

Video Article

Real Time Measurements of Membrane Protein:Receptor Interactions Using Surface Plasmon Resonance (SPR)

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Abstract

Protein-protein interactions are pivotal to most, if not all, physiological processes, and understanding the nature of such interactions is a central step in biological research. Surface Plasmon Resonance (SPR) is a sensitive detection technique for label-free study of bio-molecular interactions in real time. In a typical SPR experiment, one component (usually a protein, termed 'ligand') is immobilized onto a sensor chip surface, while the other (the 'analyte') is free in solution and is injected over the surface. Association and dissociation of the analyte from the ligand are measured and plotted in real time on a graph called a sensogram, from which pre-equilibrium and equilibrium data is derived. Being label-free, consuming low amounts of material, and providing pre-equilibrium kinetic data, often makes SPR the method of choice when studying dynamics of protein interactions. However, one has to keep in mind that due to the method's high sensitivity, the data obtained needs to be carefully analyzed, and supported by other biochemical methods.

SPR is particularly suitable for studying membrane proteins since it consumes small amounts of purified material, and is compatible with lipids and detergents. This protocol describes an SPR experiment characterizing the kinetic properties of the interaction between a membrane protein (an ABC transporter) and a soluble protein (the transporter's cognate substrate binding protein).

Video Link

The video component of this article can be found at <https://www.jove.com/video/51937/>

Introduction

Protein-protein interactions (PPI); the formation and dissociation of protein complexes, are key events in many biological processes (e.g., replication, transcription, translation, signaling, cell-cell communication). Semi-quantitative studies of PPI are often performed using pull-down or immuno-precipitation experiments. However, these (and similar) techniques are limited in the range of affinities that can be measured (low micromolar and higher affinity) due to the washing steps that are inherent to such techniques. Such "end-point" techniques cannot identify transient or low affinity interactions that are often of great biological consequences. In addition, the temporal resolution of such approaches is extremely limited, usually orders of magnitude slower than the rates of the reaction. SPR overcomes these shortcomings due to its heightened sensitivity and superior temporal resolution^{1,2}. SPR is a label-free method, and as such molecular interaction can be measured (as long as the mass changes can be detected). In addition to PPI, it has been extensively used to characterize protein-ligand, protein-drug (or small molecule), protein-DNA, and protein-RNA interactions³⁻⁵. Results are recorded and plotted in real time, which enables rapid modification of experimental conditions and flexible experimental design.

The physical principle behind SPR based instruments is the utilization of an optical system that measures a change of the refractive index on the sensor surface upon mass changes². One of the interacting partners (hereafter ligand) is immobilized onto a polymer matrix chip and the second molecule (hereafter analyte) is flowed over the surface. Analyte binding to the ligand alters the mass on the chip surface. This mass change is directly and proportionally related to changes in the refractive index of the surface. The results are plotted in real time and presented as response units (RU) as a function of time. Such a plot is termed a sensogram (e.g., **Figure 1**). Since SPR follows the complete time course of the interaction, pre-equilibrium kinetic rate constants are derived, in addition to equilibrium affinities. As detailed below, the pre-equilibrium behavior of a given system holds much information, and provides a very different perspective than equilibrium alone. Once the system is calibrated, experiments are very fast and require only small amounts of protein material (microgram to nanogram amounts). Collecting the complete kinetic information of a given system can be achieved in days. The high sensitivity of SPR affords measurements that are not possible using any other technique⁶. However, this high sensitivity is a 'two-edged sword' since it can be a major source for false positive data. Any factor that affects the reflective index is recorded and plotted on the sensogram. As such, appropriate controls must be used to eliminate false-positives, and support from complementary approaches is highly advised.

Figure 1 illustrates the progression of an SPR experiment using an NTA-coated sensor chip. The sensogram in panel A shows the injection of the nickel ions over the NTA matrix (unsubtracted data), and panels B-D display the data after subtracting the signal derived from the negative

control cell. Red and blue arrows show injection start and end, respectively. Immobilization of the ligand onto the chip gradually alters the mass until injection is terminated. The stable plateau observed after termination of ligand's injection reflects the stable association of the ligand to the surface (**Figure 1B**, cycle 2). Once a stable baseline is achieved a buffer is injected over the ligand and the reference cell (**Figure 1B**, cycle 3). This injection serves as a 'blank control', and will be subtracted during analysis. Upon injection, minor changes are recorded, reflecting a flow of mass through the chip. Then in a separate cycle (**Figure 1B**, cycle 4), the analyte is injected; the gradual increase in RU represents the analyte's association to the ligand. The binding sites become gradually occupied until equilibrium is reached. As soon as injection ends, a decline in RUs reflects the complex's dissociation. From such sensograms pre-equilibrium and equilibrium rate constants can be derived (see later). **Figure 1** depicts a transient interaction between the ligand and analyte. When the interaction is more stable, the decline in the RU level is slower, reflecting a lower k_d .

Herein, we describe an SPR experiment aimed at characterizing the interaction between a membrane transporter (detergent solubilized) and its functional partner, its cognate substrate binding protein^{6,7}. The chosen model system is an ATP binding cassette (ABC) transporter, ModBC-A of *Archeoglobus fulgidus*. This system was selected for its highly reproducible results, high signal to noise ratio, and classical 'on/off' rates. Additionally, homologues ABC transporters are available to serve as important negative controls. The transporter, ModBC (ligand A) is extracted from the membrane using detergent, purified and immobilized onto the chip. Its soluble interacting partner, ModA, is the analyte. As a negative control ligand, a different ABC transporter RbsBC is used ("ligand B").

Protocol

1. Protein Sample and Buffer Preparation

1. Sample preparation: purify the proteins of interest and make sure all aggregates are removed, by injecting the protein prior to the experiment onto a gel-filtration column or by ultracentrifugation (usually 10 min at 100,000 x g is sufficient).
NOTE: Although desirable, highly pure protein preparations are not a must. SPR has been successfully used with less-than-perfect purifications and even with total extract^{8,9}.
2. Buffer preparation:
 1. Prepare the buffer of the experiment (termed "running buffer", or RB). For the protocol described here, use 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% (w/v) DDM (n-Dodecyl β -D-maltoside). Keep in mind that the choice of buffer composition can be readily modified according to the studied system.
 2. Filter all buffers and protein samples using 0.22 μ m filters. Make sure in advance that the filters are protein-compatible. In general, recently marketed SPR platforms contain an in-line de-gasser; if this is not the case, de-gas the buffers prior to use.
 3. Dialyze overnight or dilute the samples against the RB to avoid buffer mismatches. Dialysis (or any other method of buffer exchange) is especially advisable when using chemicals of high viscosity (e.g., sucrose, glycerol, DMSO). Before connecting the RB bottle to the pump inlet keep aside 10 ml of the RB in a separate tube.
3. Dilute the concentrated ligand stock into RB to a final concentration of ~20 μ g/ml. As a rule of thumb, for stable ligand immobilization, use low ligand concentrations with longer injections at low flow, as opposed to high concentrations, high flow and shorter injections.
4. Dilute the analyte into the RB to the desired concentration. Typically, test the range of concentrations for a system of unknown affinity from 10 μ M to 10 nM in ten-fold dilutions. Always start with the lower concentrations first.

2. Sensor Chip

1. Chip selection: Use a nitrilotriacetic acid (NTA) coupled chip suitable for capturing proteins with an oligo-histidine tag.
2. Chip usage:
 1. When using a new chip, take the chip out from the sheath, rinse gently with double distilled water (DDW), dry off carefully remaining liquids using delicate wipers, and make sure not to touch the gold layer surface. Place the chip back into its sheath in the correct orientation (the insertion is smooth when the chip is properly placed).
 2. When reusing a chip, take out the stored chip from the buffer, rinse thoroughly with DDW and dry gently as stated above, and place into its original sheath.
NOTE: The accumulation of non-specific junk material on the chip can affect its 'viability'. This can be monitored by comparing the RUs prior to ligand immobilization and post stripping. Though many measurements can be done using the same NiNTA chip, the issue of its longevity is not a 'clear cut' and varies greatly between different chips.
3. Chip docking: Open the sensor chip door and place the chip in its sheath. When using a new chip, input the chip's serial number to keep usage record. Close the door and press 'dock chip'.

3. Temperature Settings

1. Set the chip cell temperature to 25 °C (or the preferred experiment temperature).
2. Optional: set the samples compartment to 7 °C to keep the proteins stable throughout the experiment.

4. Solutions for Loading and Stripping

Prepare the following solutions for loading and stripping: 0.5 mM NiCl₂ in RB, 350 mM EDTA in RB (add detergent to EDTA solution to a final concentration as in RB), 0.25% SDS in H₂O, and 100 mM HCl in H₂O. When using micro-centrifuge tubes and not SPR designated tubes, do not forget to cut off the caps of the tubes.

5. Prime the SPR Instrument with RB

6. Start a New Run

1. Set the flow rate that will be used during the experiment. To obtain the results described here set the flow to 50 $\mu\text{l}/\text{min}$.
NOTE: This parameter can be modified during the experiment.
2. Define the flow paths and reference subtraction. To obtain the results described here set the paths $F_c=1$ and $F_c=2$, subtraction $F_c=2-1$.
NOTE: The program will display in real-time the subtraction of two measured cells. Keep in mind that this parameter cannot be altered during the experiment.
3. Select the suitable sample rack (it can influence the sample volume consumption).
4. Save the results file.

7. Experiment Cycles

Each experiment is composed of cycles, keep a clear record of the injections done in each cycle, and separate the ligands' loading, the buffer's blank injection, and the analyte injection into different cycles.

1. Cycle 1: Chip preparation and nickel loading
 1. Make sure the flow and paths are set according to step 6.1 and 6.2.
 2. Inject 350 mM EDTA for 1 min to wash away leftovers.
 3. Set flow to 10 $\mu\text{l}/\text{min}$.
 4. Inject 0.5 mM NiCl_2 for 2 min.
NOTE: Now the chip resin is coated by nickel ions.
2. Cycle 2: Ligands immobilization
 1. Set flow to 15 $\mu\text{l}/\text{min}$, flow path $F_c=2$.
 2. Inject ligand A until reaching RU values that are 10-20 fold of its molecular weight. For example, load a ligand of 50 kDa to 500-1,000 RUs. Keep a record of the RU value achieved.
 3. Set flow to 15 $\mu\text{l}/\text{min}$, flow path $F_c=1$.
 4. Inject ligand B (control) up to the same RU value as that of ligand A. Monitor the subtraction sensogram ($F_c=2-1$) on-line to help control the injection length.
 5. Set flow to 50 $\mu\text{l}/\text{min}$, flow path $F_c=1$ and $F_c=2$, wash the system for 5-20 min until the baseline ($F_c=2-1$) stabilizes.
3. Cycle 3: Blank injection. This injection will serve for blank subtraction, therefore include all components that will be injected with the analyte, except the analyte itself.
NOTE: Injection length can vary depending on the time it takes to reach equilibrium (signal plateaus).
 1. Set flow to 15 $\mu\text{l}/\text{min}$, flow path $F_c=1$ and $F_c=2$, $F_c=2-1$ subtraction.
 2. Insert the 'wait' command (referred hereafter as 'wait') for 30 sec (to have a stable baseline).
 3. Inject 15 sec RB.
NOTE: The duration of injection will vary between different systems, depending on their pre-equilibrium kinetic constants (mostly the k_a).
 4. Wait 120-600 sec to record the dissociation phase.
NOTE: The length of this step varies according to the k_d . The slower the dissociation the longer this step needs to be. For very slow dissociating complexes ($k_d < 10^{-4} \text{ sec}^{-1}$), wait 10-15 min and then proceed to surface regeneration (see later).
4. Cycle 4: Analyte injection
 1. Copy/paste the exact conditions used in the reference injection (cycle 3) so these two injections can be later subtracted. Change the location of the slot in the rack from the one holding the control solution to one that holds the analyte solution. Choose a concentration that is slightly above the expected K_D . If no prior knowledge is available, choose 1 μM as a good starting point.
5. Cycle 5: Buffer injection
 1. Repeat cycle 3.
6. Cycle 6: Analyte injection
 1. Repeat cycle 4.
7. Cycle 7: Chip strip off
 1. Set flow to 50 $\mu\text{l}/\text{min}$.
 2. Inject 350 mM EDTA for 1 min. Repeat this step two more times. Do not use a single injection of 3 min as this may damage the chip.
 3. Inject 0.25% SDS for 1 min. Repeat this step two more times. Do not use a single injection of 3 min as this may damage the chip.
 4. If baseline level is not reached, inject 100 mM HCl for 1 min as additional stripping step.
 5. Insert the 'end run' command that switches to standby mode.
8. Chip storage
 1. Undock the sensor chip and take it out of the instrument.
 2. Take the sensor out from its sheath, avoid touching the surface, rinse with DDW, and place in a 50 ml tube containing RB. Store at 4 $^{\circ}\text{C}$.

8. Data Analysis Using Designated Evaluation Software

1. Perform the following steps to process the raw data obtained by SPR before derivation of the kinetic parameters.
 1. Using the evaluation software, open the results file, select cycles 3-6 Fc= 2-1 (reference subtracted data).
 2. Axis shift:
 1. Display cycles 3-6.
 2. Zero the Y-axis value of the baseline (the initial 30 sec wait time in each cycle) to of all four curves.
 3. Align the X- axis of the four curves. Choose pronounced features (e.g., spikes of injection start or finish) to perfectly align the four curves along the X-axis. Set the time of the start of analyte (or control buffer) to zero.
2. Reference subtraction
 1. Subtract the background response by subtracting curve of cycle 3 from curve of cycle 4 and curve of cycle 5 from curve of cycle 6. Use the curve subtraction operation that can be found in the Y-axis shifts menu.
NOTE: It is important to perform this step as it annuls any nonspecific components (unrelated to the analyte) that may have influenced the surface refractive index.
 1. Save the subtracted curves in the project's sheet under a different name.
 2. Refer to these curves as "doubly subtracted curves": the first subtraction is the 2-1 subtraction performed on both injections, and the second is the subtraction of one curve from another.
NOTE: Such double referencing is the gold standard in SPR experiments.
 3. Overlay the subtracted curves by performing axis shift as explained before.
3. Determination of kinetics constants and model fitting
 1. Conducting a kinetic experiment
 1. Inject a series of 6-7 analyte concentrations to have a reliable of data for kinetic constants determination. Choose analyte concentrations from 10-fold above to 10-fold below the estimated K_D , in 2-3-fold dilutions. Include the "zero" concentration, later used for double reference subtraction (see above). Conduct injections in triplicates and in random order.
 2. Align and subtract all curves with respect to the Y-axis and X-axis before attempting model fitting.
 2. Model fitting
 1. Select a suitable analysis program.
NOTE: Most available fitting programs offer several models that may be applied for fitting and determination of kinetic constants.
 2. Apply the simplest model (Langmuir) assuming a reversible 1:1 interaction between the analyte and the ligand for fitting. Unless convincing data is available from other experimental methods for the existence of a more complex interaction, always use the simple Langmuir model.

Representative Results

In the system described herein, a NiNTA chip is used to immobilize the His-tagged membrane transporter^{6,7}. Being a homo-dimer, each transporter is doubly tagged, improving its binding to the NiNTA chip. Following nickel loading, ligand A (the transporter of interest) is immobilized onto Fc=2, up to ~3,500 RU (protocol cycle 2, **Figure 2A** black label). Then, using the same flow and injection duration, ligand B (the control ligand) is injected onto Fc=1, initially reaching only up to a ~3,000 RU. To make sure that similar protein amounts are immobilized on both flow cells; ligand B is further loaded, using shorter injection, while carefully monitoring the loading up to 3,500 RUs. The 'stairs' shape represents the gradual increase in mass on Fc=1 upon each injection (**Figure 2A** gray label). An on-line monitoring of Fc=2-1 helps controlling the equal ligand loading. **Figure 2B** demonstrates the Fc=2-1 subtraction, *i.e.*, subtracting the maximum RU value in Fc=2, from the step-wise increments in the RU value in Fc=1. It is important to vary the duration of the ligands' injections to achieve equal loading of the investigated and control ligands. In contrast, when injecting the analyte, the duration of the injection must be kept constant for all concentrations used. **Figure 3A** and **3B** demonstrate the responses measured by injecting a 'blank analyte' (*i.e.*, RB omitting only the analyte) in cycles 3 and 5, and the analyte injections (in cycles 4 and 6). Note that both sensograms represent the Fc=2-1 subtraction. In each of the cycles (3-6) an identical analyte injection (blank or a given concentration) is applied onto the two flow cells, immobilized with different ligands. The two repeats superimpose very well, demonstrating the high reproducibility of SPR. A response is recorded in both cases; ~3 RU for the blank versus ~40 RU for the analyte. These values represent a signal to noise ratio of ~13. To obtain the double-referenced sensograms, representing only the specific binding response, the blank injection is now subtracted from the analyte injection (~37 RU, **Figure 3C**). The sensogram recorded reveals the characteristic pattern of a transient interaction. Upon injection, the association phase is characterized by a rapid increase in the amount of ligand-bound analyte, reaching steady state after ~7 seconds. The steady state is maintained for as long as the analyte is injected. When the injection terminates, the cells are washed with RB triggering the dissociation phase. The complex rapidly dissociates, and as the analyte is washed away from the ligand the SPR signal returns to baseline.

To gain quantitative data (pre-equilibrium and equilibrium constants), a range of analyte concentrations is injected in duplicates or triplicates (see 8.3.1 in protocol). **Figure 4** shows injections of different analyte concentrations (duplicates per each concentration), keeping a constant injection length. The different analyte concentrations are injected in a random order, and the double blanked sensograms are X-axis aligned prior to analysis. To derive the affinity and rate constants, a model fitting is applied (see section 8.3.2.2 in protocol). The calculated equilibrium dissociation constant (K_D) for the ModBC-A interaction shown in **Figure 4** is $\sim 3 \times 10^{-6}$ M, with a moderately fast k_a ($\sim 10^4 \text{ M}^{-1} \text{ sec}^{-1}$) and fast k_d ($\sim 0.1 \text{ sec}^{-1}$).

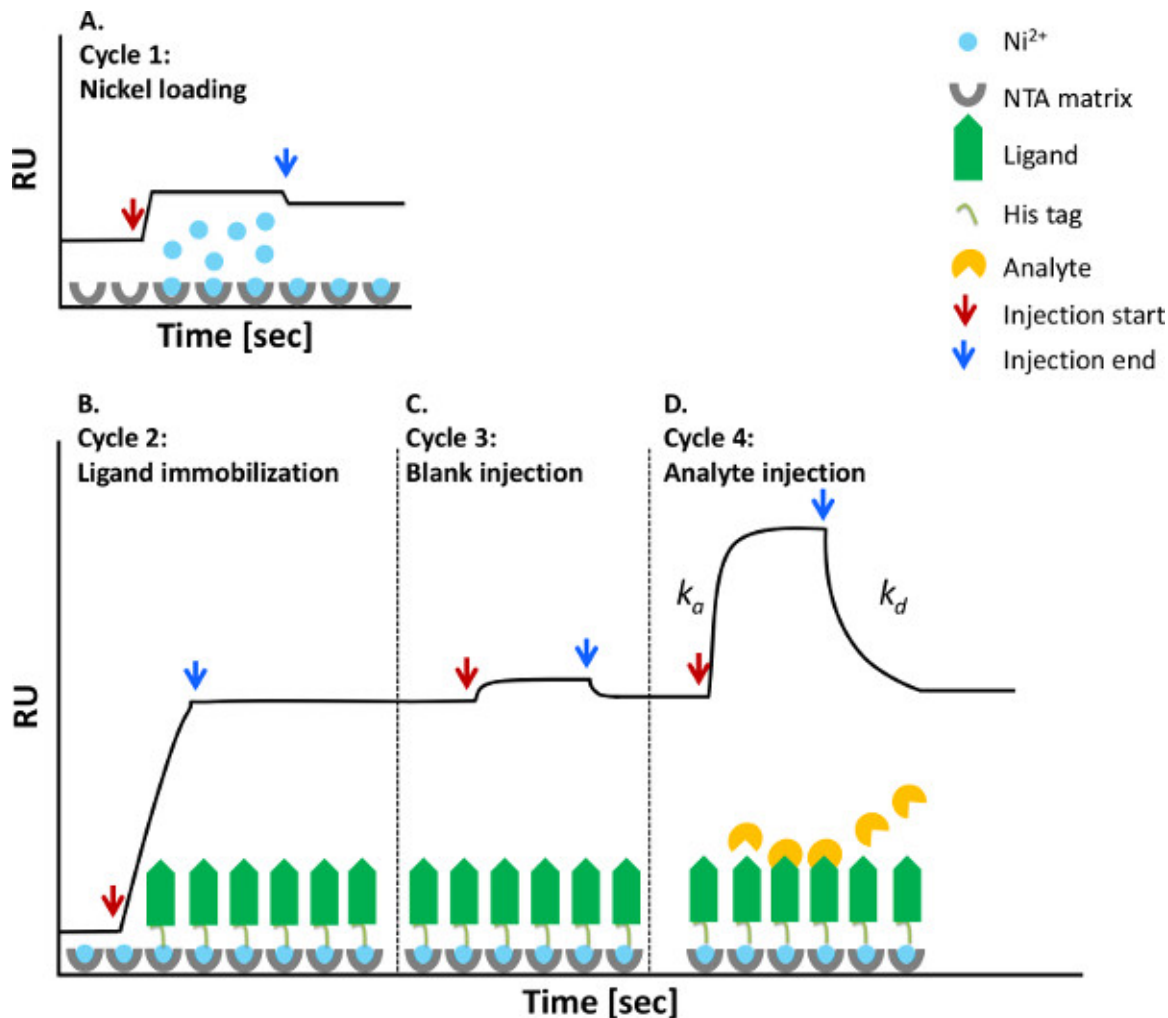


Figure 1. Illustration of the steps of an SPR experiment using a Ni-NTA sensor chip. (A) Injection of the nickel over the NTA matrix on flow cells Fc=1 and Fc=2 (shown are unsubtracted curves). The increase in mass is low (~30-50 RUs). (B-D) Illustration of the Fc=2-1 sensogram recorded during an SPR experiment. In cycle 2 as the his-tagged ligand is injected over the Ni-charged surface, mass gradually accumulates. Injection is terminated when reaching a final RU value ranging between 1,000-5,000 RU (depending on the ligand's MW). A baseline is formed, reflecting the stable binding of the ligand to the chip. In cycle 3, a blank injection is preformed, injecting the components of analyte sample excluding only the analyte. In this injection often a low response is recorded (1-5 RUs). In cycle 4 the analyte is injected using the same regime as the blank injection. The increasing RUs reflect the mass change on the surface, *i.e.*, the binding of the analyte to the ligand. The signal rapidly increases and then plateaus as the system reaches equilibrium. When the injection ends, the analyte dissociates from the ligand, resulting in a decrease in the signal and return to baseline levels.

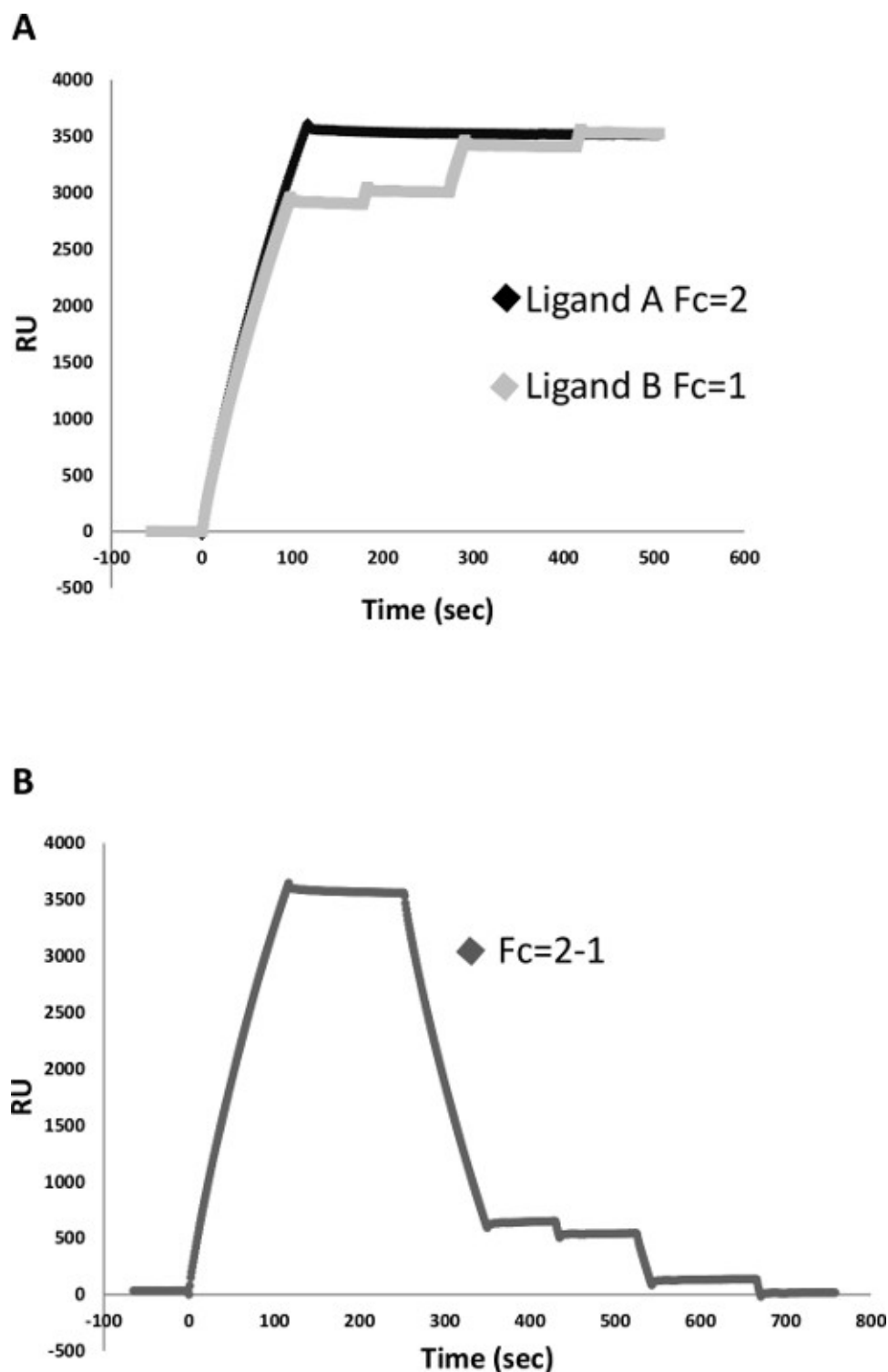


Figure 2. On line monitoring of ligand loadings. (A) Unsubtracted curves of ligand loading. The ligand of interest (ligand "A") is loaded to flow cell 2 (Fc=2, black) until reaching the desired RU level (~3,500 RU in this example). Then, the negative control ligand (ligand "B") is step-wise loaded onto flow cell 1 (Fc=1, gray) until an identical RU level is reached. (B) The two injections performed in (A) monitored in real time as an Fc=2-1 subtraction. This monitoring facilitates identical loading of ligands of interest and control.

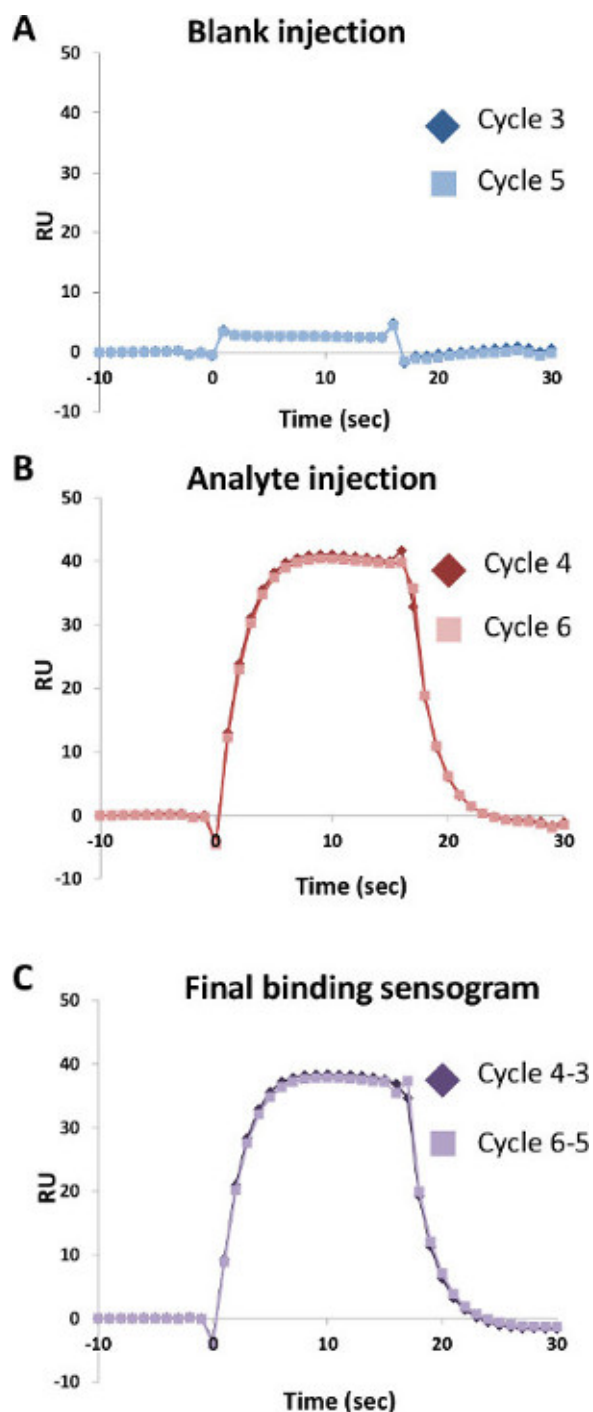


Figure 3. Double referencing. (A) Duplicates of a 2-1 subtraction (*i.e.*, $F_c=2-1$) of the blank injection. (B) As in (A), only analyte is injected. (C) Final sensograms after subtraction of the background response shown in (A) from the measured response in (B). These curves are referred to as 'double blanked' or 'double referenced'. Duplicates are of sequential cycles.

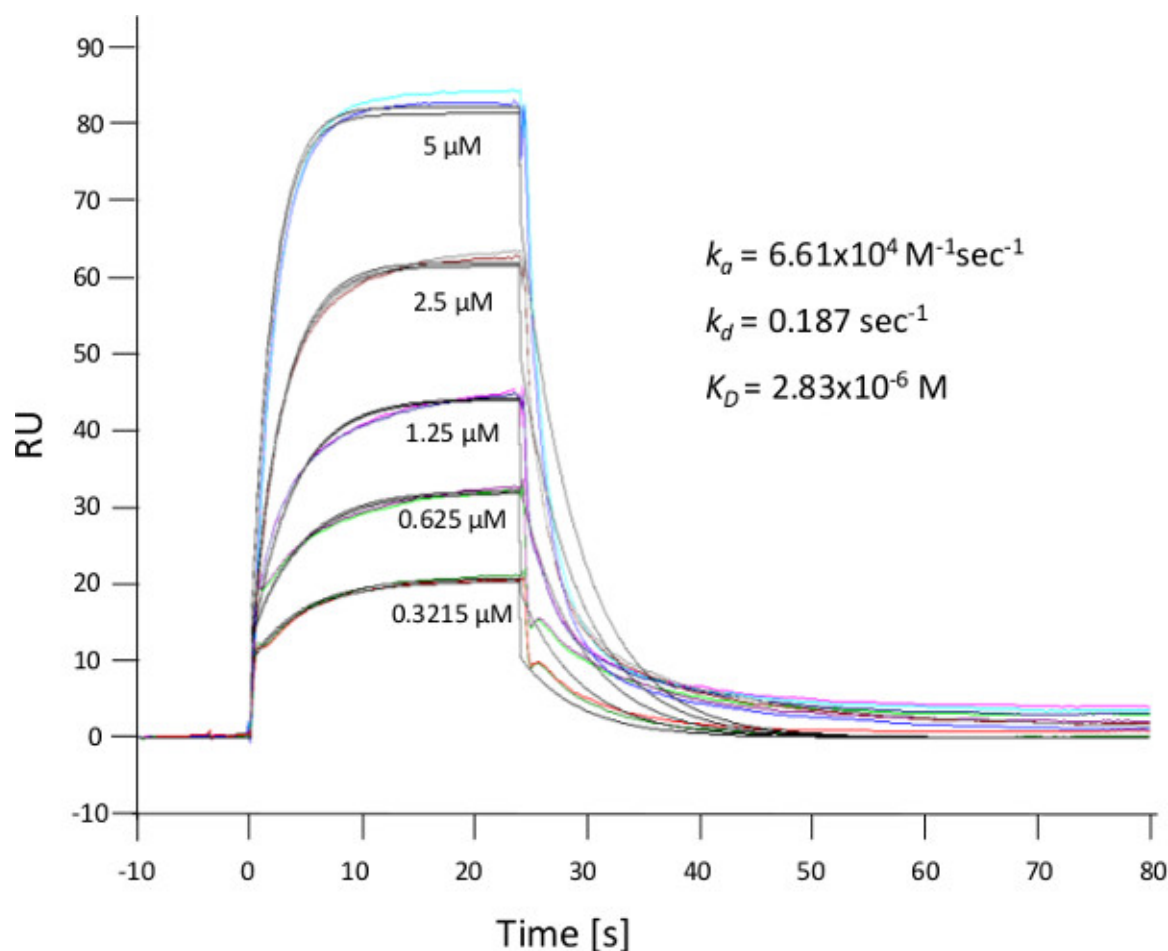


Figure 4. Determination of kinetic rate constants by injections of multiple analyte concentrations (in duplicates). The black lines are the fits that were calculated using a simple 1:1 Langmuir interaction model.

Discussion

SPR is a highly sensitive method to study molecular interactions, and is often the only approach that provides real-time monitoring of transient (yet important) interactions. An example is the transient interaction presented herein, that could not be detected by any other method (pull-down assays, liposomes sedimentation assays⁶). Moreover, while other methods are limited to equilibrium measurements (whether quantitative or qualitative), SPR is one of the only techniques that measure also the pre-equilibrium kinetics.

Its heightened sensitivity is also its caveat. False-positive sensograms are readily recorded. It is therefore recommended to establish the existence of the interaction using other methods before attempting SPR measurements. Prior knowledge of the system's characteristics (rough assessment of the K_D , stable vs. transient interaction) is very helpful in setting up SPR experiments and evaluating the functional relevance of the obtained measurements. An important issue to keep in mind before starting SPR measurements is the quality of the protein samples. SPR is highly sensitive to aggregates and these must be removed from both the ligand and the analyte preparations (by gel filtration chromatography or ultracentrifugation when possible). Another source of noise and false signals may stem from buffer mismatches between the RB and the buffer of the analyte. This may be solved by dialyzing the analyte against the RB, or by using a highly concentrated preparation of the analyte which is diluted many folds (~100-1,000-fold) into the RB. False positive data can also result from non-specific interactions of the analyte with the chip's matrix or with the negative control. These interactions are of low affinity, so sometimes can be avoided by using low analyte concentrations. In addition, inclusion of low BSA and/or detergent concentrations (0.1 mg/ml and/or 0.05-0.1% (w/v), respectively) to the RB and to the injected analyte sample can decrease nonspecific interactions. Changing the content in the negative control cell (different negative control) is another approach for dealing with nonspecific binding issues. Alternatively, using another type of chip can also affect the quality of data. Various kinds of sensor chips are commercially available, based on different chemistries. In the most commonly used sensor chip (the CM-5 chip) carboxymethylated dextran is covalently attached to a gold surface. Proteins can be covalently coupled to the sensor surface using a variety of very simple surface chemistries (most commonly amine or thiol coupling). However, in our hands, when working with membrane proteins covalent immobilization often yields artifacts and is thus not the method of choice. In the context of studying a membrane protein, it is noteworthy that there are commercial chips that are coated with lipophilic molecules. These enable the immobilization of membrane proteins onto a native-like environment. Similar sensor chips can also be prepared 'in house'. For a description of other coupling methods see^{10,11}. The protocol described herein is based on the use of Ni-NTA chip for the immobilization of a His tagged, detergent solubilized transporter (the analyte is not His-tagged to avoid analyte binding to the chip surface). In our experience^{6,12}, kinetic constants derived from experiments conducted with Ni-NTA chips are in good agreement with those determined in native-like environments. Although Ni-NTA chips are relatively expensive, their

primary advantage is that they can be stripped of ligand and re-used. Hundreds of measurements can be done per chip. Moreover, the ligand's orientation on the chip is determined by the position of the his-tag and is thus quite homogeneous.

Once an interaction has been successfully recorded by SPR, a series of controls and repeats are needed to verify the validity of the SPR signal. Verification of the following parameters may assist to validate the SPR data:

1. Specificity of the SPR signal: due to the high sensitivity of SPR, the use of appropriate negative controls is of special importance. Non-specific interaction recorded by SPR can be due to the adsorption of an analyte to the matrix. Therefore, an empty flow cell is often not the best option since a flow cell loaded with protein (the ligand) is not chemically equivalent to an empty flow cell. A better option is to use an inactive mutant variant of the ligand, one that is known not to bind the analyte. Alternatively, one can use a similarly mutated analyte. If such mutants are not available a homologous protein (but of different binding specificity) can be used¹². Examples of good negative controls are a homologous ligand (as described here), or a point mutation in one of the interacting partners that is known to abolish the association. Another recommended negative control is alteration of a post-translational modification (e.g., phosphorylation, methylation, thiol derivation) that is essential to the interaction. For example, de-phosphorylation of a tyrosine that is essential for SH2 domain interaction. Other negative controls may be system specific. When using SPR, the importance of using strict negative controls cannot be overstated.

2. Reproducibility of the SPR signal: SPR is an extremely reproducible method. When equal ligand amounts are loaded onto the biosensor chip and equal concentrations of the analyte are injected the repeats should perfectly align (see **Figure 3**).

3. Regeneration of the system: in interactions with a slow dissociation rate (k_d), the analyte must be stripped from the ligand while preserving the functional and structural integrity of the latter. If dissociation and/or regeneration are incomplete, the SPR signal will gradually decay upon subsequent injections of the analyte. Typical regenerating conditions that can be tested are high concentrations of Mg^{2+} (up to 2 M), acidic or basic conditions (e.g., 10 mM glycine pH 2.5, 10 mM NaOH), high salt (up to 4 M NaCl), or high detergent concentration (e.g., 1% DDM). However, the regenerating effectiveness of these reagents (and others) is highly system-specific and thus regeneration conditions must be tested. In some cases, the interaction is so stable and strong that regeneration is never complete, as in the case of the *E. coli* vitamin B₁₂ transport system (BtuCD-F)⁷. Under such conditions, the only option is to strip the ligand from the biosensor chip and reload a fresh batch for each injection. In cases where the dissociation of the analyte from the ligand is fast and complete surface regeneration is unnecessary (see protocol and **Figures 2-3**).

4. Membrane proteins are always purified in the presence of detergent, which can affect the SPR sensitivity and reaction rates. However, in our experience with BtuCD, the measured rates are very similar. One always needs to obtain supporting evidence using complimentary approaches such as size exclusion chromatography (SEC), pull-down assays, liposome sedimentation assay, etc. For example a correlation between SPR and other techniques has been shown for the methionine, vitamin B₁₂, and two molybdate systems^{6,12}.

Erroneous determination of the kinetic rate constants often arises during the fitting process. As a rule of thumb, always apply the simple Langmuir 1:1 interaction model for analysis kinetic experiments. The more complex models make additional assumptions of conformational changes, bivalent analyte, or sample heterogeneity. These assumptions should be made only if supported by evidence from other experimental approaches. Note that applying these models will most likely result in better fitting and lower χ^2 values, but this should not be viewed as a testimony for their reliability. The higher degrees of freedom afforded by the more complex models are usually responsible for the better fitting, rather than their mechanistic relevance.

One of the advantages of SPR is its broad chemical compatibility, especially with respect to detergents that are used to solubilize and purify membrane proteins. However, sucrose and glycerol (and other viscous solutions) that are often added during the purification process of membrane proteins have dramatic effects on the RU. It is therefore recommended to avoid these if possible, or at least reduce their concentration. When using viscous solutions, avoiding buffer mismatch becomes essential. Adding lipids to a detergent containing RB can amplify the binding signal by as much as ten-fold. However, such an endeavor is expensive (for most lipids) and tends to shorten the life span of the biosensor chips.

The tips and troubleshooting listed above may help while calibrating a new system. However, a given system often requires specific tweaking (e.g., choice of detergent, salt, pH) to achieve optimal results.

SPR has long been recognized as a powerful and unique approach to measure molecular interactions. In recent years the availability of various SPR platforms and their declining prices has made SPR more approachable. It is rapidly becoming the gold standard approach to study protein-protein interactions, target protein-drug interactions, and in discovering lead small molecule inhibitors.

Disclosures

All authors declare that they have no competing financial interests.

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