Guide to Ligand Immobilization on the ProteOn™ XPR36 System

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Surface plasmon resonance (SPR) has revolutionized the study of biomolecular interaction by providing a platform that does not require the ligand or analyte to be labeled. SPR measures the interaction between a ligand immobilized to the surface of a sensor chip and an analyte in solution. This measurement takes place in real time, providing kinetic, equilibrium, and concentration data. Performing interaction analysis on an active and stable ligand surface is key to generating robust data. The ProteOn XPR36 protein interaction array system is a multiplexed SPR instrument that utilizes novel fluidics to monitor the interaction of up to six ligands and six analytes. This allows for the simultaneous study of up to 36 interactions on the surface of the sensor chip greatly increasing experimental throughput and reducing assay development time.

In the simplest SPR experiment using the ProteOn protein interaction system, a ligand is covalently immobilized to the surface of the sensor chip and interacts with an analyte present in the running buffer that flows over the surface of the sensor chip. This direct immobilization of the ligand is known as “Direct Coupling” (see SPR Experimental Methods Box).

In another commonly used method, a biomolecule is used to capture the ligand prior to analyte interaction. In this case the ligand is not covalently immobilized to the sensor chip surface but is captured through electrostatic interactions.

Both methods have different advantages depending on the type of interaction study that is being carried out (see SPR Experimental Methods Box). The major steps of each method are discussed in this guide.

SPR Experimental Methods

Direct Coupling — See Part 1

Functional groups on the ligand form covalent bonds with carboxylic groups on the surface.

- **Random coupling** — amine coupling through primary amines of the ligand; this type of coupling may interfere with binding sites
- **Specific coupling** — coupling with a single cysteine residue from the ligand, presented naturally, synthesized (for peptides), or by site-directed mutagenesis

Capturing — See Part 2

A specific recognition site is used for noncovalent capture of the ligand to a molecule covalently immobilized to the surface.

- Creates a homogeneous ligand surface (well-defined orientation)
- Increases ligand purity
- Capture needs to be stable
- Regeneration removes the ligand
- Examples: IgG captured by protein A, biotinylated protein captured by avidin

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Part 1: Direct Coupling of Ligand — Covalent Immobilization Using Amine Coupling

Major Steps for Covalent Immobilization

1. Conditioning of GLC, GLM, GLH, or NLC sensor chip (see Sensor Chip Conditions Protocol Box).
2. Activation.
3. Immobilization.
4. Deactivation.
5. Stabilization.

The ProteOn amine coupling kit contains all the reagents needed for amine coupling of proteins or peptides to the sensor chip.

Sensor Chip Conditioning Step

This procedure is recommended for new sensor chips and can generally improve data quality by cleaning the new chip surface, encouraging rapid stabilization of the baseline prior to the start of the experiment. Sensor chip conditioning is optional. Conditioning is performed following the sensor chip initialization process using ProteOn regeneration solutions (see Sensor Chip Conditions Protocol Box).

Sensor Chip Conditions Protocol

GLC, GLM, and GLH Chip Conditioning Protocol

Buffer	Experimental running buffer*
Flow rate	30 µl/min
Time	60 sec

Ligand steps, default injection quality

Injection 1	0.5% SDS
Injection 2	50 mM NaOH
Injection 3	100 mM HCl

NLC Conditioning Protocol

Buffer	Experimental running buffer
Flow rate	30 µl/min
Time	60 sec

Ligand step, default injection quality

Injection 1	1 M NaCl
Injection 2	50 mM NaOH
Injection 3	1 M NaCl
Injection 4	50 mM NaOH

* When working with buffers containing metal ions, eliminate the NaOH injections.

Activation Step — Preparing for Amine Coupling

In this step reactive groups are formed on the sensor chip surface. The ligand of interest, such as a protein, is then attracted to the surface and binds through amine coupling. Any primary amine within a protein sequence can bind (lysine residues and the N terminal).

To create these reactive groups an activation solution is applied to the surface. This activation solution consists of an equivolume mixture of two reagents, EDAC and s-NHS. These two reagents are part of the ProteOn amine coupling kit (catalog #176-2410) and are prepared by addition of 7.5 ml water to each reagent bottle to make 400 mM EDAC and 100mM s-NHS, which are stored at –20ºC until needed. It is important to make this mixture fresh every time as it has a half life of 30–60 min and should therefore be used immediately. After thawing and mixing you may dilute the equivolume activation solution prior to use depending on which application you are working with. For example, a 1:1 dilution with water is suggested when high ligand density is required, such as for small molecule applications. When a less dense ligand surface is required a 1:5 or 1:10 dilution with water may be used.

Chemistry

After addition of EDAC and s-NHS to the chip, the carboxymethyl groups react and become sulfo-N-hydroxysuccinimide esters. During the ligand injection step, the ligand preferentially binds to the esters and is amine-coupled to the sensor surface.

Immobilization Step

Many factors affect ligand immobilization, including chip type, level of surface activation, ligand concentration, size, and injection parameters such as contact time, injection flow rate, and electrostatic attraction of the ligand to the surface. Electrostatic attraction is one of the most important factors because if the ligand is not attracted to the surface, there will be very little immobilization.

Optimizing Immobilization

After amine coupling, the sensor chip surface will have an overall negative charge, the ligand therefore must have an overall positive charge. This is achieved by determining the optimal immobilization buffer. Since the ProteOn system has six ligand channels, you can easily test multiple immobilization conditions (immobilization buffers of different pH) to determine which gives the highest level of immobilization. For example, BSA has a pI of approximately 5.5. To have a positive charge the protein must be dissolved in a buffer of pH less than 5.5. Therefore, one might wish to try a series of buffers with a pH range 5.5, 5.0, 4.5, and 4.0 and monitor which resulted in the highest level of immobilization. Care must be taken to ensure that the immobilization conditions used result in an immobilized ligand that retains its activity. When using buffers of extreme pH, the ligand may be denatured or unfolded and therefore lose its activity (see Troubleshooting). Detergents may also be added into the immobilization buffer but salt should be kept to a minimum, just enough to keep the ligand soluble.

Ligand Injection Parameters

Determining the ideal ligand injection parameters is important. Flow rate and contact time can have significant effects on immobilization. Default injection parameters are 30 µl/min for 5 min. Reducing flow rate will help to increase immobilization, as will increasing the injection/contact time.

Ligand Conditions

The concentration of the ligand will also affect the total amount immobilized. Typically, concentrations of 5–100 µg/ml should be sufficient to attain a good level of immobilization.

The ligand stock buffer should have a high concentration of the ligand so that when it is diluted with the immobilization buffer any salts or other additives present in the stock buffer will also be diluted. A good recommendation for ligand stock buffer is 0.5–1 mg/ml. Avoid (or minimize) any other amine-containing compounds (or any strong nucleophilic groups) such as azide or Tris buffer as these amines will compete with the ligand amines.

Guidelines for Immobilization Levels

Choosing what level of ligand immobilization to use is dependent on the type of interaction under study. However, "less is more" is a good guide and this is generally followed for kinetic binding measurements. With a high density surface, mass transport issues and crowding effects may result in altered kinetics (see Troubleshooting).

An easy way to help determine which ligand level to use is to calculate the theoretical R_{max} of the interaction to be studied.

Using R_{max} to Determine Ligand Immobilization Levels

The theoretical R_{max} is the maximum analyte response, assuming all of the ligand is active, ligand is 100% pure, and all binding sites are available. When using amine coupling, assume that all ligand binding sites will not be available after immobilization since this is a random coupling of the ligand to the sensor chip and therefore the ligand is not present in a homogenous orientation at the sensor chip surface (see Figure 1).

$$R_{max} = \frac{MW_A}{MW_L} \times R_L \times n$$

R_{max}, maximum theoretical response of the analyte for a given ligand level.
R_L, amount of ligand immobilized.
MW, molecular weight.
n, stoichiometry of the reaction.

Fig. 1. Determining theoretical R_{max}. The standard analyte response that gives the best data is between 100–200 RU.

Example of Using R_{max} in Protein-Protein Interactions

To study the interaction of the ligand (protein A, 10 kD) with the analyte (protein B, 5 kD) and achieve a 200 RU response of protein B, assuming maximum ligand activity, purity, and correct orientation, 400 RU of protein A must be immobilized. However, it is usual to immobilize twice as much, for example, 800 RU of protein A, to account for the fact that not all protein A ligands may be active (see Figure 2 and Immobilization Protocols Box).

$$200\ RU = \frac{5\ kD}{10\ kD} \times R_L \times 1$$

$$200\ RU = \frac{1}{2} \times R_L$$

$$400\ RU = R_L$$

RU, response units.
R_L, amount of ligand immobilized.

Fig. 2. Using R_{max} in protein-protein interactions. At least 400 RU of protein A ligand (10 kD) must be immobilized for a 200 RU response of protein B (5 kD) to be seen, assuming maximum ligand activity, purity, and correct orientation.

Example of Using R_{max} in Protein–Small Molecule Interactions

To study the interaction of a small molecule analyte, (compound X, 1 kD) with the ligand, (protein Y) and achieve a 200 RU response of compound X the following formula can be used (see Figure 3 and Immobilization Protocols Box).

$$200\ RU = \frac{1\ kD}{75\ kD} \times R_L \times 1$$

$$200\ RU = \frac{1}{75} \times R_L$$

$$15{,}000\ RU = R_L$$

RU, response units.
R_L, amount of ligand immobilized.

Fig. 3. Using R_{max} for protein–small molecule interactions. At least 15,000 RU of protein Y ligand (75 kD) must be immobilized for a 200 RU response of peptide X to be seen, assuming maximum ligand activity, purity, and correct orientation. More ligand may be immobilized to counter activity and orientation effects.

Immobilization Protocols

Protein-protein

1. Activation: 1:10 or 1:5 dilution with water of the equivolume activation mixture (EDAC + s-NHS), inject for 5 min.
2. Binding: 5 min injection at 30 µl/min of 30 µg/ml protein*.
3. Deactivation: 5 min injection at 30 µl/min of EA.
4. Stabilization: PBST or regeneration solutions.

Protein–small molecule

You need a high level of activation to immobilize high protein densities.

1. Activation: 1:1 dilution with water of the equivolume activation mixture (EDAC + s-NHS), inject for 5 min.
2. Binding: 5 min injection at 30 µl/min of 50 µg/ml protein*.
3. Deactivation: 5 min injection at 30 µl/min of EA.
4. Stabilization: PBST or regeneration solutions.

* A second ligand binding injection may be performed if necessary to achieve the desired immobilization level.

Choosing a Sensor Chip for Amine Coupling

Which sensor chip is used to study the interaction will be dependent on the level of immobilization of ligand that is required and on the specific application. A complete guide to the different ProteOn sensor chips is given in the ProteOn system user manual (pp 48). In short, for protein-protein interactions, GLC and GLM chips are sufficient. For protein–small molecule interactions GLM and GLH chips are the best choice.

Deactivation

The injection of 1 M ethanolamine follows the ligand immobilization step and deactivates any unreacted sNHS ester groups. Default injection parameters are 30 µl/min for 5 min.

Stabilization

This step is generally performed to ensure that any noncovalently attached proteins that may still be electrostatically held at the sensor surface are removed prior to the analyte injection and interaction analysis.

Stabilization buffer is injected across the surface. The type of buffer ranges from running buffer to harsher solutions like 50 mM NaCl and 50 mM NaOH. Care should be taken not to use a stabilization buffer that is so harsh that the immobilized ligand is denatured. This will reduce its activity, affect the interaction analysis, and reduce analyte response. Use short injections of 30–60 sec with high flow rates of 100 µl/min to reduce this possibility. After the stabilization injection is complete, look for a stable baseline. If the baseline drifts, a second stabilization injection may be needed or a harsher buffer may be used.

Part 2: Noncovalent Ligand Capture

For some applications such as antibody screening or capturing proteins using tags, immobilization of the ligand of interest directly to the sensor chip surface may not be desired. In such a case, selective capture of the ligand from a crude sample for subsequent analysis with an analyte may be preferred (see SPR Experimental Methods Box).

Major Steps for Noncovalent Ligand Capture

1. Immobilization of capture reagent or biomolecule (includes activation, immobilization, deactivation, and stabilization — see previous section). If working with biotinylated proteins a pre-prepared sensor chip such as the NLC chip may be used.
2. Injection of solution containing ligand to be captured, such as crude hybridoma supernatant or tissue culture lysate.
3. Removal of nonspecifically captured biomolecules and stabilization of ligand capture prior to interaction analysis.

Noncovalent Ligand Capture Using Capture Proteins — Antibody Screening

In some cases, capture of the ligand of interest from a crude sample, such as a hybridoma supernatant or phage display supernatant, prior to analysis with an analyte may be the method of choice. This type of noncovalent capture is ideally suited to the ProteOn system as the 6 x 6 array allows for the rapid screening of hundreds of antibodies, see bulletin 5540 for full experimental details.

Ligand Capture Conditions for mAb Screening

To capture a mAb from a crude hybridoma supernatant, create a sensor chip that contains a relevant capture protein to capture the mAb, such as an anti-IgG antibody or protein A/G. These anti-IgG and protein A/G surfaces can be created using the direct amine coupling method described previously.

Using R_{max} to Determine Ligand Capture Conditions for mAb Screening

Consideration must be taken to ensure that enough of the mAb is captured to be able to interact with its analyte. The level of mAb captured is dependent on the amount of mAb available in the supernatant and on the efficacy and immobilization level of the capture protein. In this case, the R_{max} equation must be used twice. First, determine how much mAb must be captured to be able to see an analyte response of ~200 RU (see Figure 4A). Second, calculate how much of the protein capture anti-IgG or Protein A/G would need to be immobilized to attain the required mAb level (see Figure 4B).

A. Amount of mAb to see 200 RU of protein Z

$$200\ RU = \frac{10\ kD}{150\ kD} \times R_L \times 1$$

B. Amount of anti-IgG to see 3,000 RU of IgG

$$3{,}000\ RU = \frac{150\ kD}{150\ kD} \times R_L \times 1$$

Fig. 4. Using R_{max} for hybridoma screening interactions. Protein Z (10 kD) is a cell surface protein that is overexpressed in cancer. The mAb is captured from the supernatants by an anti-IgG antibody (150 kD) already immobilized on the surface. In this case two interactions should be considered: 1) anti-IgG and mAb and 2) mAb and protein Z. Start with the second. For 200 RU of protein Z, at least 3,000 RU of the mAb must be captured. If everything is at theoretical maximum, it means at least 3,000 RU of the anti-IgG is required.

Noncovalent Ligand Capture Using NeutrAvidin-Biotin — The ProteOn NLC Sensor Chip

The ProteOn NLC chip allows for the selective capture of ligands that contain a biotin tag, such as proteins, DNA, or liposomes. The NLC chip comes preprepared with NeutrAvidin immobilized to its surface. This chip is suitable for subsequent protein-protein and protein–nucleic acid interactions, see page 48 of the ProteOn system user manual and bulletin 5449 for more information.

Ligand Capture Conditions for NLC Chip

For each new biotinylated ligand test a range of concentrations to determine the ideal capture conditions. We suggest 20–40 µg/ml protein in a physiological buffer at 30 µl/min for 5 min, although for some proteins lower concentrations may be sufficient. When working with biotinylated DNA the recommendation is to start with a concentration of 0.5 µM and use a flow rate of 30 µl/min with a contact time of 5–10 min. Ligand capture contact time can be increased or decreased as needed by altering injection parameters. For specific application examples see bulletin 5449.

It is advisable to use the R_{max} equation to determine what level of biotinylated protein or DNA is needed to see the analyte response.

Troubleshooting

Problem	Possible Causes	Solution
Working with acidic proteins	Acidic proteins are difficult to immobilize by amine coupling as they require buffer conditions that may be denaturing, and may neutralize the activated negative sulfo groups on the chip surface and prevent attraction	Try a capture method or biotinylation of the acidic protein
Enhancing immobilization	The amount of protein immobilized is too low	Increase contact time Lower flow rate Increase protein concentration Optimize pH
Ligand immobilized but no interaction	Protein may no longer be active because the immobilization conditions are too harsh (too strong pH or salts)	Use positive control to gauge the activity of the immobilized protein
	The active site on the protein may be buried because of the random immobilization orientation	Try a capture method to ensure correct orientation
	Enzymes may only be active if immobilized in the presence of another molecule or cofactor or to protect the active binding site	Immobilize in presence of protecting molecule or cofactor
Mass transport	This occurs when the rate of diffusion of the analyte from the flow is slower than the rate of association of the analyte to the ligand	Reduce ligand density or increase analyte flow rate

Summary

The protocols and methods given in this document are meant as a foundation to create your own experimental protocols and methods specific to your individual research projects.

Exact optimal experimental conditions will vary according to your specific interaction/binding partners. We strongly recommend they be determined as this will lead to consistent, publication quality data.

For further technical considerations and discussion, please contact your local ProteOn technical support team or Field Applications Scientist.

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