Chapter 6

Protein–Ligand Interactions Using SPR Systems

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Abstract

Surface plasmon resonance (SPR) biosensor technology has become an important tool for drug discovery and basic research. SPR instruments are used for a wide variety of applications including determining the binding kinetics and affinity of an interaction, specificity studies, screening, assay development as well as concentration measurements. The interacting molecules may be proteins, peptides, lipids, viruses, nucleic acids, or small organic molecules such as fragments or drug candidates. The ease with which real time information can be obtained has changed many customer workflows in both antibody and small molecule/fragment interaction analysis, from label based and affinity/IC_{50} based workflows towards a label free and kinetic based workflow. This chapter focuses on applications for drug discovery, and outlines the experimental design for screening and selection of small molecules from a focused library. Also, determination of kinetics and/or affinity constants of selected ligands, using established SPR methodology is described, together with potential issues during assay development, running of the assay, and results interpretation.

Key words Biosensor, Surface plasmon resonance, Screening, Ligand interactions, Kinetics, Affinity

1 Introduction

The interest in applying optical biosensor technology, such as Surface plasmon resonance (SPR), in drug discovery is growing (1), driven by the remarkable development of methodology and instrument technology. By measuring changes in refractive index close to a sensor surface these biosensors allow the user to study interactions between immobilized molecules and molecules in solution, in real time and without labeling. Observed binding levels and binding rates can be interpreted in different ways to provide information on the specificity, kinetics and affinity of the interaction, thermodynamics or on the concentration of a molecule.

As a result of the increased interest in SPR for interaction analysis there are now several commercially available SPR systems with different advantages when it comes to sensitivity, throughput, sample requirements, software, ease of use, and overall performance. The applied methodology is partly overlapping for several

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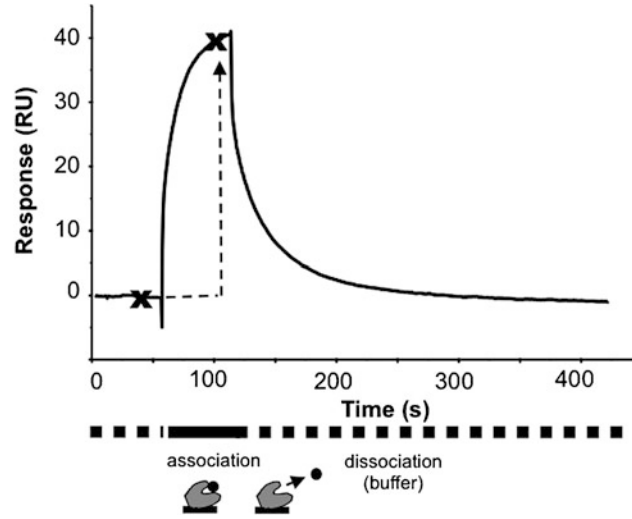


Fig. 1 Schematic illustration of a sensorgram. The bars below the sensorgram curve indicate the solutions that pass over the sensor surface. *Dotted bar* indicates running buffer, while *solid bar* indicates injection of a ligand solution resulting in binding of that ligand to a target immobilized on the chip. The shape of the sensorgram provides information about the interaction event. At time 60 s ligand is injected and starts to bind to the immobilized protein. At 120 s the ligand solution is replaced by running buffer and the ligand starts to dissociate from the target. A report point (X) records the response on a sensorgram at a specific time, typically relative to another report point. For screening and affinity analyses the report point set just before end of injection is used

systems, but our experience is mainly with Biacore™ (GE Healthcare Bio-Sciences AB) which is why the use of these systems is the focus of this chapter.

Setting up an assay with Biacore involves preparing the sensor surface by attachment of the target protein and establishment of suitable assay conditions for the ligand (*see* **Note 1**) interaction. To analyze samples, sample solution is injected over the sensor surface using automated sample handling facilities. All steps in surface preparation and analysis are monitored in a sensorgram, where changes in the molecular concentration at the sensor surface are recorded with time, Fig. 1. Experimental setup and evaluation of the results is supported by dedicated software.

Sensorgrams display the formation and dissociation of complexes over the entire course of an interaction, with the kinetics (association and dissociation rates) revealed by the shape of the binding curve. Binding responses are measured in resonance units (RU) and the response is directly proportional to the concentration of biomolecules on the surface. Report points set at specific times record obtained response levels. If the dissociation rate is rapid, the sample dissociates completely within a short time and the surface can be used directly for the next analysis. For slower dissociation

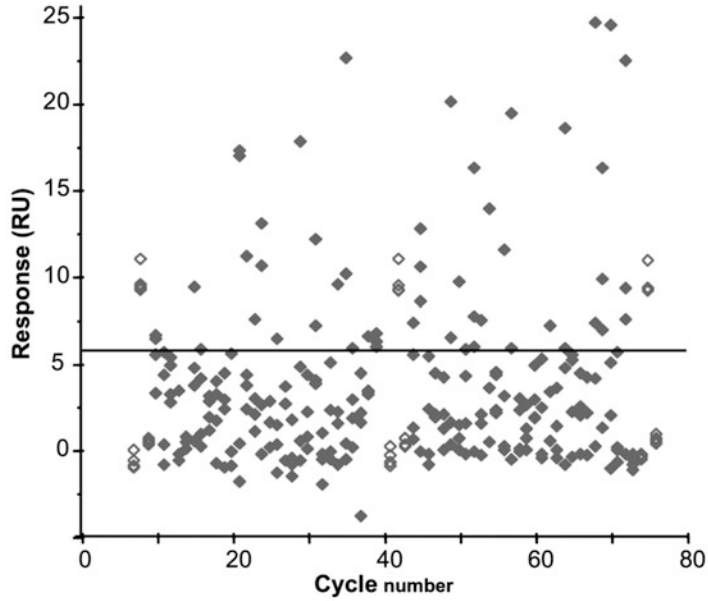


Fig. 2 Screening results from a fragment library (240 fragments with molecular weights 90–300 Da), binding to human thrombin. Screening was performed with Biacore 4000 where four different samples were analyzed in each cycle, against thrombin which was immobilized in all four flow cells. Sample concentration was 1 mM. A cut-off line was drawn based on obtaining a reasonable number of selected binders for further kinetic characterization. Controls are marked as open diamonds

rates, the surface can be washed with an injection of regeneration solution designed to remove bound ligand without affecting the protein on the sensor surface.

While true high throughput screening (HTS) with >10,000 samples is still more amenable to techniques based mainly on fluorescence (see Chapter 13), recent developments make Biacore very suitable for secondary screening of focused libraries as well as fragment screening where libraries typically contain 1,000–5,000 compounds. Figure 2 shows an example of fragment screening results.

This chapter focuses on the experimental design for screening and selection of small molecules (ligands) from a focused library, as well as the kinetic characterization of selected ligands. The protocols for characterization apply to a more basic-research style interaction study in the same way. Guidelines for assay development, running of the assays and results interpretation are given.

A quite different screening approach is the case where a ligand of interest is already at hand, and instead screening of possible target proteins is performed. Immobilization of small molecules or peptides for this approach may impose special challenges depending on the molecule. Some general guidelines for this are also given.

2 Materials

Materials for interaction studies using SPR include the protein(s) and compounds, sensor chips, immobilization reagents, buffer, and solvent correction solution. Depending on the application, regeneration and wash solutions may also be required. Materials are described along with the methods.

2.1 Selecting Buffers

Suitable running buffers for small molecule interaction studies include phosphate buffered saline (PBS) and Tris buffers. Additives such as reducing agents, cofactors necessary for target activity and glycerol may be included. It is highly recommended to include a detergent such as Surfactant P20 (GEHC) at a concentration above the critical micellar concentration (CMC) to stabilize the system; for Surfactant P20 this is typically 0.05 %. If required, dimethyl sulfoxide (DMSO) may be added to a concentration of typically 2–5 % to maintain ligand solubility, provided that the activity of the target protein is not compromised. When Sensor Chip NTA is used 50 μM EDTA should be added to the running buffer to chelate trace amounts of metal ions that otherwise may interfere with protein attachment to the sensor chip.

In general, we have found that reducing the complexity of buffer composition as far as possible often simplifies interpretation of the results. The recommendation therefore is to use the simplest buffer composition that is compatible with the requirements of the interaction. For many protein–ligand interaction studies 20 mM phosphate, 150 mM NaCl, 0.05 % Surfactant P20, 2 % DMSO, pH 7.4 (with cofactors if necessary) is a suitable buffer to try as a start. Tris buffers usually also work well.

3 Methods

Whether your aim is screening of a ligand library, kinetic characterization of ligand interactions or, the other way around, screening of protein targets over a small molecule coated surface, the first step is always preparation of your sensor surface.

3.1 Surface Preparation

3.1.1 Immobilization Level

High immobilization level is preferred for *screening* of ligands to enable detection of low level binders. For *affinity* studies one should also aim for an immobilization level that gives a clearly measurable response over the whole range of ligand concentrations so that steady state response levels can be measured with more confidence. To obtain *kinetic* data the amount of immobilized protein should be kept as low as possible (maximum ligand capacity less than 100 RU) with an acceptably measurable response. Special recommendations apply for immobilization of small molecules (see Subheading 3.1.6).

Since the SPR response is directly proportional to the mass concentration of material at the surface, the ligand binding capacity of a given target protein is related to the amount of protein immobilized as follows:

$$\text{Response}_{\max} = \text{Immobilization level} \cdot \left(\text{MW}_{\text{ligand}}/\text{MW}_{\text{immob.protein}}\right) \cdot \text{Binding stoichiometry}.$$

For example, if the target molecular weight is 30,000 Da and the ligand molecular weight is 300 Da, immobilizing 5,000 RU of protein will give a theoretical ligand binding capacity of 50 RU assuming 1:1 binding and that the protein is 100 % active. In practice, the maximum observed response is also affected by the activity of the protein, the binding kinetics of ligand binding and limitations on the maximum contact time and available ligand concentration. The theoretical binding capacity can however be useful as a guide to how much target molecule to immobilize and also as a reference for assessing the activity of the surface. Measured surface activity is typically 20–80 % of theoretical maximum response.

3.1.2 Immobilization Approaches

The success of an interaction study relies in part on using an efficient and careful method for immobilization, so that the immobilized molecule will keep a high degree of activity for the duration of the experiments.

Various immobilization chemistries can be used, directed towards different functional groups on the protein (2). Here three approaches that often result in successful immobilization of proteins are described; amine coupling of target protein to the dextran matrix of Sensor chip CM5, capture of biotinylated target to a streptavidin chip, and capture of histidine-tagged target to Sensor chip NTA. In Subheading 3.1.6 some guidelines for immobilization of small molecules and peptides are given.

The running buffer described in Subheading 2.1 can often also be used during surface preparation. For immobilization procedures involving amine groups, e.g., amine coupling, running buffers containing amines (e.g., Tris and sodium azide) should be avoided. Although DMSO does not interfere with immobilization it is often omitted in the running buffer during immobilization.

3.1.3 Amine Coupling to a Dextran Matrix

Covalent immobilization of target molecules involves activation of the sensor chip followed by injection of the protein and deactivation of excess reactive groups. The most commonly used approach is amine coupling directed towards primary amine groups on the protein (3), see Fig. 3.

Amine coupling works well for many targets and the binding site for small molecules is often available after immobilization. However, for e.g., some kinases and phosphatases it can be

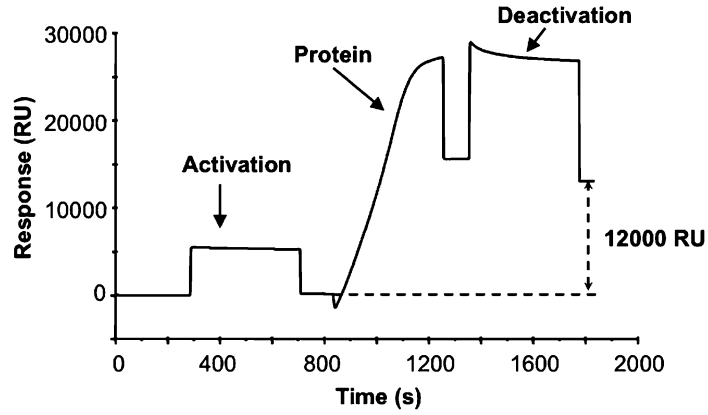


Fig. 3 Sensorgram showing amine coupling of a target protein. The first injection is activation of carboxyl groups on the dextran with a mixture of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). These reagents are mixed automatically in the instrument immediately before injection. Next, protein is injected and in this example binds to a level of ca 12,000 RU as measured from before the first injection to after the last injection. Finally any remaining active groups are deactivated by injection of ethanolamine, which also serves to remove loosely bound material. For low immobilization levels, below a few hundred RUs, the difference in binding between before and after the actual protein injection may better reflect the immobilization level. For very low levels injection of a binding molecule also helps estimate if a relevant immobilization level is obtained

beneficial to stabilize the protein by including a known binder with the target during immobilization (4, 5). The ligand concentration should then be about ten times above the K_D concentration for the known binder, to obtain close to saturation of the binding site.

Efficient immobilization of target molecules to a carboxymethylated dextran matrix, e.g., Sensor Chip CM5 or CM7, is possible from relatively dilute solutions (~10 μg/ml) thanks to *electrostatic preconcentration*. The attraction between the negatively charged dextran matrix on the sensor surface and positively charged protein molecules serves to concentrate the target in the matrix. Electrostatic preconcentration requires that the pH of the immobilization buffer is lower than the *p*I of the protein. In practice, suitable conditions for immobilization are quickly investigated using a procedure called *pH scouting*. This initial task of choosing buffer pH for immobilization on, e.g., Sensor Chip CM5 is a very uncomplicated experiment, and at the same time often very rewarding when starting with a new target protein, as it provides a lot of information in only a few minutes. The shape of the sensorgrams provides information about suitable pH together with indications about suitable protein concentration, immobilization time and occasional non-specific binding.

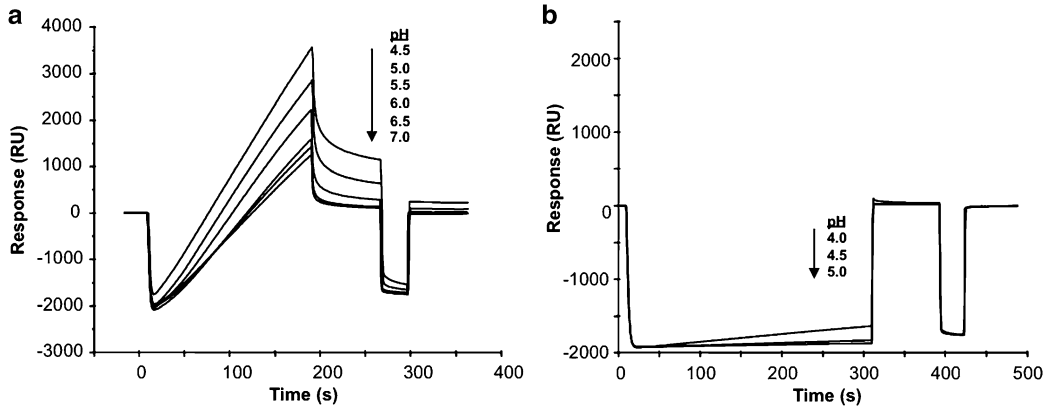


Fig. 4 Overlay plot showing results from two different pH scouting experiments. (**a**) Hemagglutinin from a human influenza subunit vaccine preparation at 5 μg/ml in buffers with indicated pH values, injected during 3 min. (**b**) A protein injected at low concentration, 0.5 μg/ml, to save material. Loosely bound protein is removed when the scouting is complete by washing with high pH (50 mM NaOH), and the surface can then be used for subsequent immobilization of protein

It is advisable to always aim for high activity of the protein and therefore to choose a buffer as mild as possible. If necessary, the time for immobilization can instead be prolonged.

For pH scouting the protein is diluted to a concentration typically in the range 1–10 μg/ml, in each of a series of low ionic strength buffers, usually in the pH range 4–7. 10 mM acetate buffers, pH 4.0–5.5 are commercially available, ready-to-use. Additional buffers are easily prepared in a "good enough" manner: 10 mM maleate, pH 6.0 and 6.5 (0.116 g maleic acid to 100 ml, pH adjusted with NaOH) and 10 mM phosphate, pH 7.0 (0.138 g $NaH_2PO_4 \cdot H_2O$ to 100 ml, pH adjusted with NaOH). The protein solutions are then injected during, e.g., 3 min over a non-activated flow cell. The flow rate is not critical, and low flow rates, e.g., 5 μl/min, may be used to save reagent, any suitable buffer can serve as running buffer. Thus, the protein is not immobilized but merely passes over the dextran matrix and the concentration of protein into the matrix is studied for different pHs. *see* **Note 2** for a few common causes of lack of preconcentration. Figure 4 shows examples of two different pH scouting experiments.

Results in Fig. 4a show that protein at all pH values displays significant preconcentration, i.e., the responses rise several 1,000 RU during 3 min, indicating that it should be possible to obtain an immobilization level of >5,000 RU for a concentration of 5 μg/ml and an immobilization time of > ca 10 min. pH 7.0 was chosen in this case. Buffers at pH 4.5, 5.0 and 5.5 all display some nonspecific binding to the dextran after the end of injection, at about 180 s. The sensorgram does not return to baseline level until after the NaOH injection at ca 270 s, when most of the remaining

bound material is washed off. If possible, conditions giving significant non-specific binding should be avoided, especially conditions leading to significant remaining binding after NaOH wash. Such non-covalently bound material may later dissociate from the surface and disturb subsequent analysis. Another immobilization buffer should then be used, or another approach for immobilization should be considered.

Results in Fig. 4b show an example where only a small amount of target protein was available and still pH scouting was valuable in determining conditions for immobilization. pH 4.0 was chosen in this case and concentration was increased for the actual immobilization.

Once suitable immobilization buffer has been established the immobilization procedure simply consists of three injections; injection of activation solution, the protein and deactivation solution, see Fig. 3. Materials for amine coupling include 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), (Amine Coupling Kit, GEHC). These are mixed (0.4 M EDC and 0.1 M NHS) in equal proportions just before injection and used for activation of carboxymethylated dextran surfaces such as Sensor chip CM5. The activity of this solution once mixed starts to decrease within minutes. 1 M ethanolamine–HCl is used for deactivation of remaining activated groups.

3.1.4 Biotinylation of the Target Molecule and Binding to Immobilized Streptavidin

The strong binding between biotin and streptavidin allows simple attachment of biotinylated molecules to streptavidin sensor chips. Although the attachment is not strictly speaking covalent, the interaction between biotin and streptavidin is of such high affinity as to be essentially irreversible. Substitution levels of around one biotin residue per protein molecule (i.e., mole equivalent) are recommended for binding to streptavidin on sensor chips (*see* **Note 3**). In general, the conditions recommended for biotinylation with commercial reagents tend to give higher substitution levels, resulting in multi-point attachment of the protein to the streptavidin chip with impairment of assay performance.

Biotinylation and subsequent binding to streptavidin is an attractive immobilization approach for sensitive targets (*see* **Note 4** for a biotinylation example). This is because neutral conditions are used both for the biotinylation step and immobilization, the latter performed by simply injecting the biotinylated target in running buffer over Sensor chip SA. 30 s to a few minutes injection at a low flow rate, e.g., 5 μl/min, is often sufficient. If only a CM5 chip is at hand, an SA chip can be prepared by amine coupling of streptavidin. This typically requires a high concentration (50–100 μg/ml) and long (20–30 min) injection time of streptavidin.

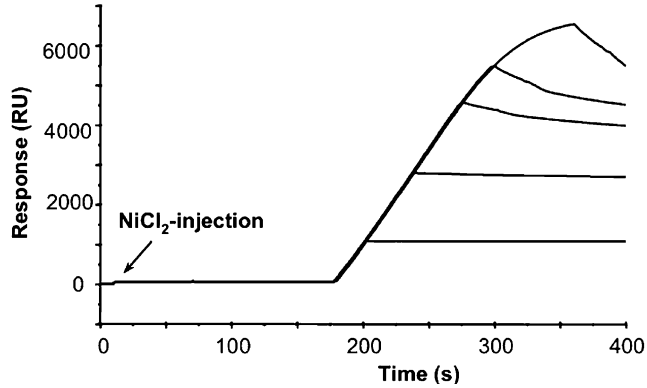


Fig. 5 Overlay plot showing binding of Histidine-tagged protein to Sensor Chip NTA. First the chip is activated by injection of 0.5 mM $NiCl_2$ (*arrow*) then histidine tagged protein is injected using five different injection times, here from 30 to 180 s. For the 30 s injection about 1,000 RU is bound to the chip and at this level binding is stable for this protein

3.1.5 Capture and Immobilization of Histidine-Tagged Proteins to Sensor Chip NTA

Poly-histidine is a widely used recombinant tag and it can chelate with Ni^{2+} ions in complex with nitrilotriacetic acid (NTA), providing a convenient approach for capturing histidine-tagged constructs on Sensor Chip NTA. This capturing approach is very attractive as it provides mild immobilization conditions for sensitive targets. It also provides homogeneous attachment points of the immobilized protein, using neutral conditions. Further, fresh histidine tagged target is used for each interaction as the Ni^{2+} is removed together with the chelated protein between each interaction cycle using a regeneration injection with EDTA. However, the latter makes capturing less attractive for screening of ligands, as more target is needed, but more attractive for kinetic characterization of hits. For *kinetics* Sensor Chip NTA is used to advantage with low capture levels.

A common mistake is to capture too much histidine tagged target; this may result in unstable binding of target to the chip. Low levels on the other hand often provide stable binding thanks to rebinding to the huge number of available NTA sites, thus may be ideal for kinetic characterization. Scouting for stable capture of histidine tagged protein to sensor chip NTA is very quick to perform. Figure 5 shows how histidine tagged tankyrase 1 (TNKS1, 5 μg/ml in running buffer) was captured during 30, 60, 90, 120, 180, or 360 s to the chip.

This protein showed stable binding level by using an injection time of 30 s, resulting in about 1,000 RU stably bound protein. With a molecular weight of 29 kDa this would give a theoretical maximum 1:1 binding level of ca 10 RU for a 300 Da compound, which proved sufficient for characterization of binding events, see Fig. 9. When capture levels are this low some further guidelines for stability may be applied (*see* **Note 5**).

Other approaches to obtain stable capture are to use more than one histidine tag on the protein if possible, or to use a tag with more than six histidines.

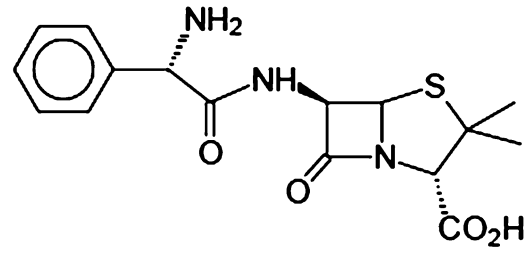
For *screening and affinity* analyses, a high level of immobilized oriented histidine-tagged target may be obtained on Sensor Chip NTA by first injecting $NiCl_2$ and then performing amine coupling of the target to carboxyl groups still available on the chip (*see* **Note 6**). Besides obtaining orientation of the target, another advantage compared to using, e.g., Sensor Chip CM5 is that near neutral buffers can be used as the immobilization buffer (often dilution in running buffer works well). Once immobilized, the same protein surface is used for all interactions.

3.1.6 Considerations for Immobilization of Small Molecules or Peptides

Immobilization of small molecules or peptides can be a valuable alternative to immobilizing the target protein, e.g., if the protein is sensitive to the regeneration conditions or if the binding characteristics of the protein are altered by coupling or modification reactions. Small molecules are in general more tolerant to regeneration conditions than the corresponding binding proteins (6). Some assay formats, in particular inhibition assays, involve immobilization of small molecules on the sensor surface. The general principles for immobilizing small molecules and peptides are basically the same as for proteins, but there are, however, some general guidelines that apply to both small molecules and peptides:

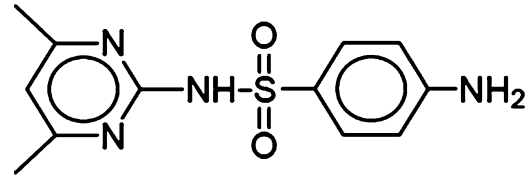
- There is a significant risk that immobilization will interfere with the binding properties of the molecule, either sterically or through alteration of the chemical properties. Care must be taken in selecting functional groups for coupling.
- The number and kind of functional groups available on a small molecule for coupling to the sensor surface is usually limited. Suitable groups for immobilization are primary amines, thiols, aldehydes, carboxylic groups and hydroxyls.
- Electrostatic pre-concentration is generally not useful for enhancing the efficiency of immobilization of small molecules or peptides. Coupling is instead performed at relatively high ligand concentrations (typically 1–50 mM) at pH 7–8.5.
- Immobilization of small molecules and peptides generally give rise to high response levels when binding protein. To achieve lower immobilization levels, required for kinetic determinations, ethanolamine, 1–95 mol %, can be added to the coupling solution.
- The amount of small molecule immobilized cannot usually be accurately assessed from the response levels since small molecules give inherently low responses. The immobilization level may therefore be estimated by measuring maximum binding level of binding protein.

Examples of Small Molecule Immobilizations



Ampicillin

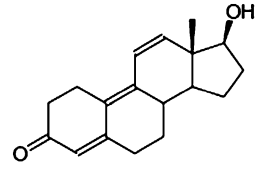
Ampicillin contains a native amine group that is sufficiently reactive to allow direct immobilization by amine coupling to sensor chip CM5. The chip is first EDC/NHS-activated for 7 min followed by 7 min injection of ampicillin dissolved to 10 mM in 50 mM sodium borate pH 8.5 (coupling is efficient at high pH, and there is no electrostatic pre-concentration effect to motivate the use of low pH buffers). Finally the chip is deactivated for 7 min with 1 M ethanolamine HCl, pH 8.5.



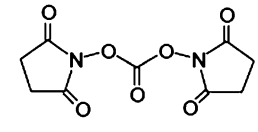
Sulfamethazine

The benzylamine group in sulfamethazine has relatively low reactivity and the compound is only sparingly soluble in aqueous buffers. Successful immobilization on sensor chip CM5 has been achieved with 7 mM sulfamethazine in 10 mM HCl pH 3.0 containing 10 % DMF (7). Immobilization was performed outside the instrument. EDC/NHS activation was performed during 18 min, sulfamethazine coupling during 3 h, and ethanolamine deactivation during 18 min.

In some cases, the molecule may need to be activated to enable immobilization. The example below illustrates activation of hydroxyl groups for immobilization on an amine surface (prepared as described in Note 7).



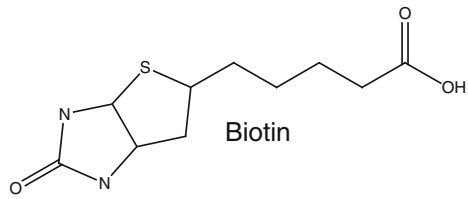
Trenbolone



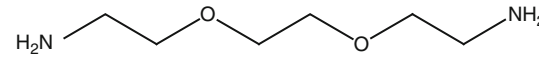
Disuccinimidyl carbonate

Modification of the hydroxyl group on trenbolone with disuccinimidyl carbonate (DSC) introduces a succinimidyl group on to the molecule which can react with amine groups on an amine-modified sensor chip surface. Modification of 10 mM trenbolone is performed outside the instrument by incubation with 40 mM DSC for 30 min in pyridine containing 40 mM 4-dimethylamin opyridine. The product is diluted 1:1 into 50 mM sodium borate buffer, pH 7 before injection for 7 min over the amine surface.

The example below shows how biotin is activated and modified with a diamine to facilitate amine coupling of biotin:



Biotin



Jeffamine

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

Further considerations for immobilizing small molecules may be found in **Note 7**.

Examples of Peptide Immobilizations

Aniline-catalyzed oxime chemistry, through oxidation of N-terminal amino acids with $NaIO_4$, was shown to be a useful tool for achieving site-directed immobilization in ref. 9. The general methods of amine coupling, thiol linkage, and biotinylation are also applicable to peptides (*see* **Note 8** for special considerations).

3.2 Screening of Ligand Libraries

Hit selection (or secondary screening) assays are aimed at identifying lead compounds that bind to the target molecule, and are designed in the first place to provide simple "yes/no" answers from analyses of moderately large numbers of compounds (1). Normally, assays will be run with single or duplicate samples for each compound at a single concentration. The data is obtained as report points (see Fig. 1) and is used to rank compounds in terms of relative binding levels that can be interpreted as yes/no binding answers. Further, the shape of the sensorgram reveals information about binding strength as well as identification of unfavorable binding properties such as super-stoichiometric binding (more than 1:1 binding) of the ligand, precipitation, promiscuous binders and binding to the reference surface, Fig. 6.

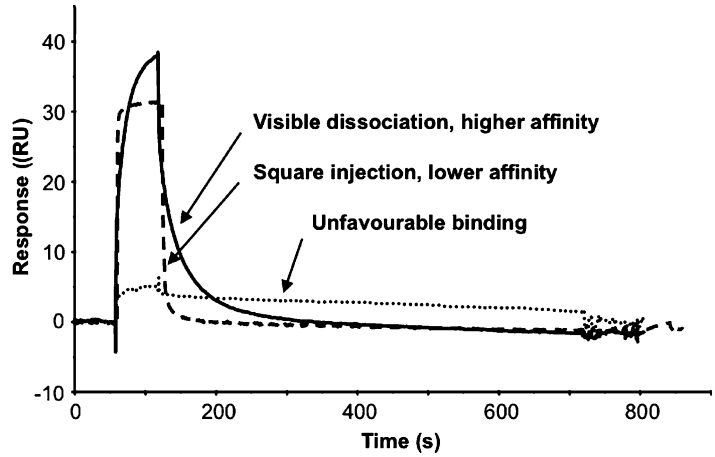


Fig. 6 Overlay plot of three sensorgrams, two displaying typical high and low affinity binding, and one sensorgram exemplifying unfavorable binding. The shape of the sensorgram provides information about the interaction event. Unfavorable binding may be displayed as a variety of different disturbed shapes

3.2.1 *Before You Start*

A prerequisite for obtaining high quality data is that the instrument has been well maintained using the recommended procedures. This is particularly important when low responses are expected as for small ligand interactions. Some libraries may contain "sticky" compounds that will cause carry over responses to the next cycle. If this is the case, an automatic extra wash injection (as described in the Instrument Manual) with 50 % DMSO in running buffer may be included after each compound injection. The extra wash procedure does not pass over the target molecule, but washes the rest of the flow system. Screening results with observed binding of negative controls could in rare cases also be caused by poor cleaning of laboratory glass ware. A quick rinse of glass ware with 50 mM NaOH followed by MilliQ™ water may help this.

3.2.2 *Run Setup*

The setup of a method for screening contains steps with the following purposes:

- *Start-up* for stabilizing the response before analyzing samples. Start-up cycles are run exactly as the samples, but with buffer as sample.
- *Solvent correction* (see Subheading 3.2.3), run at the beginning and end of the run and, e.g., every twentieth sample.
- *Samples*, injected during 30–60 s using a flow rate of, e.g., 30 µl/min, an extra wash with 50 % DMSO included if necessary.
- *Control samples*, run at the beginning and end of the run and, e.g., every twentieth sample. Run as the samples. Positive and

negative control samples should always be included when available, to provide a check on the binding activity of the surface. Use buffer as negative controls.

A reference surface is always used for the type of small molecule interactions described in this chapter. The reference serves two functions, allowing correction for bulk response changes that arise from differences in refractive index between samples and buffer, and providing a control for non-specific interaction of the sample with the reference. The choice of reference surface is to some extent determined by the design of the experiment, but most commonly a non-immobilized reference surface is used. In certain cases, immobilization of an appropriate reference protein can be very valuable in providing more stringent screening results (10).

3.2.3 Sample and Buffer Preparation

For sample preparation 1–10 mM stock solutions of compounds in DMSO are often convenient to start with. Dilution of the ligands to a concentration of 30 μM is a suitable starting concentration, if the affinity is unknown. Depending on previous knowledge of the interaction affinity, the concentration may be adjusted accordingly. In general, lower concentrations decrease the risk of artifacts such as super-stoichiometric binding, solubility issues etc. For fragment screening (fragments typically weighing <200 Da) where affinity is expected to be lower, a concentration of 200–1,000 μM is usually needed and thus also higher stock concentrations in DMSO. Which final DMSO concentration to use in the assay is dependent firstly on the DMSO sensitivity of the target protein and secondly on the solubility of the compounds, 2–5 % is typical.

It is important to match samples and running buffer as closely as possible with respect to refractive index, thus to strive for the same DMSO concentration in samples and running buffer. Even very small differences in DMSO concentration contribute significantly to the bulk refractive index (11). The need for solvent correction procedures arises because subtraction of the reference response does not exactly eliminate the contribution of bulk solution to the measured response. This is because bulk solution is excluded from the volume occupied by target molecules in the target flow cell, so the bulk contribution to the response in the target flow cell is smaller than in the reference cell. The difference is small (usually less than 10–20 RU), but it can be of the same order of magnitude as the responses expected from binding of low molecular weight compounds. In practice it is quite difficult even for a trained person to obtain a sufficiently close buffer/samples match for all samples, and a solvent correction procedure can then be applied. The solvent correction procedure simply performs a series of injections of varying DMSO concentration in running buffer; solvent correction is then applied automatically to assay results in the evaluation software, after the assay is completed, *see* **Note 9**.

Table 1
Preparation of DMSO calibration stock solutions and running buffer

	1.5 % DMSO	2.0 % DMSO	2.8 % DMSO
1.02× PBS[a]	9,800 µl	980 ml	9,800 µl
100 % DMSO	150 µl	20 ml	280 µl
Final volume	≈10 ml	1,000 ml[a]	≈10 ml

[a]Used as running buffer

Table 2
Preparation of ready-to-use DMSO calibration solutions

	1	2	3	4	5	6	7	8
DMSO concentration	2.8 %	←					→	1.5 %
1.5 % DMSO	0 µl	200 µl	400 µl	600 µl	800 µl	1,000 µl	1,200 µl	1,400 µl
2.8 % DMSO	1,400 µl	1,200 µl	1,000 µl	800 µl	600 µl	400 µl	200 µl	0 µl

Preparation of running buffer, solvent correction solutions, and samples is here exemplified for ligands diluted to 30 µM in 20 mM PBS buffer (20 mM phosphate, 150 mM NaCl, 0.05 % Surfactant P20, 2 % DMSO, pH 7.4). *See* **Note 10** for some guidelines on DMSO handling.

1. Prepare a buffer stock solution at 1.02 times the final concentration, containing all buffer components and additives except DMSO. It is important to use the same 1.02 stock buffer for samples, solvent correction solutions, and running buffer.
2. Prepare buffer containing 1.5, 2.0, and 2.8 % DMSO according to Table 1.

   The final buffer concentration will be a little higher than 1.0× in the 1.5 % DMSO solution and a little lower in the 2.8 % DMSO solution. This is intentional, since the solvent correction solutions are designed to correct for small errors in preparation of the samples, where the same variations in PBS concentration will occur.
3. Using the above two DMSO solutions, prepare a series of aliquots for the solvent correction curve, according to Table 2.

These DMSO concentrations should when injected cover a range very roughly from −500 RU to +1,000 RU relative to the baseline of the running buffer with 2 % DMSO. Use these solutions for the solvent correction cycles in the run. The solutions should be freshly prepared for each run. Note: The exact DMSO concentrations are not critical. The important factor is that the concentrations cover an adequate range, i.e., the bulk range of the samples.

To prepare 1 ml 30 μM sample solution in buffer with 2 % DMSO from a 10 mM compound stock, use, e.g., 3 μl sample stock, 17 μl DMSO, and 980 μl 1.02× buffer stock.

3.2.4 Regeneration

If the ligand dissociation is sufficiently rapid, regeneration can be achieved simply by waiting for complete dissociation. Although each sample will take a little longer to analyze this is often preferable to spending time on optimizing regeneration, a task that may be rather challenging for small molecule interactions. For kinetics, using the single cycle kinetics (SCK) format may omit the need for regeneration of high affinity binders altogether, see Subheading 3.3.1. Regeneration requires washing with a solution that favors dissociation. For *protein–protein* interactions many protein surfaces (e.g., antibodies) can be regenerated using acidic conditions such as 10 mM glycine-HCl buffers at pH 1.5–3. Other conditions that have proved useful include ethylene glycol at concentrations up to 100 %, 0.5–3 M $MgCl_2$, high pH (10–50 mM NaOH) and high ionic strength. For *regeneration of ligands* from target proteins, however, we have found that low pH rarely works, while high pH, ethylene glycol, or $MgCl_2$ may be more successful. To investigate the effect of a certain regeneration solution it is more reliable to study the repeated binding level of a known binder than to study the changes in baseline. In general, baseline changes should be viewed in relation to the immobilization level and as such are usually secondary in importance in comparison to repeated binding level of a positive control. The flow rate for regeneration is not a critical parameter; 10–30 μl/min is often used, together with an injection time of 30 s.

3.2.5 Example of Screening Results

The screening evaluation itself is based on reference subtracted data obtained as report points. However, further data adjustment is also usually applied, that is solvent correction, molecular weight adjustment, and possibly also surface activity adjustment, see Fig. 2 for a results example. Reference subtraction and solvent correction is applied first for all data, in Biacore systems this is performed automatically by the software. Molecular weight adjustment is necessary for valid comparison of responses from ligands of different molecular weights. Surface activity adjustment compensates for drift in the performance of the assay as revealed by repeated control sample measurements, and should be applied if assay drift is significant.

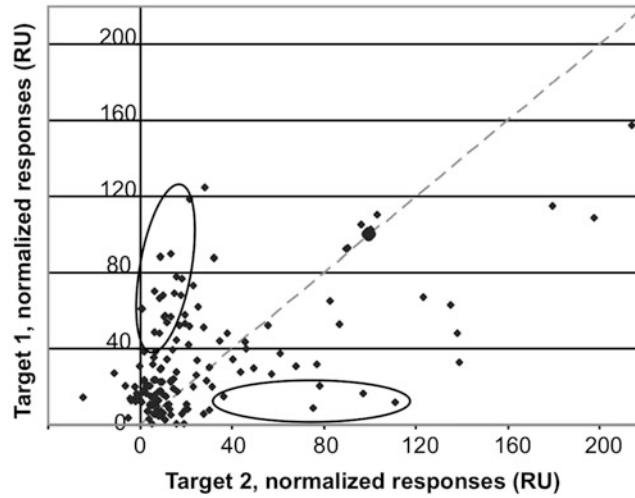


Fig. 7 Compounds screened at a single concentration, 30 μM, in 20 mM PBS, 0.05 % Surfactant P20, 2 % DMSO, pH 7.4, using Biacore T200. The compounds were injected for 60 s simultaneously over two different target proteins. Response levels were plotted against each other for visualization of selective binders. Responses were reference subtracted, solvent corrected and adjusted for differences in molecular weights. Finally data was normalized against a positive control (set to 100, *large diamond*) that was analyzed at intervals during the run. Compounds showing selective binding to respective target (*circled*) were selected

Figure 7 shows another result, this time from screening of a focused ligand library consisting of 180 compounds. The ligands were injected simultaneously over two different target proteins, immobilized in different flow cells. The histidine tagged target proteins were immobilized via capture to Sensor Chip NTA and subsequent amine coupling (5 μg/ml in 20 mM PBS, 0.05 % Surfactant P20, pH 7.4) to 5,000 RU (*see* **Note 6**).

From these results compounds showing selective binding to the respective target were chosen for further kinetic characterization. Compounds displaying visible dissociation according to Fig. 6 were also selected, showing a desired higher stability of binding.

3.3 Kinetics and Affinity Determinations

The experimental principles for determination of binding kinetics and affinity are similar. Both measurements are best approached through determination of binding characteristics over a range of ligand concentrations. The kinetic evaluation procedure determines association and dissociation constants by fitting the experimental data to a 1:1 interaction model between ligand A and target protein B (12):

$$A + B \underset{k_d}{\overset{ka}{\rightleftharpoons}} AB.$$

Kinetic constants are obtained by fitting sensorgram data to a set of rate equations that describe the 1:1 binding mechanism. Affinity constants may be calculated as the ratio of the kinetic constants, $K_D = k_d/k_a$, or determined from steady-state response levels obtained through injection of different ligand concentrations. In practice, these approaches are generally applicable in different situations that are often mutually exclusive: kinetic analysis can be used for sensorgrams that contain kinetic information, but do not necessarily reach steady state. Affinity determination from steady state measurements is most useful for interactions where the kinetics are too fast to measure with confidence but steady state is reached with relatively short injection times.

3.3.1 Kinetics Determinations

In practice to obtain kinetic information immobilization levels need to be low. In general, target immobilization levels that correspond to a maximum binding capacity for the small molecule, R_{max}, of 5–50 RU are suitable for most purposes. As the immobilization level is decreased the binding kinetics is more pronounced and becomes easier to interpret, see Fig. 8.

The range of ligand concentrations for optimal kinetic determination is related to the affinity constant of the interaction. The optimal ligand concentration series for robust kinetics should: (a) give rise to sensorgrams that for the highest concentrations approach the saturation level, (b) display curvature during injection and (c) display a significant drop of the signal during dissociation. For compounds displaying a visible dissociation phase from screening, a wide concentration range of 2.4, 12, 60, 300, and 1,500 nM may be used as a start. It is often advisable to include more than one blank injection, e.g., one blank run before, and one after the concentration series, to be able to choose the best blank for subtraction, in case minor changes to the target occur during the run. Include 1–3 start-up cycles with buffer (run exactly as the ligand samples) first in the run to stabilize the system. 1 min injection time and 10 min dissociation time may be used as a start before the need for regeneration has been explored.

An alternative approach is to inject all concentrations in sequence in the same cycle, so called *single cycle kinetics* (*SCK*) (13). The ligand does not need to dissociate between injections, rather the next concentration can be injected immediately. After the last injection the ligand is allowed to dissociate, see Fig. 9. For high affinity binders like the one in Fig. 9b this format eliminates the need of regeneration, which can be a huge advantage. Blank cycles are run exactly as the ligand cycle with the same number of injections.

The same recommendations apply as for multi-cycle kinetics regarding concentrations. To obtain high quality data it is important to include a blank cycle run exactly as the ligand cycle. Double referencing is then performed during evaluation i.e., reference flow

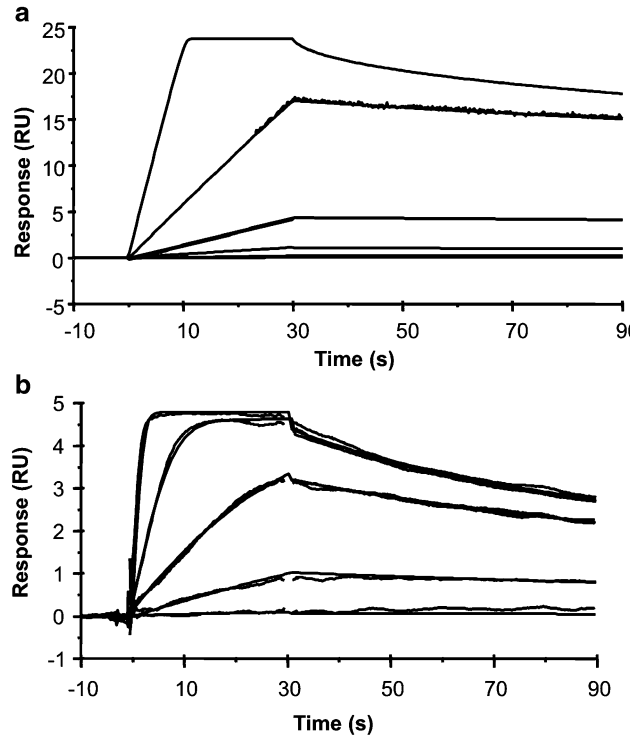


Fig. 8 The compound melagatran interacting with immobilized thrombin, data obtained in Biacore T200. A multi cycle kinetics approach was used where each concentration is injected in separate cycles. In this case the thrombin was regenerated (not shown) using 2 M $MgCl_2$ (**a**) The binding rate for this interaction is highly limited by diffusion of the molecule down to the target protein on the surface (so called *mass transfer limitation*). As soon as a molecule arrives in the matrix it is immediately bound and thus the concentration close to the surface is not the same as the injected concentration. This is seen as sensorgrams displaying mainly straight binding lines up to time 30 s (the association phase), and it is caused by too high immobilization level. These straight line sensorgrams do not contribute with any kinetic data (**b**) The immobilization level has been decreased (note the scale on the *y*-axis), diffusion is no longer limiting the binding rate, the curves during the association phase display more curvature, and kinetic data is obtained

cell data are subtracted and data from blank cycle is also subtracted. Advantages with SCK are that it saves time and the need for regeneration may be omitted altogether. Once a suitable concentration range has been established injection times may be shortened to, e.g., 30 s and for difficult interactions only 2–3 concentrations may still be sufficient to obtain kinetic data.

3.3.2 Affinity Determinations

Most commonly when injecting a small molecule, the sensorgram is displayed as a square injection, lacking a visible association and dissociation phase. The affinity is then in the μM to mM range

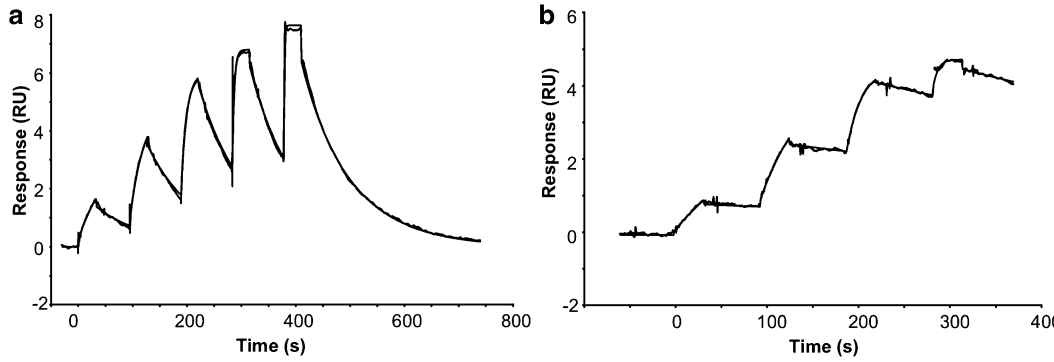


Fig. 9 Two sensorgrams showing single cycle kinetics estimation of a small compound binding to two different tankyrases. About 1,000 RU histidine tagged tankyrase was first captured stably to Sensor Chip NTA. Kinetic evaluation was then performed for the concentration series 0.06, 0.18, 0.56, 1.67, and 5 μM, injected during 30 s and data fitting well to a 1:1 interaction model after reference and blank cycle subtractions. (**a**) The two highest concentrations are visibly approaching the saturation level and the compound dissociates down to baseline after the last injection. K_D was about 70 nM with k_a 1.6 $\times$ 10^5 1/ms and k_d 0.012 1/s. (**b**) For this compound data for the highest concentration was omitted, being disturbed. Still, also here saturation is approached and the dissociation time although short is sufficient for an estimate of the dissociation rate: K_D was about 13 nM with k_a 1.1 $\times$ 10^5 1/ms and k_d 0.001 1/s. Use of the single cycle kinetic format here omitted the need for regeneration of this stably bound compound

and determination of affinity constants from steady-state response levels can be obtained by measurement over a series of ligand concentrations. The immobilization level for the target is less critical for affinity measurements, since the steady-state response is not affected by mass transport limitations. Higher immobilization levels are in fact preferable for affinity measurements since ligand response values will be higher and steady state response levels can be measured with more confidence.

As for kinetics determinations, the range of ligand concentrations for optimal affinity determination is related to the affinity constant of the interaction. The optimal ligand concentration series for robust affinity should cover a range of about 0.1–10 times the expected K_D concentration, and should give rise to sensorgrams that approach saturation level for the highest concentrations. In practice for low affinities, in particular for fragments, the used concentration is often limited by solubility issues and/or a limited amount of compound. Affinity determinations from concentration series below the K_D concentration can still be made if an R_{max} value (saturation level) can be set during evaluation. The R_{max} level may be estimated theoretically from the immobilization level (including estimated potential activity loss), or by use of a known binder injected to saturating levels. R_{max} is thus locked as a constant and a reliable fit of steady state responses may be found. Figure 10 shows an example of an affinity determination where saturation was approached using the selected concentration series and affinity was estimated through fitting to a steady state model.

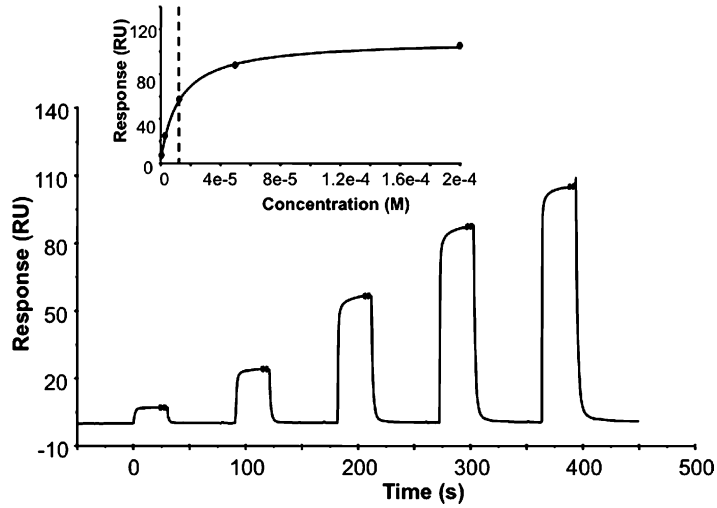


Fig. 10 Sensorgram showing single cycle affinity determination of a small compound binding to 5,000 RU immobilized target protein. Affinity was estimated for the concentration series 0.78, 3.12, 12.5, 50, and 200 μM, data fitting well to the steady state interaction model after reference and blank cycle subtractions. K_D was estimated to 12 μM (*dotted line*). The two highest concentrations are approaching saturation level which is a good sign (best seen in the insert). To confirm the affinity value one should perform several determinations. For this molecule, showing a remaining small upward slope, prolonging the injection time from 30 to 60 s is a good idea to ensure that steady state has been obtained

Finally it is worth considering the purpose of the kinetic/affinity experiment. For many purposes an estimate is sufficient to, e.g., compare different ligands of interest and the hunt for an exact number is overkill. Even just visually comparing, e.g., dissociation rates may give you the information you need about the ligand binding.

4 Notes

1. Much of the Biacore literature, including the product documentation from GE Healthcare, uses the term "ligand" for the interacting partner immobilized and "analyte" for the partner injected in solution. The current chapter adopts the accepted terminology in drug discovery and development work, where "ligand" refers to a small molecule binding to a target. In this sense, the ligand is the molecule injected in solution over a target protein immobilized on the sensor chip.
2. Lack of preconcentration effect may be caused by any of the following: (a) pH is too high or too low, (b) too much salt remains after dilution into the immobilization buffers. As a rule of thumb at least a five to tenfold dilution of a protein, from a stock solution containing physiological salt levels

(0.15 M), is required for the ionic strength to be low enough not to shield electrostatic interactions (exactly how much depends on the protein and the stock buffer content), (c) the protein concentration is too low. However, by zooming in on also very low levels of preconcentration, just visually comparing curves may still make it possible to conclude which pH is more suitable. And a slight increase in concentration and/or a prolonged immobilization time (up to ca 30 min if necessary) often gives adequate immobilization.

3. The commercially available *N*-hydroxysuccinimide (NHS)-based biotinylation reagents can differ regarding water solubility, length and characteristics of the spacer between NHS and biotin, etc. Biotinylation reagents with longer and hydrophilic spacers may result in increased capture levels on the sensor chip and improved protein–ligand availability. We have used the following reagents successfully for biotinylation and binding of active protein to streptavidin: EZ-Link™ (sulfo-)NHS-LC-biotin, EZ-Link™ (sulfo-)NHS-LC-LC-biotin (Thermo Fisher Scientific Inc), and ChromaLink™ Biotin 354S (Solulink, Inc). It is absolutely essential to remove all free biotin and for this PD Spin Trap G-25, Illustra NAP-5 (GEHC), or Protein Desalting Spin Columns (Thermo Scientific) work well.

   To achieve the substitution level distributed around 1, a molar ratio of 1.5:1 between biotin-reagent and protein is recommended and often works well. This ratio minimizes the amount of contamination from free biotinylation reagent and also minimizes the risk of influencing the binding activity of the protein. Recommended protein concentrations are 0.1–1.0 mg/ml in reaction volumes of 50–100 µl suiting a desalting micro-spin column, with a total reaction volume of 100 µl (*see* **Note 4** for a biotinylation example). The minimum amount of protein that can be biotinylated is set by the choice of the separation/desalting technique used for separation of remaining free biotin from the biotinylated protein. If small volume micro-spin columns or micro-dialysis tubes are used, it is possible to biotinylate ~10 µg protein (~100 µg/ml), depending on the protein.

   Some proteins are more difficult to biotinylate than others and some are more sensitive to covalent modification. This can sometimes motivate optimizing the degree of biotinylation. For optimization, different molar ratio of the biotin reagents (in our hands 0.5–5 mol equivalents) may be evaluated by measuring the level of binding of biotinylated protein to immobilized streptavidin.

4. Example of biotinylation of the protein Human Epidermal growth factor Receptor 2 (HER-2), molecular weight 96,800 Da:

(a) 25 μg (2.59×10^{-10} mol) HER-2 in 50 μl 0.1 M sodium-borate buffer pH 8.5 was pipetted to an Eppendorf vial (PBS, pH 7.2–7.8 also works).

(b) 1.5 molar excess of biotin, 3.88×10^{-10} mol, thus 3.9 μl of a 0.1 mM sulfo-NHS-LC-biotin solution in MilliQ water was added to the protein and incubated for 60 min at room temperature.

(c) Very careful removal of any remaining free biotinylation reagent is important, free biotin being a common cause of "no binding" of the biotinylated protein. If a column is used, it may be necessary to perform more than one buffer change. In this example, to avoid double passages and accompanying dilution of HER-2, a modified single passage through an Illustra NAP-5 column (GEHC) was used, eluting a narrower peak of the protein, perhaps losing a minor portion of the protein but avoiding free biotin. First the sample was applied to the column (54 μl), then 650 μl assay running buffer was added and finally elution of biotinylated protein was performed by addition of another 600 μl running buffer.

(d) The concentration after passage was 45 μg/ml (theoretically at 100 % recovery after passage), the protein was diluted to 30 μg/ml and injected during 3 min over Sensor Chip SA (GEHC), resulting in 2,900 RU bound HER-2.

5. For extremely low capture levels, assay stability benefits from minimizing any possible disturbances. Thus, use the same flow rate for all injections ($NiCl_2$, capture of histidine-tagged protein, the ligand and regeneration). A compromise to save histidine-tagged reagent and at the same time obtain reliable kinetic data is to use, e.g., 15 μl/min for all injections. This flow rate is sufficiently high to avoid mass transfer limitations for most small molecules. Also, avoid using Extra Wash with 50 % DMSO for kinetics determinations at these low levels, or place the extra wash after the last ligand injection for each compound.

6. Amine coupling of target protein to Sensor Chip NTA starts with conditioning of the chip. For this 0.35 M EDTA is injected for 60 s followed by Extra Wash with running buffer to get rid of remaining traces of EDTA. This EDTA solution is preferably prepared from ready-to use EDTA solution (Sigma). From solids the pH needs to be adjusted to dissolve all EDTA. Inject 500 μM $NiCl_2$ in running buffer for 60 s followed by Extra Wash with running buffer with 3 mM added EDTA, to get rid of any remaining traces of $NiCl_2$ in the flow system. Inject EDC/NHS for 7 min. Inject histidine tagged target protein, typically 5–50 μg/ml in running buffer using injection time of, e.g., 7 min. In rare cases, the protein concentration/injection time needs to be increased significantly. Deactivate

with ethanolamine for 7 min. The flow rate, as for any immobilization, is not critical for any of the injections, typically 5–10 µl/min is used.

7. Especially for small molecules the following should also be considered:
   (a) The solubility of many organic compounds is limited in aqueous buffers, so that it may be difficult to achieve sufficiently high concentrations in coupling buffer. Solutions for immobilization are often best prepared by dissolving the small molecule in a water-miscible solvent (e.g., DMSO or dimethyl formamide, DMF) and diluting the solution in immobilization buffer. Amine coupling has been performed successfully in the presence of up to 50 % DMSO, DMF or acetonitrile. Check the respective Instrument Handbook for compatibility of the Biacore flow system with organic solvents.
   (b) Small molecules are usually inherently "sticky" in their nature and may, due to the high concentration used in immobilization, be adsorbed to tubing and channels in the flow system. Residual amounts of small molecule adsorbed to the flow system may interact with injected binding protein, and diminish or in worst case totally quench the binding to the immobilized molecule on the chip. One way to overcome this is to immobilize the small molecule outside of the instrument flow system, either by dispensing molecule solution directly to the sensor chip, outside of the instrument (avoid high concentration of organic solvent, 10 % of organic solvent is in most cases sufficient to dissolve the molecule that should be immobilized), or by using the Surface Prep Unit (only available for Biacore 3000) for immobilization. If immobilization is done in the instrument, extensive wash of flow system is recommended. A useful washing solution is 50 % isopropanol in 1 M NaOH and 1 M NaCl.
   (c) To obtain immobilization to selected flow cell/s only, selected flow cells can be activated in the Biacore instrument, followed by undocking the chip. The molecule solution is then dispensed directly to the chip outside the instrument, followed by deactivating the chip by dispensing ethanolamine solution.
   (d) In some cases, the small molecule may need to be activated to enable immobilization. Activation of carboxylic groups with EDC/NHS or hydroxyls with DSC is examples of this.
   (e) Modification of the sensor surface to introduce new functional groups extends the range of chemistries that can be used for immobilization. One alternative is to immobilize

a diamine (e.g., ethylendiamine or 1,8-diamino-3,6-dioxaoctane) compound using amine coupling on Sensor Chip CM5 to create a surface carrying free amine groups. This allows, for example, immobilization via carboxyl groups on the molecule, by an "inverted" amine coupling procedure where the small molecule is activated with EDC/NHS and reacts with amine groups on the surface. Activation is first performed by injection of EDC/NHS for 7 min followed by 7 min injection of 0.1 M ethylenediamine dissolved in 50 mM sodium borate pH 8.5. Finally the chip is deactivated for 3 min with ethanolamine. Other diamines or other functional groups can be introduced using similar principles. Note that the amine surface is not stable and should be used directly after preparation.

(f) Small molecules which do not contain a native group amenable to immobilization can be modified to introduce such a group (14). While modification may require expertise in organic chemical synthesis, this approach has some inherent advantages:

- The functional group used for immobilization can be chosen to suit a selected immobilization chemistry. Side reactions with other functional groups on the molecule can in this way be reduced or eliminated.
- A spacer, such as 1,8-diamino-3,6-dioxaoctane can be introduced between the small molecule and immobilization site, which can often help to reduce steric interference in the interaction with the protein.
- The position of the molecule modification can be chosen to minimize adverse effects on the interaction with protein.

8. Especially for peptides the following should also be considered:

(a) Acidic ($pI < 3$) peptides may be electrostatically repulsed from the carboxymethylated sensor surface at neutral or slightly acidic amine coupling pH's (pH 4–5.5). Preconcentration can be facilitated by using acidic (pH < 3) coupling buffer or a coupling buffer with high ionic strength (e.g., 1 M NaCl).

(b) To facilitate amine coupling of peptides without affecting the binding properties, use of existing or introduction of ε-amino group of the C- or N terminal lysine is preferred.

(c) As an alternative, if the peptide contains lysines that participate in binding, a cysteine can be added at the C or N-terminus of the peptide. The cysteine can then be used in so called "Ligand thiol coupling" to reactive disulfides at the sensor chip.

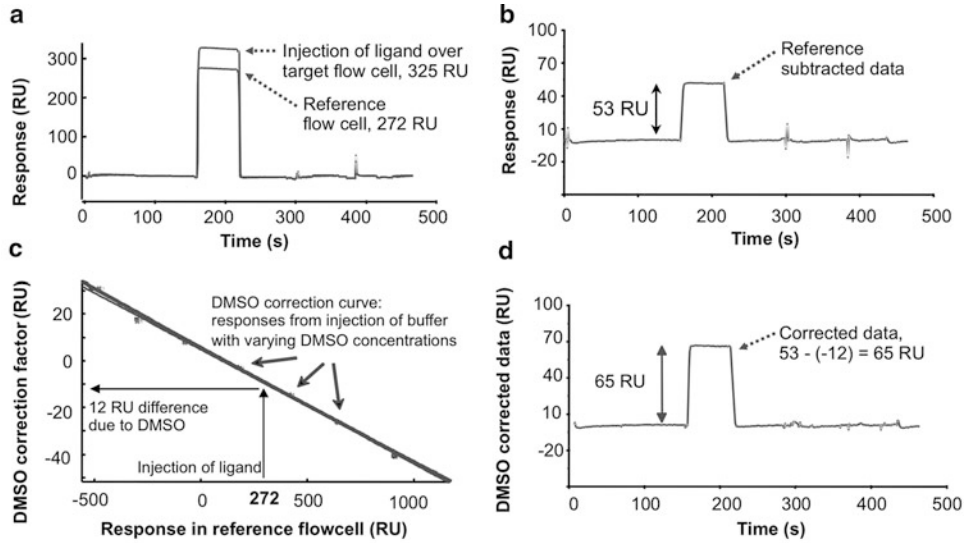


Fig. 11 (**a**) Injection of a ligand over reference and target flow cell. A high response level is obtained since the DMSO concentration of sample and assay buffer was not exactly matched in sample preparation. (**b**) Reference data subtracted from target data. (**c**) A series of eight calibration solutions (running buffer with different DMSO concentrations) were run at intervals during the experiment. The responses of the calibration solutions, obtained from the reference surface, covered a range from ca −500 to +1,000 RU relative to the baseline. A DMSO calibration curve was created by plotting difference in response between target and reference flowcell (DMSO correction factor) versus response in reference flow cell. (**d**) Ligand responses were corrected for DMSO bulk differences via the calibration curves

(d) Peptides can be also be modified with biotin to facilitate capture to streptavidin, using Sensor Chip SA or Biotin CAPture kit www.gelifesciences.com/biacore.

9. Figure 11 shows the principle for how solvent correction is applied by the software.
10. Do not store DMSO in polystyrene vessels. Make sure that all plastic equipment used in handling the solutions is resistant to DMSO. Polypropylene (used for, e.g., Eppendorf tips, vials, and micro-plates) is preferred. Prepare all solutions carefully. Keep all vials and microplates covered with correct caps and foil respectively to avoid evaporation of samples, and note that DMSO in itself is hygroscopic.

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