

AUTOLAB APPLICATION NOTE

Autolab ESPRIT

Ligand Immobilization Strategies: Preparation and considerations

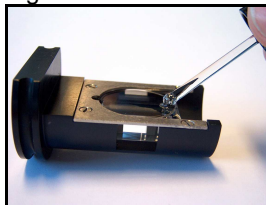
To ensure optimal results using the SPR technology a number of choices have to be made. These include choice and placement of the measurement chip, surface priming, composition of buffers, immobilization procedures, regeneration conditions and stabilization runs.

Below some assistance for practical application and proper use of the ESPRIT is given.

Choice and placement of the sensor chip

Two items need to be considered when choosing which of the molecules involved in the interaction has to be immobilized. Firstly the available reaction schemes, either covalently (amine, ligand thiol, surface thiol, or aldehyde) or via affinity (streptavidin-biotin, NTA/His-tagged, lipid layer, or antibody-antigen). Secondly, the presence of a suitable coupling group in the ligand far-away from its binding region, e.g. a primary amine in a peptide (N-terminal, lysine). In a protein these groups are readily available, however, small ligands contain fewer reaction sites and it is advisable to attach a spacer to increase the distance between coupling group and binding epitope. In this note only interactions in a water environment are considered like protein-protein or protein-small molecule. To investigate such interactions a suitable high quality hydrophilic surface is needed. Both Biacore and Xantec provide sensor chips with e.g. carboxymethylated dextran surfaces, to be positioned inside the ESPRIT on top of a slider with a glass prism (see figure 1).

Figure 1.



cuvette. Leakage may arise from wrong positioning of the cuvette, or dissolution of the glue using too aggressive solvents (see the sensor chip manual for their applicability), or leakage can occur from application of higher temperatures. Be sure to press the cuvette carefully but firmly against the chip surface. SPR gold disks are larger in size and not glued (see figure 2.). However, their dextran density (in case the disks come from Xantec) on top of the gold is less thus possibly leading to a lower binding capacity. Practically, it highly depends on the interaction under study, which type and manufacturer of sensor chips should be best suited for the job.

Priming prior to immobilization

A new sensor surface has to be primed to rewet the dry dextran layer. This pre-soaking is essential for efficient and reproducible coupling, stable baselines, and it removes contaminants. Several solutions can be used alternating and with different contact times. A well tested guideline may be a repetitive sequence of running buffer, 100 mM HCl, buffer, 100 mM NaOH, buffer, all in one minute intervals lasting 1

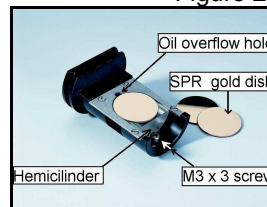
hour. In certain cases also SDS (0.2 %) and 10 mM phosphoric acid can be used. **Caution:** SDS may stick to the dextran and disturb the immobilization procedure to follow. SDS causes extra matrix swelling, which may lead to higher but variable coupling loads and this is not always advisable [1]. A gentler alternative for SDS could be overnight soaking in buffer to fully swell the dextran matrix.

Buffer compositions and isoelectric point of the coupling ligand

To immobilize effectively with higher coupling yields it is essential to increase the ligand concentration close to the sensor surface during reaction. Therefore, the ligand should be dissolved in a low salt buffer, e.g. 10 mM Na-Acetate. This provides an environment where the ligand pre-concentrates to the surface by electrostatics. The negatively charged carboxylated hydrogel can interact with positively charged residues in the ligand, thus attracting the ligand towards the chip. By choosing a pH that is 0.5 to 1 lower than the isoelectric point (pI) of the ligand a positive charge is generated [1]. However, in strategies where this is impossible, e.g. ligands with pI values below 4, a high salt buffer may be used: 100 mM Na-Borate, 1 M NaCl, pH 8.3 [2]. Salt shields the negative charge in the ligand. Be aware that under these conditions the concentration of the ligand needs to be 10-100 fold higher. The optimum pH for the EDC/NHS chemistry lies around 8.

Pre-concentration levels for proteins simply can be examined prior to surface activation by changing the buffer pH. The surface can be regenerated afterwards for a new test. Small molecules will not show sufficient shifts in plasmon signals during pre-concentration; the optimal concentration has to be evaluated from the R_{max} values (see appl....) after coupling. Therefore, prior knowledge of their pI value is useful. Realise that compared to proteins in

Figure 2.



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general small molecules are more difficult to couple. However, if small molecules are used as analyte they are difficult to detect (tiny SPR shift due to small mass change). Very dense protein immobilized surfaces are needed for such a set-up.

Normal experimental running buffer consists of 10 mM Hepes, 4 mM EDTA, 150 mM NaCl, pH 7.4, filtered over 0.2 μm , and supplemented with 0.005% Tween-20. This buffer is well designed for most interaction studies, because its composition is physiological and prevents non-specific binding (high salt and surfactant). Other buffer pH values can be used [3].

Immobilization of the ligand

In most immobilization schemes EDC/NHS is the surface activating reactant of choice. EDC is very sensitive and should be used from frozen (-20°C) aliquots. Defrost, mix with NHS, and use immediately. Present this solution to the sensor surface, either manually (pipetting) or via an immobilization sequence routine, without air bubbles entrapped that could lower coupling efficiency. Under conditions of pre-concentration the ligand should be in a concentration range of 50-500 $\mu\text{g/ml}$. The coupling efficiency is linked with pH: the pH should be between pH 3.5 and 8.5, and a lower pH requires a higher ligand concentration. Below pH 3.5 the dextran carboxylic groups become protonated. Also the ligand contact time can be important. Some ligands react fast (one minute), while others are less effective. Contact times longer than 30 minutes are useless due to the short half-life of the activated NHS-ester. **Important:** with high affinity and fast interactions the bulk solution can be depleted from free analyte in a diffusion layer just above the surface [1]. Lowering the ligand capacity on the surface can decrease this mass transport limitation effect. More reliable binding data and faster kinetics

are obtained. Make an effort for R_{max} values not higher than 80 m^2 . Begin with low ligand concentrations. The EDC/NHS activation can be repeated at least three times on the same chip to increase the loading.

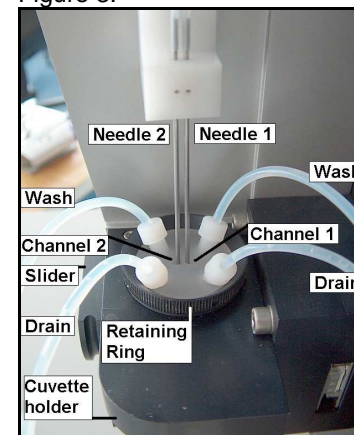
Stabilization conditions prior to final interaction analysis

When the ligand is finally covalently coupled the interaction can be tested. If indeed binding is observed it is vital to obtain stable baselines. Therefore after each binding experiment the surface should be completely regenerated. Very often in the first twenty experiments variable results will be obtained. This effect may reside from unstable swollen hydrogels, or non-specifically bound ligand still not fully removed after coupling that now slowly diffuses from the surface, or it is the bound analyte that is not completely removed by the regeneration procedure. To find the best regenerative conditions depends on the stability of the attached ligand. Start mildly with 50-100 mM HCl or 10 mM NaOH [1]. If this is not effective enough add to these solutions 0.2-0.5% SDS [2]. Another mild solution is 50-100 mM glycine pH 2-3. Use more harsh conditions only if the ligand can withstand them. One should attempt to achieve stable and constant baselines and R_{max} values that only slowly decrease in time.

Qualitative analysis of the SPR signals is most practical using a reference signal to subtract every signal effect, like those induced by bulk or temperature that is not related to the interaction of interest. **Caution:** A surface activation procedure changes the overall charge on the dextran, leading to differences in non-specific binding between measuring and reference channel. Therefore, a good reference channel is synchronized with everything the measuring channel experiences including the immobilization step, however, without introducing a

ligand. It should be emphasized, single channel measurements with independent control measurements are also possible, e.g. in case of very complex interactions. Examples in [1-3]. (See Figure 3.)

Figure 3.



References

1. Fischer MJE, Kuipers C, Hofkes RP, Hofmeyer LJF, Moret EE, de Mol NJ, *Biochim Biophys Acta* 1568: 205-215, 2001.
2. De Mol NJ, Plomp E, Fischer MJE and Ruijtenbeek R, *Anal Biochem*, 279: 61-70, 2000.
3. De Mol NJ, Gillies MB and Fischer MJE, *Biorg Med Chem* 10: 1477-1482, 2002.