand. The amount bound before the deactivation step can provide useful information for troubleshooting purposes (*see* Section 7.2).

The immobilization level only indicates the amount of material attached to the surface. You should also test the analyte binding capacity by injection of analyte, as explained in the next section.

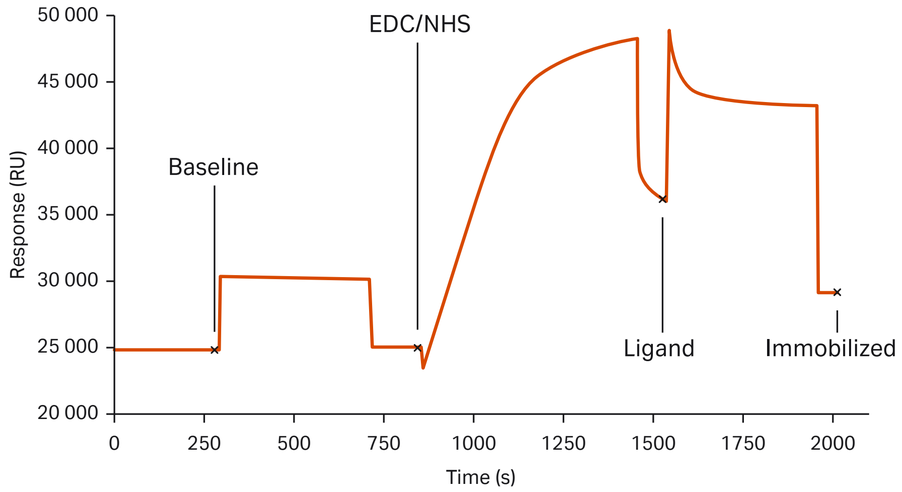

Fig 4.3. Schematic sensorgram from a typical amine coupling, illustrating the distinction between the amount of ligand bound and the amount immobilized.

Surface activation itself results in a small change in response, on the order of 100 to 400 RU for activation with EDC/NHS, but this can usually be ignored in measuring the amount of ligand immobilized. For low immobilization levels, measuring the amount bound in relation to the response before and after ligand injection (instead of to the baseline before surface activation) may be more appropriate. 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 immobilization levels are low, the results of immobilization can only be reliably assessed in terms of the analyte binding capacity of the ligand immobilized on 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 analyte aggregation.

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 (*see* 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 chemistry used to attach the ligand to the sensor surface, the first step in most covalent immobilization procedures is activation of the carboxyl groups on the surface with EDC/NHS to obtain reactive succinimide esters (Fig 4.4). Recommended concentrations are normally 0.2 M EDC and 0.05 M NHS (final concentrations after mixing EDC and NHS in a 50:50 ratio).

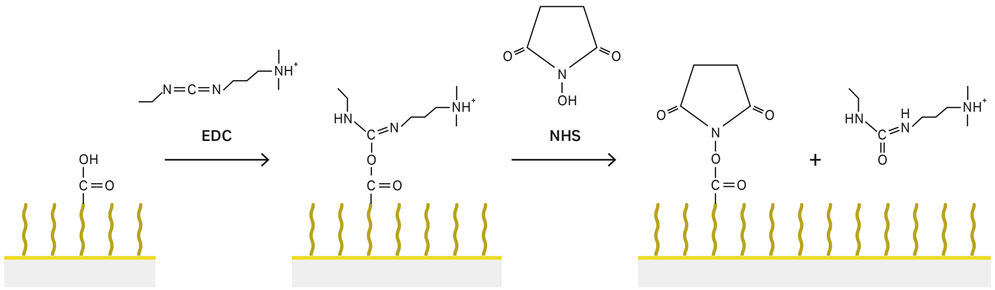

Fig 4.4. Activation of carboxyl groups with EDC/NHS.

The succinimide esters react spontaneously with amine and other nucleophilic groups, allowing direct immobilization of molecules containing such groups. The most common form of immobilization reaction using this activation chemistry is amine coupling.

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 be used in a thiol-disulfide exchange reaction to immobilize thiol-containing ligands (ligand thiol coupling, *see* Section 4.7.1). Another is the introduction of hydrazide groups which can react with *cis*-diols obtained by oxidation of aldehyde-containing molecules (*see* Section 4.8).

4.4 Amine coupling

4.4.1 Chemistry

Amine coupling chemistry is the most widely applicable approach for attaching proteins and other biomolecules covalently to the sensor surface. With this method, carboxyl groups on the sensor chip surface are first activated with EDC/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 in the ligand (Fig 4.5).

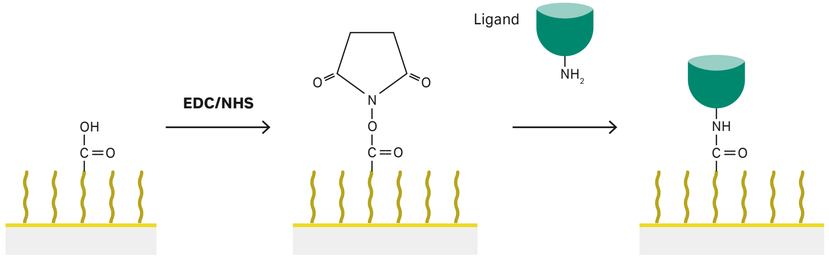

Fig 4.5. Amine coupling of ligands to the sensor surface.

Most proteins contain several amine groups, and 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, which results in accompanied loss of activity with attachment. Ligand activity is sometimes negatively affected by the low pH needed for amine coupling. In such cases, the ligand can be attached using an alternative coupling chemistry or a capture approach.

4.4.2 Required solutions

EDC, NHS, and ethanolamine are included in Amine Coupling Kit from Cytiva.

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 10 to 50 µg/mL in immobilization buffer

4.4.3 Typical immobilization protocol

Injection	Flow rate	Contact time
1. EDC/NHS (activates the surface)	10 µL/min	7 min
2. Ethanolamine (does not pass over the surface, performed automatically after EDC/NHS activation)	Wash	–
3. Ligand	10 µL/min	7 min
4. Ethanolamine (deactivate excess reactive groups)	10 µL/min	7 min

The typical immobilization protocol can be implemented as the default predefined methods and wizard templates in all Biacore™ systems. If the resulting immobilization levels are too high, test the following:

- Reduce ligand concentration.
- Reduce ligand contact time. It is however desirable for reproducible immobilization that binding during the ligand injection approaches a plateau.
- Reduce EDC/NHS activation time.
- For immobilization levels typically below 500 RU use a mixture of 20% EDC and 80% NHS instead of the standard 50:50 mixture, and at the same time reduce the activation time to 30 s. Do not change the ligand concentration and contact time if you use this approach.
- Some Biacore™ systems include an ***Aim for immobilized level*** option in the immobilization wizard template, which attempts to achieve a specified level of immobilization. This function is described in the documentation for the respective systems.

4.4.4 Sensorgram

A schematic sensorgram for amine coupling is shown in Figure 4.6.

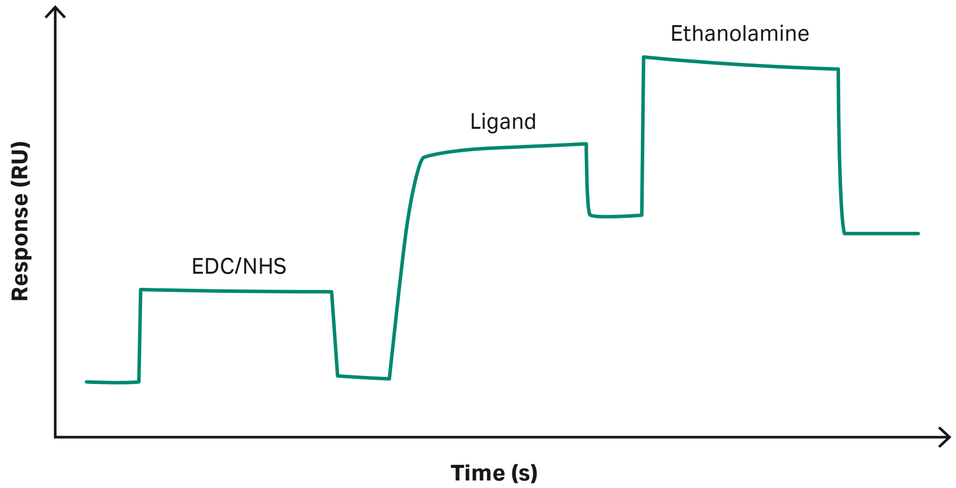

Fig 4.6. Schematic sensorgram showing the results of amine coupling.

4.5 Reversed amine coupling

Reversed amine coupling involves attachment of a diamine compound (e.g., ethylene diamine) to the sensor chip surface using standard amine coupling procedures, followed by immobilization of ligand via carboxyl groups on the ligand molecule. The amine surface created in this way is not stable and should be used directly after preparation.

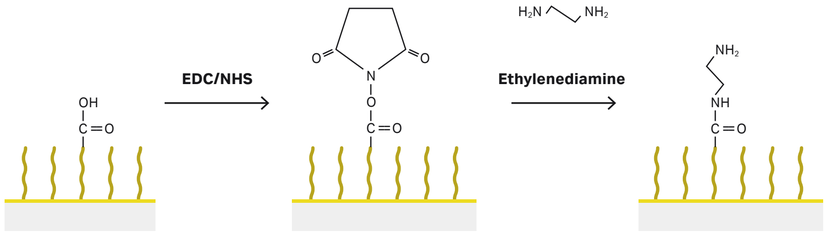

Fig 4.7. Introduction of amine groups on the sensor chip surface.

Preparing an amine surface by attachment of ethylenediamine

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

This method is particularly useful for immobilization of small molecules. The small molecule is diluted in PBS buffer and mixed with EDC and NHS. Contact times and molar ratios between EDC/NHS and the small molecule need to be optimized for each individual case, depending on the application and nature of the small molecule. Below is an example of typical settings that can be used as a starting point.

Coupling of a small molecule to an amine surface

Small molecule in PBS buffer	5 mM final concentration
EDC	5 mM final concentration
NHS	1 mM final concentration
Contact time	420 to 1800 s

4.6 Micelle-mediated coupling of negatively charged ligands

Coupling of negatively charged ligands to a negatively charged Biacore™ sensor chip requires alternative methods. CTAB (hexadecyltrimethylammonium bromide) is a positively charged detergent that forms micelles in aqueous solutions at concentrations above CMC. The negatively charged ligand is added to the micelles and interacts electrostatically. The micelles then carry the ligand to the EDC/NHS activated surface and the ligand is coupled to the surface by standard amine coupling chemistry. This method is useful for coupling, for example, oligonucleotides and acidic proteins. In the example below, micelle-mediated coupling was used to couple a 20-mer oligonucleotide to Sensor Chip CM5. The settings used in this example can be used as starting points for development of a coupling method for your protein.

Parameter	**Setting**
Flow rate	5 µL/min
Running buffer	HBS-N
Coupling buffer	HEPES, pH 7.4

Injection	**Contact time**
EDC/NHS (activation)	7 min
Ligand (CTAB 0.6 µM + protein 10 µM)	10 min
Ethanolamine (deactivation)	3 min

4.7 Thiol coupling

Thiol coupling can help you to immobilize ligands in a defined orientation, since there are often fewer potential attachment sites than with amine coupling (in some cases just a single site). Thiol coupling can also be a valuable approach if the ligand is inactivated by amine coupling because 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 preconcentration properties.

One approach to thiol coupling utilizes exchange reactions between thiols and active disulfide groups. The active disulfide 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, Fig 4.8).

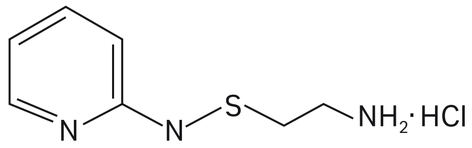

Fig 4.8. PDEA Thiol coupling reagent, 2-(2-pyridinyldithio)ethaneamine hydrochloride.

An alternative approach relies on reaction of thiol groups on the ligand with maleimide reagents introduced on to the sensor chip surface (*see* Section 4.7.3).

4.7.1 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.

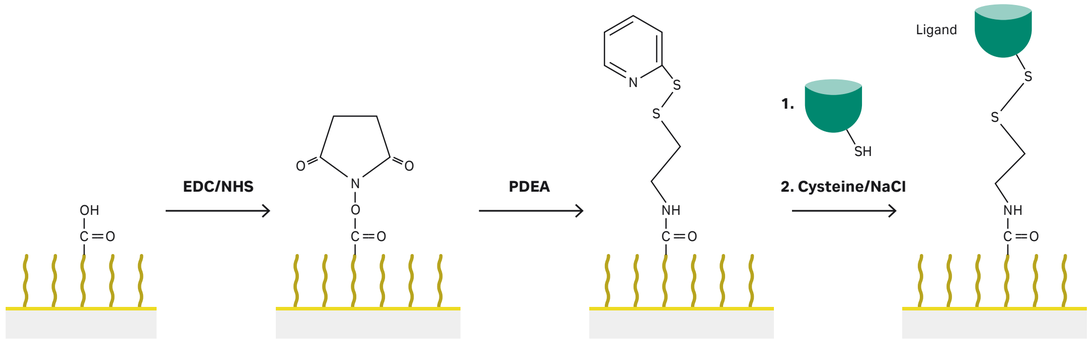

Fig 4.9. Ligand thiol coupling of ligands to the sensor chip surface.

Required solutions

Reagents for ligand thiol coupling are available in Thiol Coupling Kit from Cytiva. PDEA is also available as a separate product from Cytiva.

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
PDEA	80 mM 2-(2-pyridinyldithio)ethaneamine in 0.1 M sodium borate, pH 8.5. Use within 1 h of preparation.
Cysteine/NaCl	50 mM cysteine and 1 M NaCl in 0.1 M sodium acetate, pH 4.0
Ligand	Typically 10 to 50 µg/mL in immobilization buffer

Typical immobilization protocol

Injection	Flow rate	Contact time
1. EDC/NHS (activate the surface)	10 µL/min	2 min
2. Ethanolamine (does not pass over the surface)	Wash	–
3. PDEA (introduce reactive disulfide groups)	10 µL/min	4 min
4. Ligand	10 µL/min	7 min
5. Cysteine-NaCl (deactivate excess reactive groups)	5 µL/min	4 min

The typical immobilization protocol is set as the default method and wizard template in most Biacore™ systems.

Sensorgram

A schematic sensorgram for ligand thiol coupling is shown in Figure 4.10.

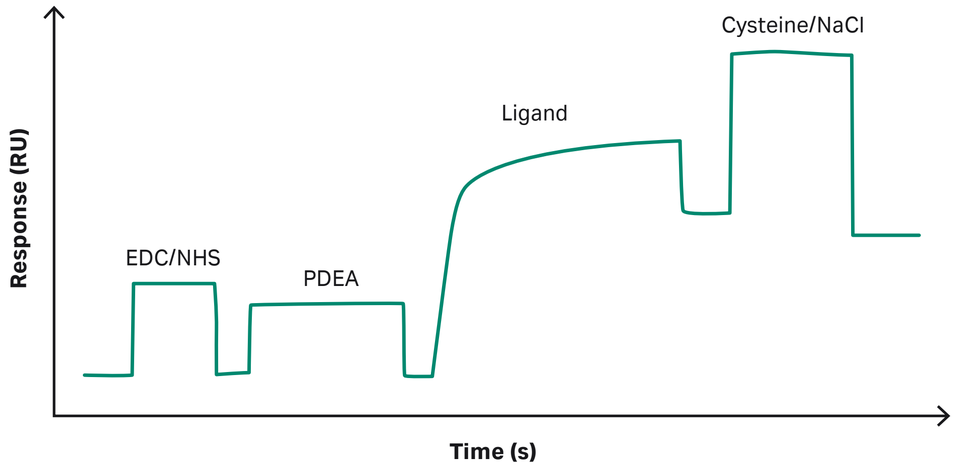

Fig 4.10. Schematic sensorgram showing the results of ligand thiol coupling.

Additional ligand thiol methods

Thiol groups for ligand thiol immobilization can be introduced into ligands by modification, for example, using Traut's reagent (2-iminothiolane) to modify amine groups (1).

4.7.2 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 (Fig 4.11).

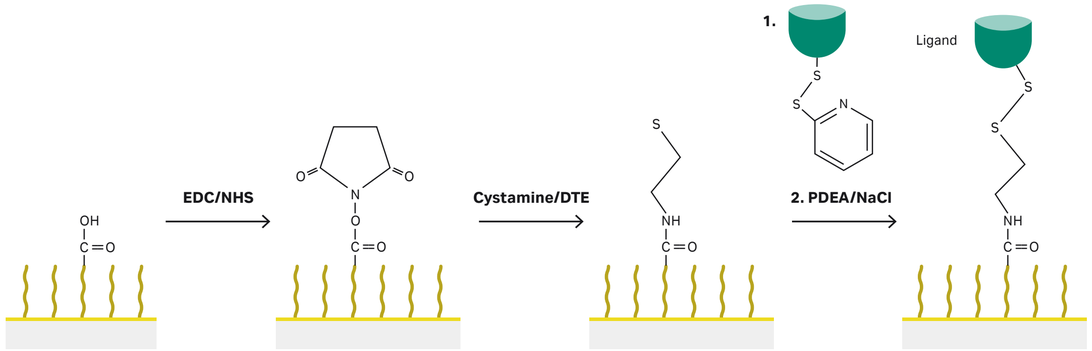

Fig 4.11. Surface thiol coupling of ligands to the sensor surface.

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 of ligand in 0.5 mL of 0.1 M morpholino-ethanesulfonic acid (MES) buffer, pH 5.0 at 25°C.
2. Add 0.25 mL of 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 of 0.4 M EDC (final EDC concentration 13 mM).
4. Mix and incubate for 10 min at 25°C. If the ligand is not stable at room temperature, the modification may be performed on ice for 1 h.
5. Remove the excess reagents on a buffer exchange column equilibrated with a suitable buffer.

Under these conditions, PDEA reacts with carboxyl groups on the ligand (Fig 4.12). 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.

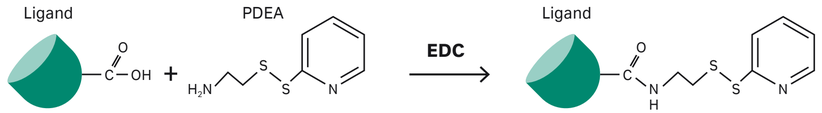

Fig 4.12. Reaction with PDEA introduces reactive disulfide groups onto carboxyl residues in the ligand.

Determining the degree of modification

For proteins modified with PDEA, the degree of modification can be important for immobilization. The degree of modification can be determined approximately by reduction of the disulfide 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 of protein solution, add 50 µL of 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:

$$c_{TP} = \frac{A2_{343} - A1_{343}}{\varepsilon_{343,\,TP} l} \qquad c_{prot} = \frac{A_{280,\,prot}}{\varepsilon_{280,\,prot} l}$$

$$A_{280,\,prot} = A_{280} - (c_{TP}\varepsilon_{280,\,TP} l) \qquad \text{molar degree of modification} = \frac{c_{TP}}{c_{prot}}$$

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 ($8.08 \times 10^3\ M^{-1}cm^{-1}$)
$\varepsilon_{280,TP}$	is the molar extinction coefficient for thiopyridone at 280 nm ($5.1 \times 10^3\ M^{-1}cm^{-1}$)
$\varepsilon_{280,prot}$	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.

Required solutions

Reagents for surface thiol coupling and ligand modification with PDEA are available in Thiol Coupling Kit from Cytiva. PDEA is also available as a separate product from Cytiva.

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
Cystamine	0.04 M cystamine dihydrochloride in 0.1 M sodium borate, pH 8.5
DTE	0.1 M dithioerythritol in 0.1 M sodium borate, pH 8.5. Dithiothreitol (DTT) may also be used.
PDEA/NaCl	20 mM 2-(2-pyridinyldithio)ethaneamine and 1 M NaCl in 0.1 M sodium acetate, pH 4.0
Ligand	Typically 10 to 50 µg/mL in immobilization buffer

Typical immobilization protocol

Injection	Flow rate	Contact time
1. EDC/NHS (activate the surface)	10 µL/min	2 min
2. Ethanolamine (does not pass over the surface)	Wash	–
3. Cystamine (introduce disulfide groups)	10 µL/min	3 min
4. DTE (reduce disulfides to thiols)	10 µL/min	3 min
5. PDEA-modified ligand	10 µL/min	7 min
6. PDEA-NaCl (deactivate excess reactive groups)	10 µL/min	4 min

The typical immobilization protocol is set as the default method and wizard template in most Biacore™ systems.

Sensorgram

A schematic sensorgram for surface thiol coupling is shown in Figure 4.13.

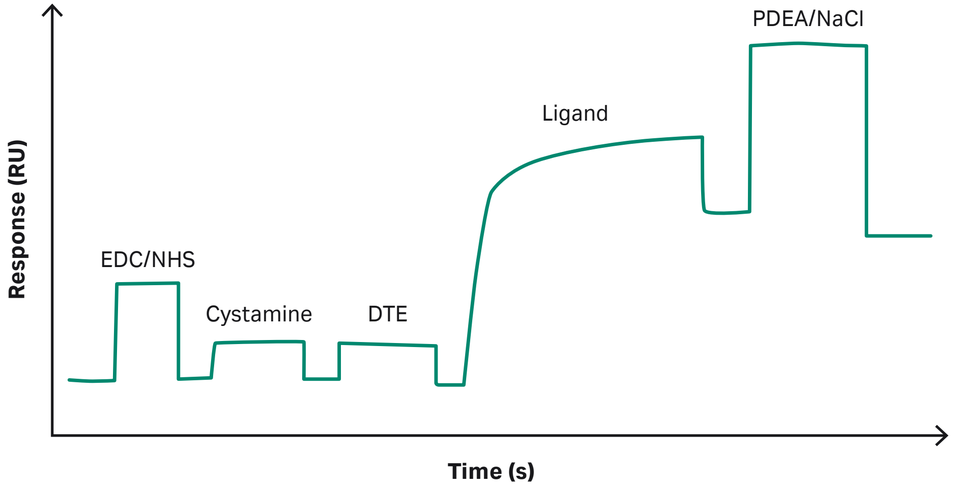

Fig 4.13. Schematic sensorgram showing the results of surface thiol coupling.

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 of heterobifunctional reagents that are activated with NHS include N-succinimidyl-3-[2-pyridyldithio]-propionate (SPDP) and 4-succinimidyl-oxycarbonyl-methyl-α-[2-pyridyldithio]-toluene (SMPT). Aldehyde groups (created by oxidation of *cis*-diols, *see* Section 4.8) can be modified with N-[ε-maleimido-caproic acid]hydrazide (EMCH).

4.7.3 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, N-[ε-maleimido-caproic acid]hydrazide (EMCH) and N-[γ-maleimidobutyryl-oxy]sulfo-succinimide ester (sulfo-GMBS) (Fig 4.14), are discussed below.

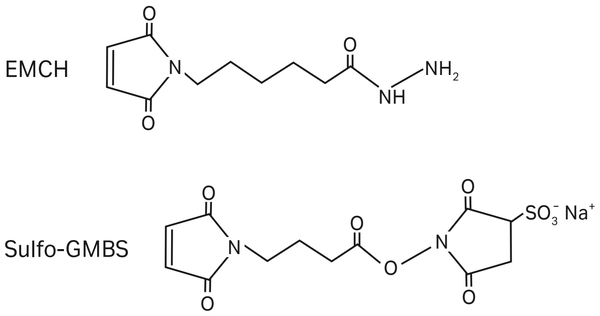

Fig 4.14. Reagents that can be used for maleimide coupling.

EMCH coupling

EMCH is first coupled to the surface via the terminal hydrazide group after activation of the sensor chip surface with EDC/NHS. Thiol groups on the injected ligand then react with the maleimide group to form a stable thioether linkage (Fig 4.15).

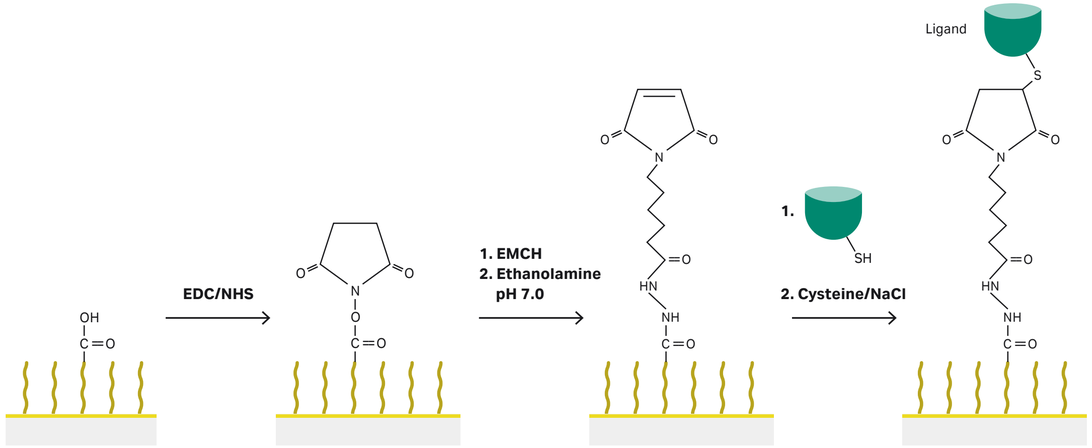

Fig 4.15. Maleimide coupling using EMCH.

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

Required solutions

EDC, NHS, ethanolamine pH 8.5, cysteine, and 1 M NaCl in 0.1 M sodium acetate, pH 4.0 are available in Thiol Coupling Kit from Cytiva.

EDC	0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water
NHS	0.1 M N-hydroxysuccinimide in water
Ethanolamine, pH 7.0	1 M ethanolamine-HCl in 0.1 M sodium phosphate, pH 7.0
Ethanolamine, pH 8.5	1 M ethanolamine, pH 8.5
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 to 50 µg/mL in immobilization buffer

Typical immobilization protocol

Injection	Flow rate	Contact time
1. EDC/NHS (activate the surface)	5 to 10 µL/min	6 to 7 min
2. Ethanolamine pH 8.5 (does not pass over the surface)	Wash	–
3. EMCH (introduce maleimide groups)	5 to 10 µL/min	3 min
4. Ethanolamine, pH 7.0[1] (deactivate excess reactive groups)	5 to 10 µL/min	3 min
5. Ligand	5 to 10 µL/min	6 to 7 min
6. Cysteine/NaCl (deactivate excess maleimide groups)	5 to 10 µL/min	4 min

[1] Use ethanolamine at pH 7.0. Do not use the ethanolamine pH 8.5 from the Amine Coupling Kit as this will destroy the maleimide reagent on the dextran matrix.

The typical immobilization protocol is set as the default predefined method and wizard template in most Biacore™ systems.

Sensorgram

A schematic sensorgram for maleimide coupling using EMCH is shown in Figure 4.16.

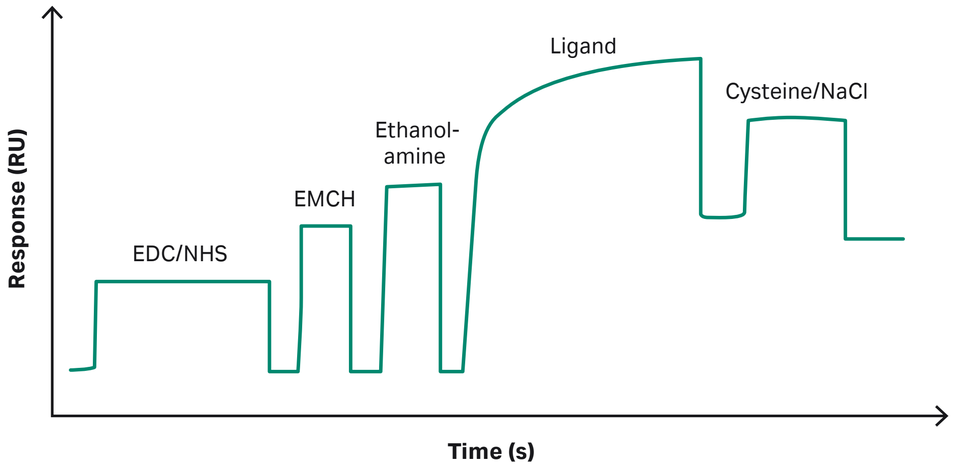

Fig 4.16. Schematic sensorgram showing the results of maleimide coupling using EMCH.

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 (Fig 4.17).

Note: *the amine surface is not stable and should be derivatized with sulfo-GMBS directly after preparation.*

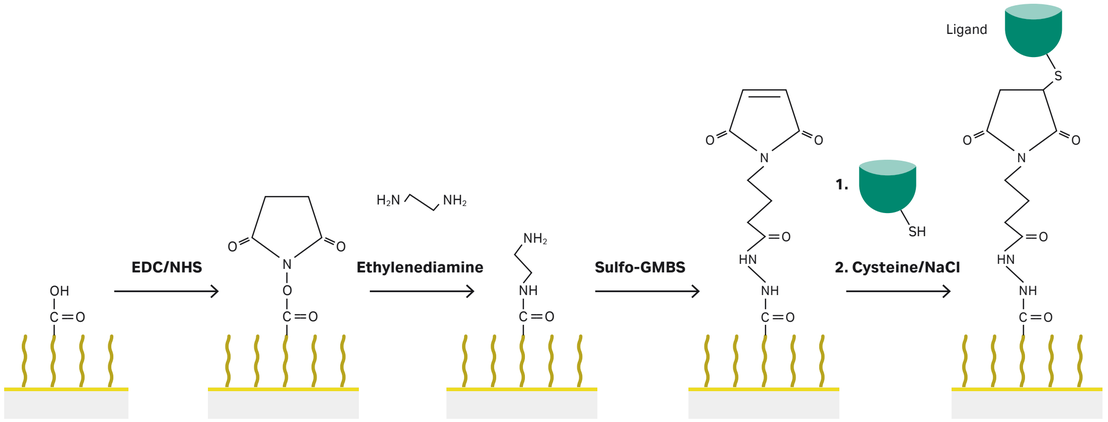

Fig 4.17. Maleimide coupling using sulfo-GMBS.

Required solutions

EDC, NHS, ethanolamine, cysteine and 1 M NaCl in 0.1 M sodium acetate, pH 4.0 are available in Thiol Coupling Kit from Cytiva.

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
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 to 50 µg/mL in immobilization buffer

Typical immobilization protocol

Injection	Flow rate	Contact time
1. EDC/NHS (activate the surface)	5 to10 µL/min	6 to 7 min
2. Ethanolamine (does not pass over the surface)	Wash	–
3. Ethylenediamine (introduce amine groups)	5 to10 µL/min	6 to 7 min
4. Sulfo-GMBS (introduce maleimide groups)	5 to10 µL/min	4 min
5. Ligand	5 to10 µL/min	6 to 7 min
6. Cysteine/NaCl (deactivate excess maleimide groups)	5 to10 µL/min	4 min

Sensorgram

A schematic sensorgram for maleimide coupling using sulfo-GMBS is shown in Figure 4.18.

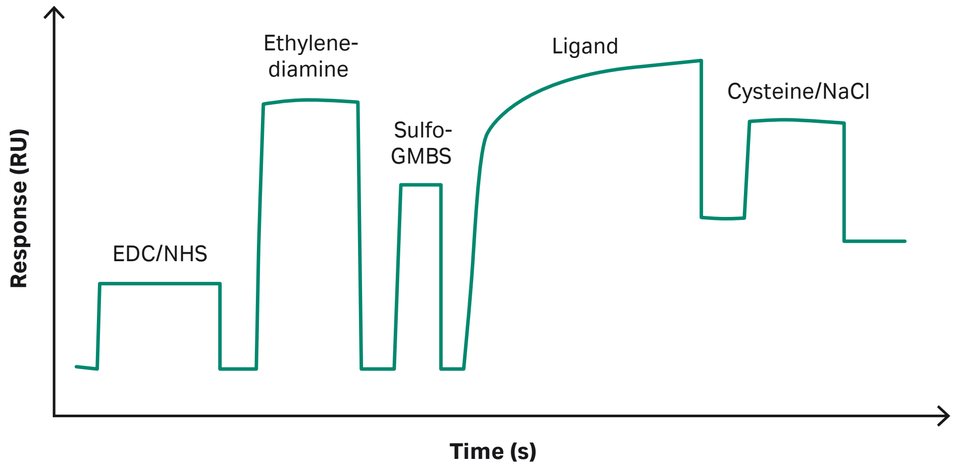

Fig 4.18. Schematic sensorgram showing the results of maleimide coupling using sulfo-GMBS.

4.8 Aldehyde coupling

4.8.1 Chemistry

Ligands containing aldehyde groups (either native or introduced by oxidation of *cis*-diols) can be immobilized after activating the surface with carbohydrazide. The chemistry of aldehyde coupling is summarized in Figure 4.19.

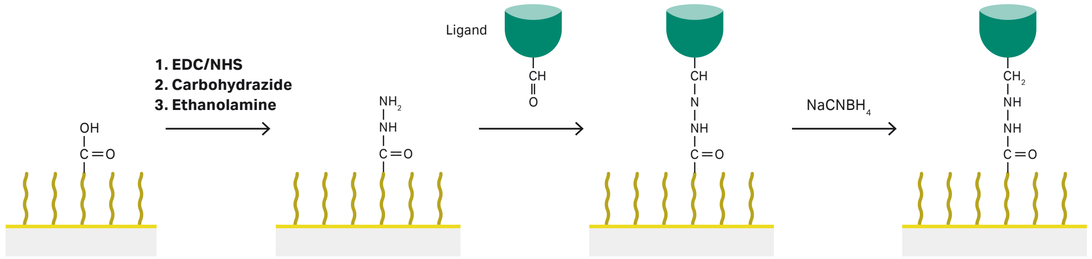

Fig 4.19. Aldehyde coupling of ligands to the sensor surface.

Aldehyde coupling provides an alternative approach for immobilizing glycoproteins and other glycoconjugates which can be difficult to couple using amine or thiol coupling. This method is particularly suitable for ligands containing sialic acid, since these residues are very easily oxidized to aldehydes using sodium metaperiodate.

4.8.2 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 (2). 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
- Amersham™ NAP™-5 column or other desalting column from Cytiva
- Type I water quality

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 min on ice.
- Stop the reaction by desalting the mixture on a desalting column equilibrated with buffer B. Elute the ligand in buffer B. Store the oxidized ligand in the refrigerator.
- The degree of oxidation is most easily estimated by testing immobilization to a carbohydrazide activated sensor chip surface. Unoxidized ligand is preferably used as a 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 (3). In many cases, penultimate galactose residues can be exposed by treatment with neuraminidase before being oxidized with galactose oxidase.

4.8.3 Required solutions

EDC, NHS, and ethanolamine are included in Amine Coupling Kit from Cytiva.

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
Carbohydrazide	5 mM carbohydrazide in water
Cyanoboro-hydride*	0.1 M sodium cyanoborohydride in 0.1 M sodium acetate, pH 4.0

Warning

*Cyanoborohydride is very toxic and should be handled with extreme care. Please refer to Safety Data Sheets for more information.

4.8.4 Typical immobilization protocol

Injection	Flow rate	Contact time
1. EDC/NHS (activate the surface)	10 µL/min	3 min
2. Ethanolamine (does not pass over the surface)	Wash	–
3. Carbohydrazide (introduce hydrazide groups)	10 µL/min	7 min
4. Ethanolamine (deactivate excess reactive groups)	10 µL/min	7 min
5. Ligand (immobilize the ligand)	10 µL/min	7 min
6. Cyanoborohydride (stabilize the bond)	2 µL/min	20 min

The typical immobilization protocol is set as the default method and wizard template in most Biacore™ systems.

4.8.5 Sensorgram

A schematic sensorgram for aldehyde coupling is shown in Figure 4.20.

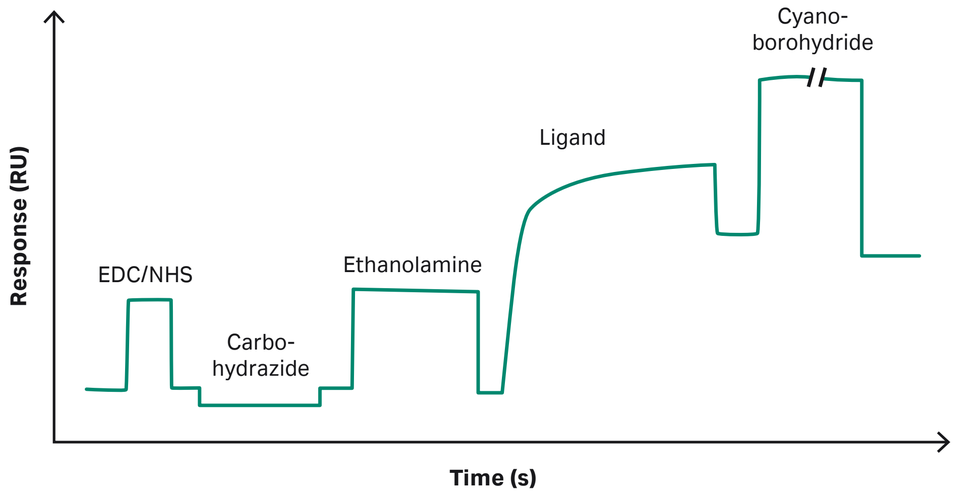

Fig 4.20. Schematic sensorgram showing the results of aldehyde coupling.

4.9 Ligand modification for capturing

For capture approaches, the capturing molecule is normally attached to the sensor surface using one of the covalent methods described above. The 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 attached capturing molecule is generally a more reliable approach.

Several capture 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 capture site. Capture using Sensor Chip SA, Sensor Chip NA, or Biotin CAPture Kit are exceptions to this generalization since biotin is usually introduced into the ligand by chemical techniques *in vitro*.

4.9.1 Biotinylation for streptavidin-biotin capture

A range of methods and commercial reagents for ligand biotinylation are available. The choice of method will depend mainly 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 attachment and analysis.

Substitution levels around one biotin residue per ligand molecule are recommended for capture to biotin binding molecules. In general, conditions recommended for biotinylation of proteins with commercial reagents tend to give higher substitution levels, resulting in multipoint attachment of the ligand to the surface with consequent impairment of assay performance. Suggested procedures for ligand biotinylation may be found on cytiva.com/biacore.

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′-hydroxy-azo-benzene-2-carboxylic acid) are generally easy to perform but have low sensitivity and require relatively large quantities of biotinylated ligand (4). More sensitive assays such as enzymatic, chemiluminescent, or fluorescent detection are valuable when the amount of ligand is limited. A detailed description can be found in the Lab procedure Biotinylation for Steptavidin/NeutrAvidin-biotin capture on Biacore™ sensor chips.

It is crucial that excess biotinylation reagent is removed from the ligand before capture on Sensor Chip SA and Sensor Chip NA or when using Biotin CAPture Kit. Free biotin in the ligand solution will compete for biotin binding sites which will result in reduced ligand capture level. 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.

4.10 References

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05

Hydrophobic adsorption methods

Working with liposomes and membrane proteins can be challenging. Most biophysical techniques were optimized for water-soluble proteins in an aqueous environment, where membrane proteins show poor stability. But there are several tools and techniques available to help you overcome this problem.

In this chapter you will find procedures for attaching lipid membranes and vesicles to the sensor chip surface (Fig 5.1).

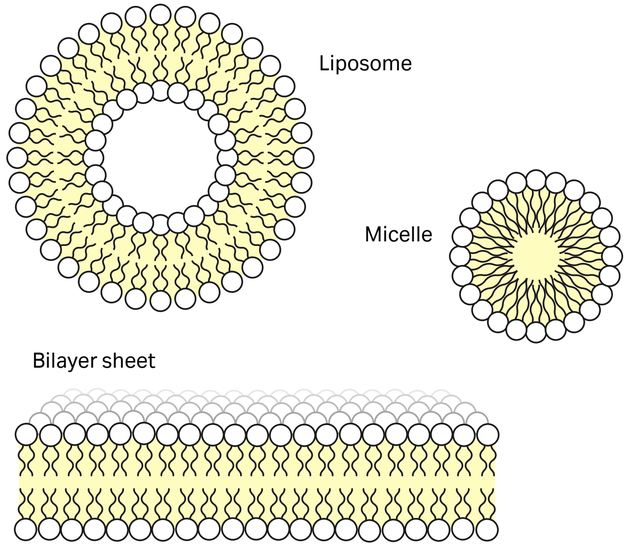

Fig 5.1. Illustration of various forms of lipid membranes and vesicles.

5.1 Monolayer adsorption on Sensor Chip HPA

Sensor Chip HPA has a flat hydrophobic surface which can be coated with a monolayer of lipids. This setup allows you to study peripheral membrane proteins. The flat hydrophobic surface consists of long-chain alkane thiol molecules attached directly to a gold film. User-prepared liposomes adsorb spontaneously to the surface to form a supported lipid monolayer, with polar head groups directed out towards the solution. Molecules associated with membrane surfaces can be incorporated into the monolayer and used as ligands.

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 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 also be detergent-free.

Instrument cleaning and maintenance

Ensure that the Biacore™ SPR 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.

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 room temperature.

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 larger liposomes and give better coverage of the sensor surface. Membrane-associated proteins may be included with the liposomes, although incorporation of transmembrane proteins is not appropriate since the lipids adsorb to the sensor surface in a monolayer (half-membrane) structure (2).

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 sensor chip package, docking the sensor 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 min injection of 40 mM octyl glucoside (n-octyl β-D-glucopyranoside) in water.
2. Inject the liposome preparation at a low flow rate (2 to 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 to 3 h 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. Maximum responses reached are usually in the region of 1500 to 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 multilayered structures. Wash the surface with a short injection (0.5 to 1 min) of 10 to 100 mM NaOH to remove loosely bound structures.

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 min 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

The surface of Sensor Chip L1 consists of a carboxymethylated dextran matrix modified with lipophilic groups for capture of lipid vesicles with and without membrane proteins.

The binding process involves diffusion of the vesicles to the surface and incorporation of the lipophilic structures on Sensor Chip L1 into the lipid membrane, noncovalently anchoring the vesicle. You can capture liposomes, nanodiscs, and other lipoprotein particles nonspecifically by hydrophobic interaction on Sensor Chip L1. The surface provides sites for attachment of lipid membranes 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. A concentration of 0.5 mM with respect to phospholipid is usually sufficient. 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 at least two 30 s injections of detergent such as 20 mM CHAPS (3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate) or 40 mM octyl glucoside in water, or with a mixture of 2 parts isopropanol and 3 parts 50 mM NaOH. It is important to wash the needle and flow system (including the sample line in the IFC) thoroughly. Refer to instructions for the respective Biacore™ SPR system for guidelines on how to wash the needle and flow system.
2. Inject the liposome preparation at a low flow rate (2 to 10 µL/min). Adequate liposome adsorption is usually achieved 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. Allow buffer to pass over the surface for 5 to10 minutes after the liposome preparation to stabilize the formation of the liposome layer.
3. For surfaces prepared with pure liposomes, a short injection of 10 to 100 mM NaOH can be used to remove loosely bound structures and stabilize the baseline. ***Note***: This treatment may damage liposomes containing additives such as cholesterol, ceramides, or proteins.
4. Perform several 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 5000 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 and capture using antibodies directed against specific liposome constituents (2, 3). Proteoliposomes can also be covalently immobilized using standard coupling chemistry to attach the protein component to the sensor chip surface (4).

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 attached to the surface instead of being passively carried in the adsorbed liposomes, generally giving 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 (GPCR) (5).

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 (Fig 5.2).

The running buffer used for OSR should be 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 through the use of a consecutive injection such as ***Dual*** command available in newer Biacore™ SPR systems. Refer to the respective system documentation for details.

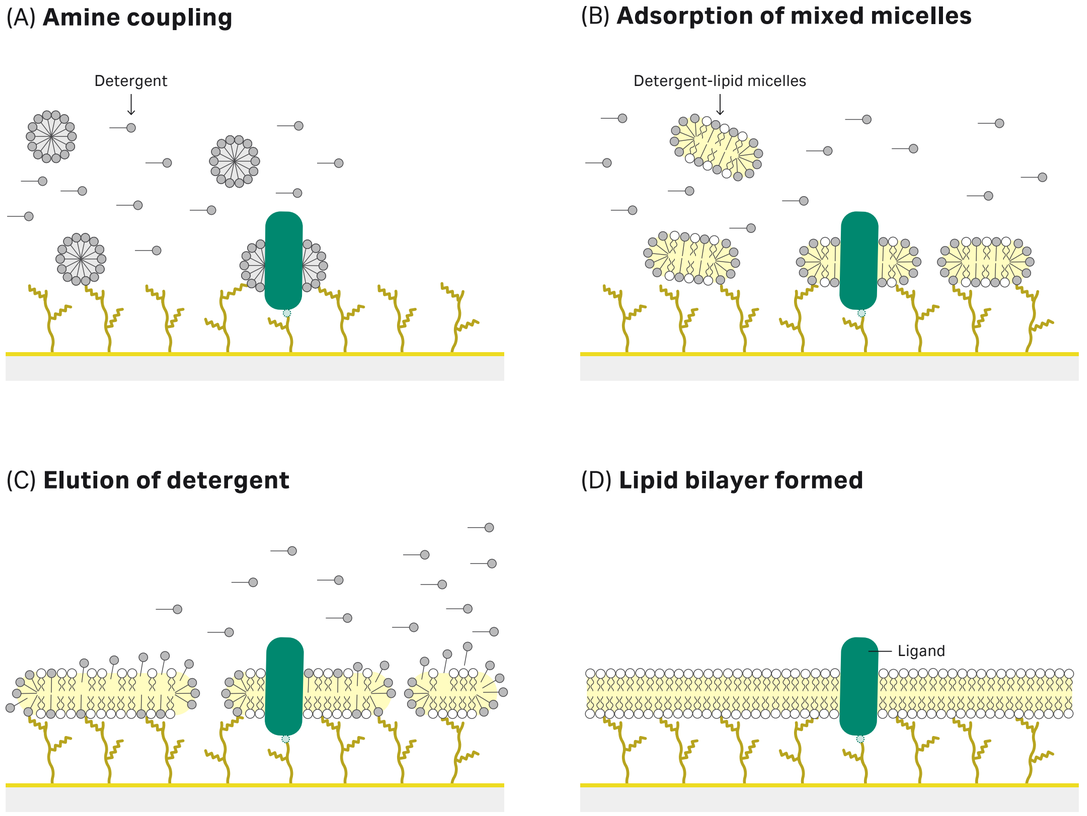

Fig 5.2. OSR on Sensor Chip L1 resulting in the formation of a reconstituted lipid bilayer covering the sensor chip surface.

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 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 to 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 h.
3. Prepare solutions of octyl glucoside at 20 to 40 mM in HBS buffer by dilution from a 0.5 M octyl glucoside stock solution and 10× concentrated HBS.
4. Add the detergent solution to the lipid and shake every 10 min for at least 45 min 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 to 50 mM CHAPS. Inject each preparation for 1 to 8 min at 5 to 10 µL/min. Regenerate the surface with two 1 min injections of 50 mM octyl glucoside between each test. The optimal lipid-detergent ratio is the clear solution that gives the highest response in the test injection (Fig 5.3). Responses up to about 3000 to 6000 RU above baseline may be expected for POPC micelles. Levels may vary with other liposome types, for example, anionic lipids often give lower response levels.

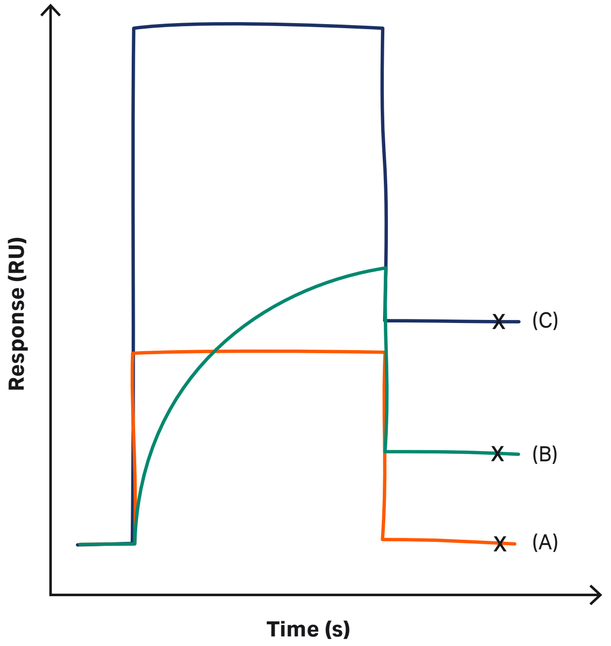

Fig 5.3. Schematic sensorgrams illustrating preparations with different content of octyl glucoside and POPC injected over Sensor Chip L1. The response level at the marked report point indicates the amount of lipid stably adsorbed to the surface. (A) Lipid does not adsorb from a detergent-rich sample. (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 capture 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 to 2 min) 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.

5.5 Analyzing membrane proteins

The strategies that you can use when preparing liposomes and membrane proteins for analysis using Biacore™ SPR system can broadly be divided into:

- Solubilized proteins
- Soluble protein domains and variants
- Membrane-like environments
- Immobilized whole cells or cell membranes

You can, in principle, attach solubilized proteins using any of the standard immobilization techniques. The specific properties of your protein of interest will determine the best method and the optimal conditions to use.

It is becoming increasingly common to capture tagged or biotinylated membrane proteins on the sensor surface as a method of attachment. This also enhances the specificity of attachment when working with natural membrane preparations. Useful affinity tags when working with membrane proteins include His tag or 1D4 tag.

Adding detergent in the running buffer when working with detergent-solubilized proteins may help prevent loss of protein functionality. To avoid sensorgram artefacts, detergent is typically added in a lower concentration in the running buffer than in the buffer used for solubilization. However, this must be tested on a case-by-case basis.

On the other hand, detergent-free running buffer is required for assays in membrane-like environments, as detergents may interfere with the lipid structures. Membrane-like environments include, for example, proteoliposomes and reconstituted membranes, Styrene Maleic Acid Lipid Particles (SMALPs), Salipro technology, virus-like lipoparticles (VLPs), and nanodiscs.

It is possible to use whole cells in Biacore™ SPR systems to study membrane proteins but maintaining sensitivity and specificity can be a challenge. So, in practice, whole cells or membrane preparations are not suitable for direct binding assays using low molecular weight analytes. However, inhibition assays may be used.

You can find more information in the Working with liposomes and membrane proteins in Biacore™ systems, and in the Sensor Chip HPA and Sensor Chip L1 Instructions for use.

When selecting run parameters for your assay, the same general rules apply as when working with soluble proteins. Our application guides found here contain helpful information.

5.6 References

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06
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 (*see* 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 binding activity from the surface will impair the performance of your assay and shorten the useful lifetime of the sensor chip.

You do not always need to use a regeneration solution to regenerate the surface. If your analyte dissociates fast enough, all the analyte may be removed within a reasonable time simply by allowing buffer to pass over the surface. This is immediately evident from the sensorgram since the response returns to baseline after the analyte 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 essentially 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. Regeneration Scouting Kit available from Cytiva provides reagents suitable for regeneration scouting with most protein ligands.

6.1 Regeneration scouting strategy

6.1.1 General considerations

Regeneration scouting is a procedure for finding suitable conditions for regeneration, which can later be refined if necessary and tested more extensively. Scouting is performed by testing a few (five are recommended) repeated cycles of analyte binding and regeneration with each condition and examining trends in the response levels within each condition. Scouting should start with a previously unused sensor surface immobilized with ligand.

Follow these general guidelines for regeneration scouting:

- 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 with sensor chip type, immobilization chemistry, immobilization method, and ligand level.
- 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, or insufficient regeneration will more easily go undetected. In addition, regeneration conditions established at low analyte binding levels may not necessarily be optimal for higher levels.
- Test regeneration conditions using analyte that reflects the experimental samples if possible. This is particularly important if your samples are complex mixtures such as cell culture medium or body fluids, where binding of nonanalyte 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 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 do not usually 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 microenvironment on the surface of the sensor chip.

Low pH, such as 10 mM glycine-HCl at pH 1.5 to 3.0 available as ready-to-use solutions from Cytiva, is often appropriate for regenerating protein surfaces. Other conditions which have proved useful include:

- high pH (1 to 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%)
- ethylene glycol at concentrations up to 100%

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

Always make sure that the regeneration solution you choose 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 a major factor determining suitable regeneration conditions, the optimum conditions are also affected by 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. For example, in a test study using anti-myoglobin antibodies as ligand and myoglobin as analyte, we found optimal regeneration with glycine-HCl on Sensor Chip CM3 required pH values 0.2 to 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.

Coupling conditions

Different immobilization conditions for the same chemistry (e.g., using different contact times for activation with EDC/NHS) lead to differences in charge properties of the surface, even at the same ligand density. Optimal regeneration conditions may be affected.

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 for your application. This ensures that the optimal 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 results are also 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. Regeneration Scouting Kit from Cytiva, provides scouting solutions suitable for a range of applications.

1. Use a new sensor chip and immobilize approximately the same amount of ligand that you will use 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. Use a report point just after the end of the analyte injection to evaluate analyte responses levels.
3. Inject the mildest regeneration solution that you intend to test. Use a short contact time, 30 to 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 to 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 3.0 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.

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 reports on the efficiency of regeneration in the previous cycle (Fig 6.1).

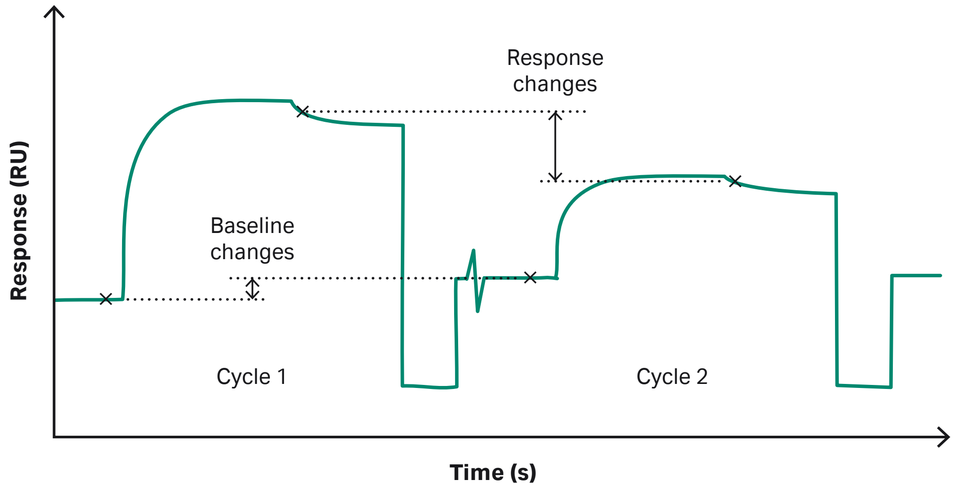

Fig 6.1. Regeneration scouting is assessed by following the effects of regeneration attempts on the analyte binding capacity and baseline levels. The measurements from Cycle 2 in the scouting procedure report on the efficiency of the regeneration attempt in Cycle 1.

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.*

The baseline response during repeated cycles with the same regeneration conditions reveals whether material accumulates on the surface, while the analyte response reveals whether the surface retains analyte binding capacity.

Regeneration conditions that do not remove analyte sufficiently lead to an increase in baseline between cycles. As more of the surface becomes permanently occupied the analyte binding response may decrease. Conditions which are too harsh may remove all bound analyte but result in a loss of analyte binding capacity as the ligand activity deteriorates (Fig 6.2).

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 on their own but may provide complementary information to aid in interpretation of the analyte response. The following general guidelines apply:

- The analyte binding response should be constant. A falling trend in analyte 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 ideally remain constant. An increasing baseline indicates that material is accumulating on the surface (regeneration is too mild). Depending on the demands of the experiment, this may be acceptable if sufficient binding capacity remains available.
- 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.

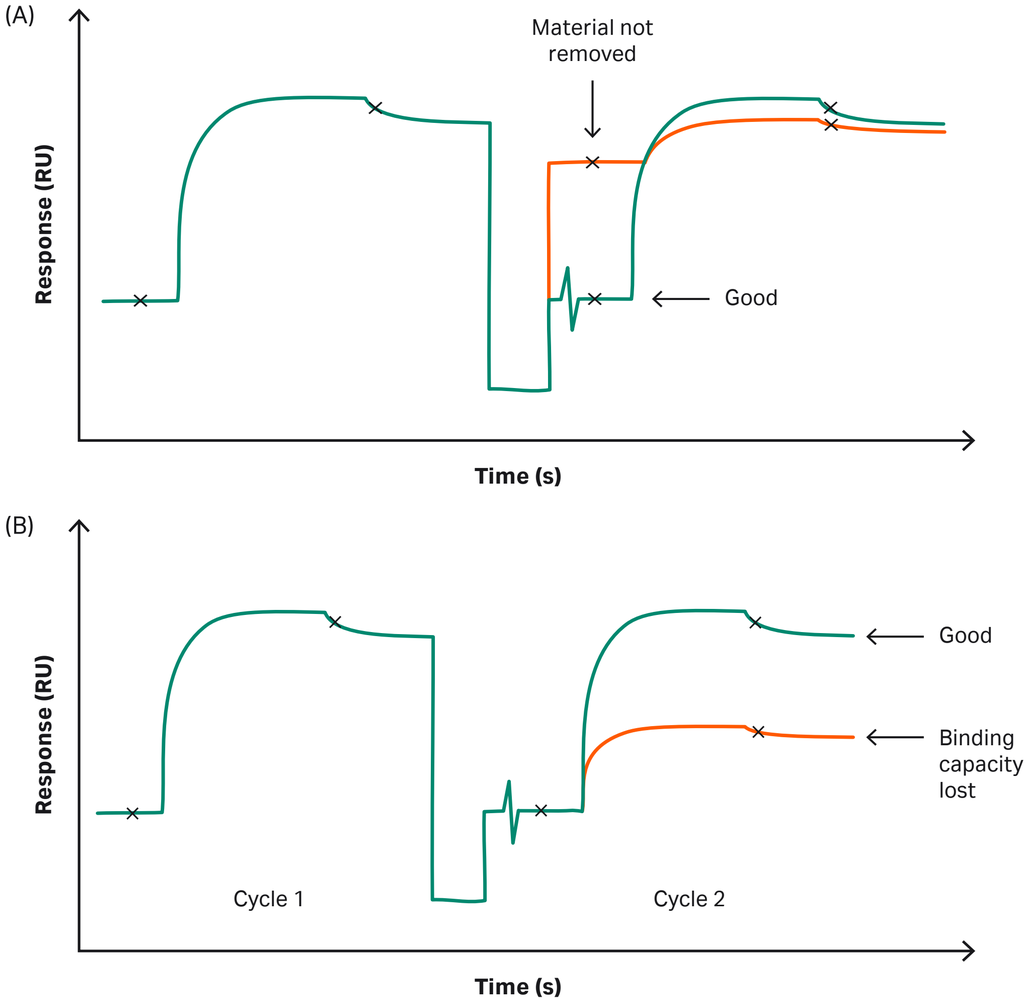

Fig 6.2. Efficient regeneration removes all bound analyte. Inefficient regeneration leaves analyte on the surface seen as an increase in the baseline (A). The analyte response in the second injection reveals whether the ligand is still fully active (B).

Figure 6.3 illustrates the general principles of regeneration scouting assessment in terms of response plotted against cycle.

- The first condition tested (A) is too mild. The analyte response is consistently low and the baseline is consistently increased, indicating that analyte bound in the first cycle is not removed.
- The second condition (B) shows some improvement but the progressive increase in analyte response indicates that binding capacity is not completely restored and regeneration is still too mild.
- Condition (C) gives a stable analyte binding and a stable baseline.
- Condition (D) results in a decrease in analyte binding response, which indicates that the regeneration is too harsh.

In conclusion, C is the optimal condition of the four conditions tested.

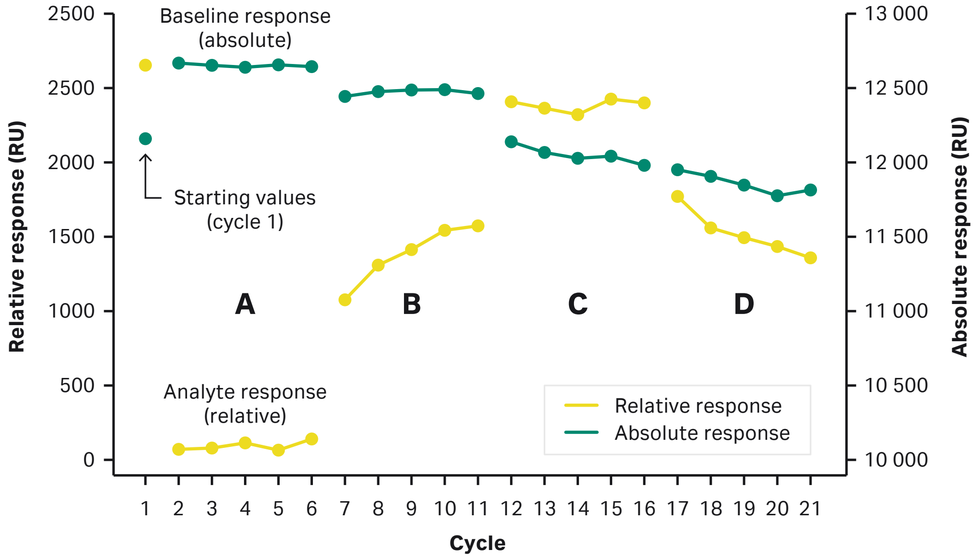

Fig 6.3. General principles of scouting for regeneration conditions.

Some further considerations in assessing regeneration behavior include:

- The first cycle on a newly prepared sensor chip often shows a analyte response that differs from subsequent cycles. Ignore the first cycle if this is observed.
- 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.
- 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 sensorgrams of the analyte binding from the regeneration scouting cycles can help to reveal changes in the interaction behavior that might be missed in a trend plot (e.g., 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.

6.3 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. We recommend a minimum of 20 cycles.

The acceptance criteria 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 nonbinders. As a general recommendation, good regeneration conditions should give an analyte response that does not vary by more than 5% to 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 to 25 rather than cycles 1 to 20.

6.4 Suggested regeneration solutions

This section offers some recommendations for regeneration solutions based on the literature and our experience. 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 to 1.5).

Other proteins

Although the range of protein-protein interactions studied with Biacore™ SPR systems is very broad, experience has shown that a relatively small set of conditions provides suitable regeneration. Many surfaces can be regenerated using one of the following solutions:

- 10 mM glycine-HCl pH 3.0 to 1.5 (available ready-to-use from Cytiva)
- 50% to 100% ethylene glycol
- 1 to 100 mM NaOH
- 1 to 4 M $MgCl_2$
- 0.5 to 5 M NaCl
- 0.02% to 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 3.2.6). In some cases, regeneration can also be performed simply by injecting buffer without the metal ion required for binding. The same principle can be applied to interactions that require the presence of, for example, a cofactor.

When the interaction requires a high salt concentration, regeneration may be performed by lowering the salt concentration or using only ultrapure water in the regeneration step.

6.4.2 Nucleic acids

Hybridized oligonucleotides can usually be regenerated with freshly prepared 1 mM HCl or by using a mixture of guanidine hydrochloride and NaOH.

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

6.4.3 Serum and plasma

Nonspecific binding can be a problem with samples in serum or plasma. An injection of 50 mM NaOH is usually effective in removing nonspecific material. If the specific analyte is not removed by this treatment, use two consecutive injections, one of NaOH and one directed at the analyte.

6.4.4 Membrane-associated ligands

Lipid monolayers on Sensor Chip HPA

Regeneration of Sensor Chip HPA surface is directed towards removing bound analyte from the ligand. Regeneration 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 extremes of pH such as 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. Cytiva does not guarantee the performance of Sensor Chip HPA treated in this way.

Supported lipid bilayers

Proteoliposomes 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. The same approach as for ligands covalently attached to CM-series and other sensor chips can be used but make sure that regeneration solutions do not contain detergents or organic solvents.

For surfaces prepared with pure liposomes, the lipid can be removed with a 10 to 30 s injection of 2:3 isopropanol:50 mM NaOH. 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 (on surface reconstitution) 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.

6.4.5 Small molecules

Small molecules are generally more tolerant of harsh conditions than large molecules so 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 to 100 mM NaOH in 30% acetonitrile. This solution is not stable and must be prepared fresh each day.
- 20 to 100 mM NaOH containing 0.5% Surfactant P20 or 0.05% SDS
- 10 mM glycine-HCl pH 3.0 to 1.5 (available ready-to-use from Cytiva)
- 1 to 4 M $MgCl_2$

6.4.6 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 to 100 mM HCl
- ~ 0.1% trifluoroacetic acid
- ~ 1 M formic acid
- ~ 1 M ethanolamine-HCl at pH 9.0 or higher

Denaturing agents such as urea or guanidine hydrochloride can be useful if the ligand is resistant to the conditions. For example, 6 M guanidine hydrochloride with 0.25 M NaOH is recommended for regeneration of Sensor Chip CAP (included in Biotin CAPture Kit).

In particularly stubborn cases, or when several components from the sample may bind to the surface, a mixture of different regeneration solutions (e.g., acidic and chaotropic conditions) may prove fruitful. The control software for most Biacore™ SPR systems support regeneration with two or more consecutive injections. This approach can in some cases be an alternative to mixed reagents. Using two consecutive regeneration pulses of the same solution has proved successful in some cases, as has optimization of regeneration contact time.

07

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 not lost during storage. For recommendations on storage and reuse of sensor chips refer to Chapter 8.

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 to 40 µL/min) and, if possible, a contact time that is long enough for the binding curve to approach saturation. 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 (Fig 7.1). The maximum analyte binding capacity compared with the theoretical value (*see* 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.

Determining maximum binding capacity empirically requires that sufficiently high concentrations of analyte are used. The concentration should be several times higher than the steady-state dissociation constant of the ligand-analyte complex (at a concentration equal to the dissociation constant, the maximum binding that can be achieved corresponds to 50% of the maximum binding capacity for a simple 1:1 binding). An alternative approach is to estimate the binding capacity (R_{max}) by analyzing kinetic or steady-state affinity measurements. Details are described in Kinetics and affinity measurements with Biacore™ systems.

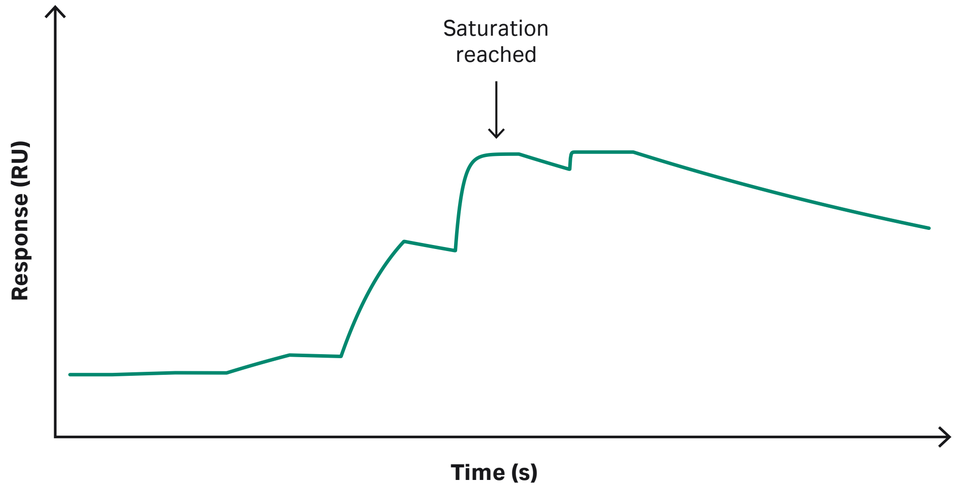

Fig 7.1. Repeated injections of analyte without regeneration can be used to estimate the maximum analyte binding capacity of the surface.

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 preconcentration of ligand on the surface is not satisfactory (Fig 7.2):

- Test preconcentration at different pH values. Generally, pH values down to about 3.5 can be used. For pH 3.5 to 4.0 citrate buffers should be used. If preconcentration 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 5.5 to 7.2 and MES buffers at pH values in the range of 5.5 to 6.7.
- 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, that is, when the ligand injection has not reached saturation.
- Increase the ligand concentration.

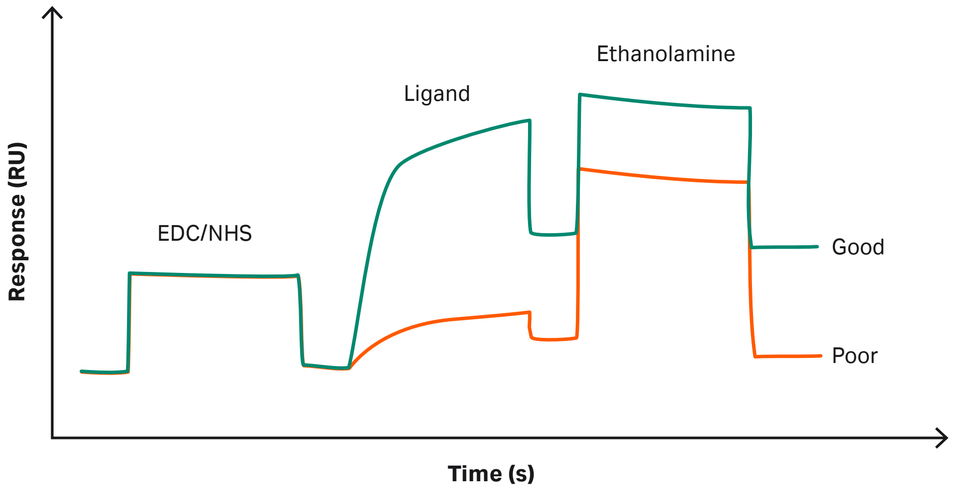

Fig 7.2. Inadequate preconcentration of ligand to the surface is seen as a poor increase in response during and after the ligand injection. This illustration shows a sensorgram for amine coupling.

If ligand is preconcentration on the surface but is not immobilized (Fig 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.

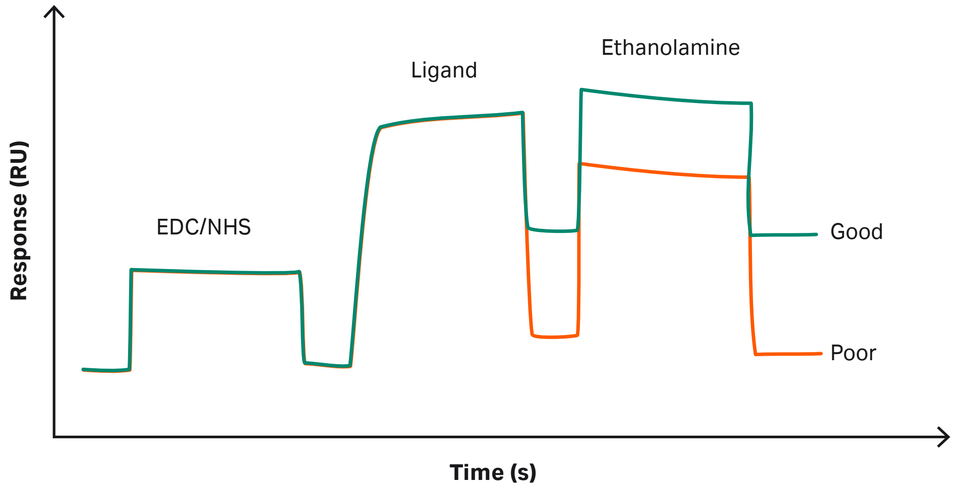

Fig 7.3. Inadequate immobilization of ligand is seen as an increase in response during ligand injection but a low level over baseline after the injection.

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 during ligand preparation or as a result of 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. For particularly difficult systems, two alternative approaches may be considered:

- 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 nonspecific binding

For some applications, particularly those where samples are complex mixtures such as serum or unfractionated cell extracts, response arising from other sources than binding of analyte to ligand can complicate the interpretation of the results. Such responses may arise from binding of nonanalyte molecules in the sample to the ligand or from nonspecific binding of analyte or nonanalyte molecules to the sensor chip surface matrix. 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 nonspecific binding of sample components to the sensor surface but can incur higher levels of unwanted binding to the ligand.

Problems with nonspecific 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, nonspecific 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 nonspecific binding, and inclusion of salt in the running buffer is always recommended. Other buffer components may have a significant effect on nonspecific binding in individual cases.

Positively charged (basic) molecules tend to bind nonspecifically to carboxyl groups on the sensor surface. This may be counteracted, if necessary, by using buffers with higher pH and higher ionic strength and/or sensor chips with lower carboxymethylation levels (e.g., Sensor Chip C1, Sensor Chip CM4, or Sensor Chip PEG).

A powerful technique for dealing with nonspecific 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 at a separate site from 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.

Buffer composition

Always use buffer containing 0.15 M salt whenever possible. Additional salt up to 0.5 M has been found to reduce nonspecific binding considerably.

Choice of sensor surface

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

In some applications sensor chips with a flat surface (no dextran matrix) work better, that is, Sensor Chip C1 and Sensor Chip PEG. Sensor Chip PEG is recommended for crude samples especially with serum and plasma compared to Sensor Chip C1.

If a sensor chip with dextran matrix is preferred, immobilization of aminomethyl-polyethyleneglycol (aminomethyl-PEG) to Sensor Chip CM5 prior to ligand immobilization has been found to reduce the level of nonspecific binding. To use this approach, activate the surface with a 10 min injection of 0.05 M EDC/0.2 M NHS. Follow the activation with an injection of 1 to 5 mM aminomethyl-PEG in 10 mM sodium borate, pH 8.5 for 5 to 10 min. Immobilize the ligand using the chosen chemistry directly after the aminomethyl-PEG injection.

The immobilization capacity of the surface is reduced after immobilization of aminomethyl-PEG, and a balance must 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 nonspecific binding further but at the expense of a reduction in ligand immobilization capacity.

The ligand immobilization chemistry can influence the level of nonspecific 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 nonspecific binding.

Where alternative ligands are available (e.g., monoclonal antibody ligands), the choice of ligand may have a decisive effect on the extent of nonspecific binding. In many cases, immobilization of Fab'_2 fragments is preferable to the use of intact antibodies, eliminating binding of sample components to the Fc portion of the antibody.

Sample additives

For sensor chips with a dextran matrix, soluble carboxymethyl-dextran added to the sample at 0.5 to 1 mg/mL can compete for molecules that bind to the dextran on the sensor surface without interfering with the analyte-ligand interaction. Dextran suitable for this purpose is available as NSB Reducer from Cytiva.

For Sensor Chip PEG unwanted binding from anti-PEG antibodies can be avoided by adding PEG (M_r 1000 to 5000) to the sample dilution buffer to a final PEG concentration of approximately 5 mg/mL. For plasma assays, avoid EDTA in sample dilution buffer as this may increase nonspecific binding.

In some cases, addition of "ligand mimics" to the sample can help to reduce unwanted binding to the immobilized ligand. An example of this approach is the addition of polyclonal antibody preparations (at 100 to 200 µg/mL) from nonimmune animals to counteract nonspecific binding to immobilized monoclonal antibodies.

08

Storing and reusing sensor chips

Sensor chips are supplied in individually sealed packages in a nitrogen atmosphere. 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 later. Decreased ligand activity is expected after each storage and reuse cycle of the chip. This chapter describes procedures for the storage of used chips. Wet storage is recommended for most ligands, while dry storage may be used for low molecular weight ligands.

Important

Due to mechanical tolerances immobilized chips should be reused with the same instrument as used for the immobilization. Reusing sensor chips with immobilized ligand is not recommended for Biacore™ 4000 and Biacore™ 8K series. Precise and reproducible positioning of the sensor chip in relation to the IFC cannot be guaranteed in these systems as previously immobilized areas on the surface may not line up exactly with the flow cells when the chip is reused.

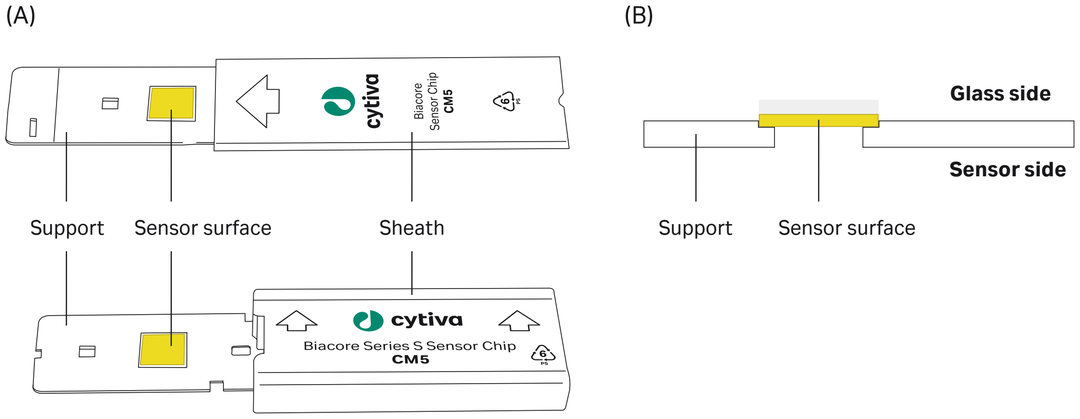

Fig 8.1. (A) Sensor chip support, sensor surface, and sheath, showing the orientation of the chip support in the sheath. (B) Side view of the chip support, indicating the glass and sensor sides.

8.1 Recommended procedure for wet storage

Materials

- Running buffer
- Saline neutral buffer, for example, HBS-EP+ buffer
- 50 mL polypropylene test tube

Storage

1. Undock the sensor chip and remove it from the instrument.
2. Remove the sensor chip support from 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 store it in a refrigerator.

Reuse

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 or dry the sensor surface itself.*** The glass sensor chip is fixed on the support so that the sensor surface is recessed with respect to the support (Fig 8.1).
4. Re-insert the support into the sheath with the glass side towards the label on the cassette. Do not force the support into the sheath. Insert the sensor chip in the instrument and dock it. Equilibrate the system with buffer by running the ***Change solutions*** or ***Prime*** software commands depending on Biacore™ SPR system.

Note: *For optimal performance, the steps mentioned for storage and reuse respectively should be performed consecutively with no waiting time in between.*

8.2 Recommended procedure for dry storage

Materials

- Silica gel with humidity indicator
- 50 mL polypropylene test tube

Storage

1. Undock the sensor chip and remove it from the instrument.
2. Remove the sensor chip support from 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 of indicating silica gel. Cap the tube securely and store it in a refrigerator.

Reuse

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 sheath, with the glass side facing the label on the cassette. Do not force the support into the sheath.
3. Insert the sensor chip in the instrument and dock it. Equilibrate the system with buffer by running the ***Change solutions*** or ***Prime*** command depending on Biacore™ SPR system.

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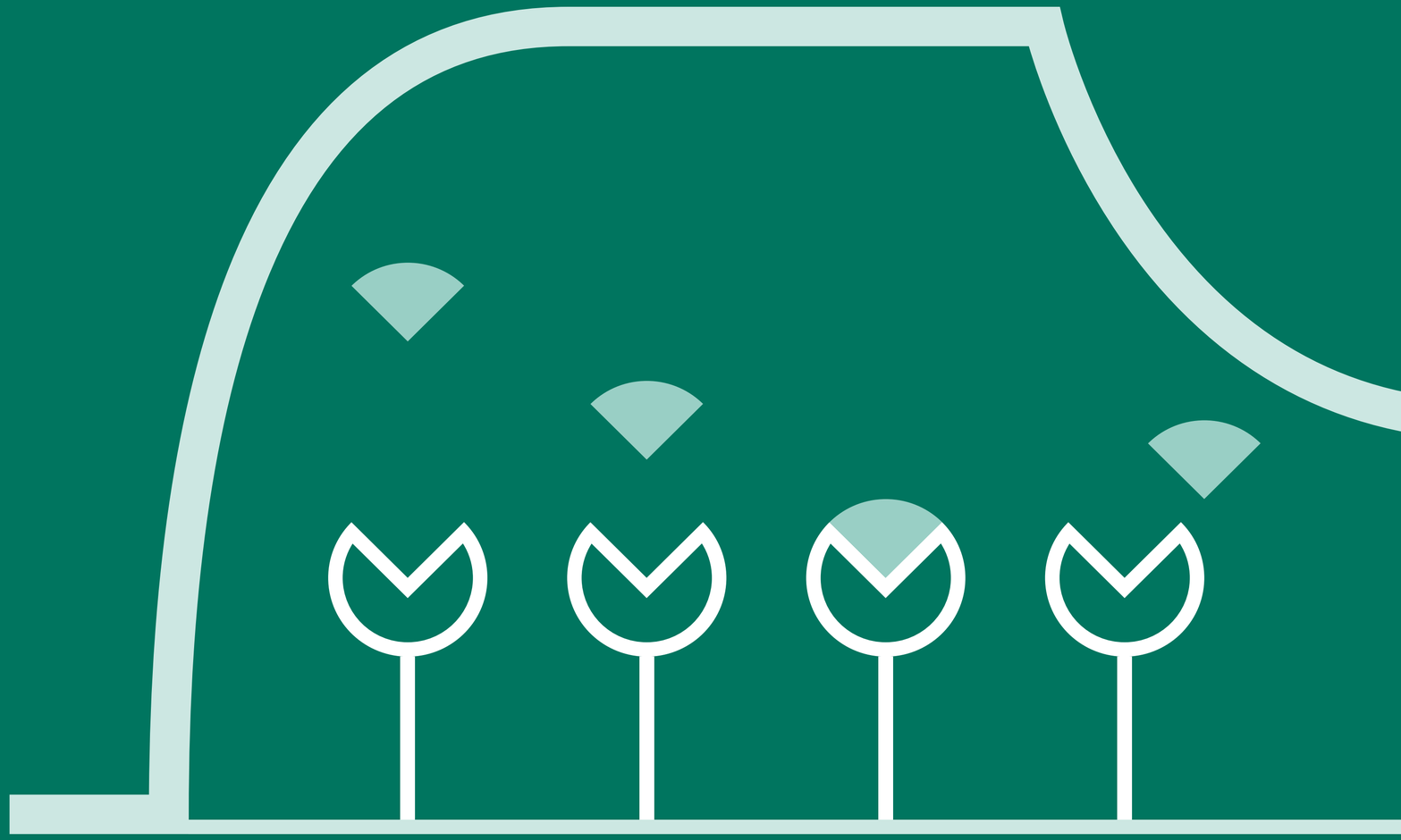

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