Surface plasmon resonance[1]

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[1] van der Merwe, P. (2001). Surface Plasmon Resonance. In S. E. Harding & B. Z. Chowdhry (Eds.), Protein-Ligand Interactions: hydrodynamic and colorimetry (Vol. 2, pp. 137–170). Oxford University Press, USA.

1. Introduction

Since the development almost a decade ago (1,2) of the first biosensor based on surface plasmon resonance (SPR), the use of this technique has increased steadily. Although there are several SPR-based systems (3-5), by far the most widely used one is the BIAcore (1,2), produced by BIAcore AB, which has developed into a range of instruments (Table 1). By December 1998 over 1200 publications had reported results obtained using the BIAcore. It is likely that it would be even more widely used were it not for its high cost and the pitfalls associated with obtaining accurate quantitative data (5-8). The latter has discouraged many investigators and led to the perception that the technique may be flawed. This is unjustified because the pitfalls are common to many binding techniques and, once understood, they are easily avoided (4,8,9). Furthermore, the BIAcore offers particular advantages for analysing weak macromolecular interactions, allowing measurements that are not possible using any other technique (4,10). This Chapter aims to provide guidance to users of SPR, with an emphasis on avoiding pitfalls. No attempt is made to describe the routine operation and maintenance of the BIAcore, as this is comprehensively described in the BIAcore instrument manual

(see Section 9.3). Although written for BIAcore users, the general principles will be applicable to experiments on any SPR instrument.

2. Principles and applications of Surface Plasmon Resonance.

2.1. Principles

The underlying physical principles of SPR are complex (see Section 9.1). Fortunately, an adequate working knowledge of the technique does not require a detailed theoretical understanding. It suffices to know that SPR-based instruments use an optical method to measure the refractive index near (within ~300 nm) a sensor surface. In the BIAcore this surface forms the floor of a small flow cell, 20-60 nL in volume (Table 1), through which an aqueous solution (henceforth called the *running buffer*) passes under continuous flow (1-100 $\mu L.min^{-1}$). In order to detect an interaction one molecule (the *ligand*) is immobilised onto the sensor surface. Its binding partner (the *analyte*) is injected in aqueous solution (*sample buffer*) through the flow cell, also under continuous flow. As the analyte binds to the ligand the accumulation of protein on the surface results in an increase in the refractive index. This change in refractive index is measured in real time, and the result plotted as response or resonance units (RUs) versus time (a *sensorgram*). Importantly, a response (*background response*) will also be generated if there is a difference in the refractive indices of the running and sample buffers. This background response must be subtracted from the sensorgram to obtain the actual binding response. The background response is recorded by injecting the analyte through a control or reference flow cell, which has no ligand or an irrelevant ligand immobilized to the sensor surface. One RU represents the binding of approximately 1 pg protein/mm^2. In practise >50 pg/mm^2 of analyte binding is needed. Because is it very difficult to immobilise a sufficiently high density of ligand onto a surface to achieve this level of analyte binding, BIAcore have developed sensor surfaces with a 100-200 nm thick carboxymethylated dextran matrix attached. By effectively adding

a third dimension to the surface, much higher levels of ligand immobilisation are possible. However, having very high levels of ligand has two important drawbacks. Firstly, with such a high ligand density the rate at which the surface binds the analyte may exceed the rate at which the analyte can be delivered to the surface (the latter is referred to as mass transport). In this situation, mass transport becomes the rate-limiting step. Consequently, the measured association rate constant (k_{on}) is slower than the true k_{on} (see Section 7.2). A second, related problem is that, following dissociation of the analyte, it can rebind to the unoccupied ligand before diffusing out of the matrix and being washed from the flow cell. Consequently, the measured dissociation rate constant (apparent k_{off}) is slower than the true k_{off} (see Section 7.2). Although the dextran matrix may exaggerate these kinetic artefacts (mass transport limitations and re-binding) they can affect all surface-binding techniques .

2.2. Applications

This section outlines the applications for which SPR is particularly well suited. Also described are some applications for which it is probably not the technique of choice. Of course, future technical improvements are likely to extend the range of applications for which the SPR is useful.

2.2.1. What SPR is good for

2.2.1.1. Evaluation of macromolecules

Most laboratories studying biological problems at the molecular or cellular level need to produce recombinant proteins. It is important to be able to show that the recombinant protein has the same structure as its native counterpart. With the possible exception of enzymes, this is most easily done by confirming that the protein binds its natural ligands. Because such interactions involve multiple residues, which are usually far apart in the primary amino acid sequence, they require a correctly folded protein. In the absence of natural ligands monoclonal antibodies (mAbs) that are known to bind to the native protein are an excellent means of assessing the structural integrity of the recombinant protein. The BIAcore is particularly well suited to evaluating the binding of

recombinant proteins to natural ligands and mAbs. Setting up an assay for any particular protein is very fast, and the data provided are highly informative.

2.2.1.2. Equilibrium measurements (affinity and enthalpy).

Equilibrium analysis requires multiple *sequential* injections of analyte at different concentrations (and at different temperatures). Because this is very time-consuming it is only practical to perform equilibrium analysis on interactions that attain equilibrium within about 30 min. The time it takes to reach equilibrium is determined primarily by the dissociation rate constant or k_{off}; a useful rule of thumb is that an interaction should reach 99% of the equilibrium level within $4.6/k_{off}$ seconds. High affinity interactions (K_D < 10 nM) usually have very slow k_{off} values and are therefore unsuitable for equilibrium analysis. Conversely, very weak interactions (K_D >100 μM) are easily studied. The small sample volumes required for BIAcore injections (<20 μL) make it feasible to inject the very high concentrations (>500 μM) of protein required to saturate low affinity interactions (11).

Equilibrium affinity measurements on the BIAcore are highly reproducible. This feature and the very precise temperature control makes it possible to estimate binding enthalpy by van't Hoff analysis (12). This involves measuring the (often small) change in affinity with temperature (Section 7.4). Although not as rigorous as calorimetry, much less protein is required.

2.2.1.3. Kinetic measurements

The fact that the BIAcore generates real-time binding data makes it well suited to the analysis of binding kinetics. There are, however, important limitations to kinetic analysis. Largely because of mass transport limitations it is difficult to measure accurately k_{on} values faster than about 10^6 $M^{-1}s^{-1}$. This upper limit is dependent on the size of the analyte. Faster k_{on} values can be measured with analytes with a greater molecular mass. This is because the larger signal produced by a large analyte allows the experiment to be performed at lower ligand densities, and lower ligand densities require

lower rates of mass transport. For different reasons measuring k_{off} values slower than 10^{-5} s^{-1} or faster than ~1 s^{-1} is difficult. Because the BIAcore is easy to use and the analysis software is user-friendly, it is deceptively easy to generate kinetic data. However *obtaining accurate kinetic data is a very demanding and time-consuming task, and requires a thorough understanding of binding kinetics and the potential sources of artefact* (Section 7.2).

2.2.1.4. Analysis of mutant proteins

It is possible using BIAcore to visualise the capture of proteins from crude mixtures onto the sensor surface. This is very convenient for analysing mutants generated by site-directed mutagenesis (13,14). Mutants can be expressed as tagged proteins by transient transfection and then captured from crude tissue culture supernatant using an antibody to the tag, thus effectively purifying the mutant protein on the sensor surface. It is then simple to evaluate the effect of the mutation on the binding properties (affinity, kinetics, and even thermodynamics) of the immobilised protein. This provides the only practical way of quantifying the effect of mutations on the thermodynamics and kinetics of weak protein/ligand interactions (15).

2.2.2. What SPR is *not* good for.

2.2.2.1. High throughput assays.

The fact the BIAcore can only sample can be analysed at a time, with each analysis taking 5-15 min, means that it is neither practical nor efficient for high throughput assays. Automation does not solve this problem because the sensor surface deteriorates over time and with re-use. Blockages or air bubbles in the microfluidic system are also common in long experiments, especially when many samples are injected.

2.2.2.2. Concentration assays.

The BIAcore is also unsuitable for concentration measurements, because these require the analysis of many samples in parallel, including the standard curve. A second problem is that, for optimal

sensitivity, concentration assays require long equilibration periods.

2.2.2.3. Studying small analytes.

Because the SPR measures the mass of material binding to the sensor surface, very small analytes (M_r <1000) give very small responses. The recent improvements in signal to noise ratio have made it possible to measure binding of such small analytes. However a very high surface concentration of active immobilised ligand (~1 mM) is needed, and this is difficult to achieve. Furthermore, at such high ligand densities accurate kinetic analysis is not possible because of mass-transport limitations and re-binding (Section 7.2). Thus only equilibrium analysis is possible with very small analytes, and then only under optimal conditions. This assessment may need to be revised as and when future improvements are made in the signal to noise ratio.

3. General principles of BIAcore experiments

3.1. A typical experiment

A typical SPR experiment involves several discrete tasks.

- Prepare ligand and analyte.
- Select and insert a suitable sensor chip.
- Immobilise the ligand and a control ligand to sensor surfaces.
- Inject analyte and a control analyte over sensor surfaces and record response.
- Regenerate surfaces if necessary.
- Analyse data.

While the ligand and analyte could be almost any type of molecule, they are usually both proteins. This chapter focuses on the analysis of protein-protein interactions.

3.2. Preparation of materials and buffers

BIAcore experiments are frequently disrupted by small air bubbles or other particles passing through

the flow system. Usually these can be flushed out and the experiment repeated, wasting only time and reagents. Occasionally, however, the damage is irreversible necessitating the replacement of an expensive sensor chip or integrated fluidic cartridge. This can be minimised by following a few simple rules (Section 9.2.1).

3.3. Monitoring the Dips

The output from the photo-detector array that is used to determine the surface plasmon resonance angle (θ_{spr}, Section 9.1) can be viewed directly as 'dips'. The current BIAcore documentation does not describe how to view and interpret dips, and so this information is supplied here (Protocol 1).

Protocol 1. Normal and abnormal 'dips'

Viewing the dips

1. Enter the service mode on the BIAcore control software by simultaneously pressing the c*ontrol*, *alt*, and *s* keys.
2. When the dialog box appears requesting a password ignore this and press the *enter* or *return* key. An additional *Service* menu will appear on the menu bar.
3. Select *View dips* from this menu.
4. A graph appears similar to the one in Figure 1 showing the amplitude of light reflected off the sensor surface (Reflectance) measured over a small range of angles. The angle of the minimum reflectance (θ_{spr}) is calculated by fitting a curve to all this data, thereby enabling θ_{spr} to be measured at a far greater resolution than the resolution at which the data are actually collected.

Normal dip

The important feature of a normal dip is its depth (Figure 1, Dip1). Generally it bottoms out at a Reflectance of ~10000. When the refractive index above the sensor surface increases (e.g. because of protein binding), the dip shifts to the right while maintaining its shape and depth and (Dip 2).

Abnormal dip

There are two main types of abnormal dip.

1. The shallow dip (Dip 3). The θ_{spr} is measured along a section of the sensor surface rather than at

a single point. Refractive index heterogeneity on the surface gives heterogenous θ_{spr} values. When averaged the result is a shallow dip that does not reach below 10000 RUs. Heterogeneity can be the result of differences in the amount of material immobilised along the surface, in which case the dip is usually slightly shallow, or the result of small air bubbles or particles in the flow-cell, in which case the dip is very shallow.

2. No dip (Dip 4). A large change in the refractive index beyond the instrument dynamic range (Table 1) will shift the θ_{spr} so much that no dip is evident. Usually this is the result of air in the flow cell.

While slight shallowness of the dip is acceptable, and is common after coupling large amounts of protein, more severe abnormalities should not be ignored. Attempts should be made to return dips to normal by flushing the flow cells with buffer and/or regenerating the sensor surfaces. If this is ineffective a new sensor surface should be used.

4. Ligand

4.1. Direct versus indirect immobilisation

The most challenging step when setting up SPR experiments is immobilising of one of the proteins (the *ligand*) to the sensor surface without disrupting its activity. Immobilisation can either be direct, by covalent coupling, or indirect, through capture by a covalently coupled molecule.

The major advantage of *direct covalent immobilisation* is that it can be used for any protein provided that it is reasonably pure (>50%) and has a pI > 3.5. However, it has three important drawbacks. Firstly, because they usually have multiple copies of the functional group that mediates immobilization, proteins are coupled heterogeneously and sometimes at multiple sites. Secondly, direct coupling often decreases or completely abrogates binding to analyte. And thirdly, directly-coupled proteins are difficult to regenerate (see below).

Indirect immobilisation has the disadvantage that it can only be used for proteins that have a suitable binding site or tag for the covalently coupled molecule. However, it has four important advantages,

which make it the method of choice in most cases. Firstly, proteins are seldom inactivated by indirect coupling. Secondly, the protein need not be pure. It can be captured from a 'crude' sample. Thirdly, all the molecules are immobilised in a known and consistent orientation on the surface. Finally, using appropriate buffers it is often possible to dissociate selectively the non-covalent ligand/analyte bond, thereby enabling the ligand surface to be re-used – a process termed ‘regeneration’.

4.2. Covalent immobilisation

4.2.1. A general approach

Instead of replicating the detailed protocols in the BIAcore literature (see Section 9.3), I will suggest a general approach (Protocol 2) to covalent coupling, discussing several aspects in detail.

Protocol 2. An approach to covalent coupling of a protein

1. Select the coupling chemistry (Section 4.2.2).
2. Prepare the protein (Section 4.2.3).
3. Optimize the pre-concentration step (Section 4.2.4).
4. Couple the protein (Section 4.2.5).
5. Evaluate the activity of the immobilised protein (Section 4.5).
6. Establish conditions for regeneration (Section 4.2.6).
7. Adjust the immobilization conditions (Section 4.2.7).

4.2.2. Choice of chemistry

There are three main types of coupling chemistry, which utilize, respectively, amine (e.g. lysine), thiol (cysteine) or aldehyde (carbohydrate) functional groups on glycoproteins (Table 3). All covalent coupling methods utilize free carboxymethyl groups on the sensor chip surface. They can therefore be used for any of the sensor chips that have such carboxymethyl groups (See Table 2). If the protein to be immobilised has a surface-exposed disulphide or a free cysteine, ligand-thiol

coupling is probably the method of choice. Failing this, amine coupling should be tried in the first instance. If amine coupling inactivates the protein (as assessed by ligand and/or mAb binding), aldehyde coupling can be attempted, provided that the protein is glycosylated. Detailed protocols are available from BIAcore for all coupling techiques (see Section 9.3). Only amine coupling is described here in some detail.

4.2.3. Prepare the protein

Only a modest amount (5-10 μg) of protein is needed. The major requirement is that the protein is pure and has a high level of activity. Because direct coupling is relatively indiscriminate, all protein in the preparation will be coupled. Thus if the preparation is contaminated by other proteins or is partially active, the level of binding observed will be proportionally decreased. Because the protein needs to be diluted into the pre-concentration buffer (Section 4.2.4) to a final concentration of 20-50 μg/ml, the stock should be fairly concentrated (> 0.5 mg/ml). If the protein is in solutions that are strongly buffered or contain high salt concentrations, primary amine groups or sodium azide, then these must either be dialysed out or much larger dilutions (1:100) made. In the latter case the protein will need to be at higher concentrations (>2 mg/mL).

4.2.4. Pre-concentration

The purpose of pre-concentration is to concentrate the protein to very high levels (>100 mg/mL) within the dextran matrix, thereby driving the coupling reaction. Without pre-concentration far higher concentrations of protein would need to be injected to get equivalent levels of coupling. Pre-concentration is driven by an electrostatic interaction between the negatively charged carboxylated dextran matrix and positively charged protein. To this end the protein is diluted into a buffer with a low ionic strength (to minimize charge screening) with a pH below its isoelectric point or pI (to give the protein net positive charge). However amine coupling is most efficient at a high pH because

activated carboxyl groups react better with uncharged amino groups. Thus the highest pH compatible with pre-concentration is determined empirically (Protocol 3). Electrostatically-bound protein should dissociate rapidly and completely when injection of running buffer resumes, both because the proteins net positive charge will decrease and because electrostatic interactions will be screened by the high ionic strength of the running buffer. Incomplete dissociation suggests that the interaction was not purely electrostatic, perhaps because of the binding, at low pH, of a denatured form of the protein.

Protocol 3. Determining the optimum pre-concentration conditions for a protein

1. Dilute the protein to a final concentration of 20-50 μg/mL (final volume 100 μL) into pH 6.0, 5.5, 5.0, 4.5 and 4.0 pre-concentration buffers (see Section 9.2.2).
2. Start a manual BIAcore run using a single flow cell (flow-rate 10 μL/min)
3. Inject 30 μL of each sample, beginning at pH 6.0 and working down.
4. If no electrostatic interaction is observed continue with protein samples diluted in pH 3.5 and 3.0 pre-concentration buffers.
5. Use the highest pH at which >10000 RU of protein binds electrostatically during the injections.
6. Check that all the bound protein dissociates after the injection. If not it suggests that not all the protein was bound electrostatically and that the protein denatures irreversibly at that pH.

4.2.5. Amine coupling

There is a standard protocol for amine coupling (Protocol 4). The first step is to activate the carboxymethyl groups with N-hydroxysuccinimide (NHS), thus creating a highly reactive succinimide ester which reacts with amine and other nucleophilic groups on proteins. The second (coupling) step is to inject the protein in pre-concentration buffer, thereby achieved high protein concentrations and driving the coupling reaction. The third (blocking) step, blocks the remaining activated carboxymethyl groups by injecting very high concentrations of ethanolamine. The high

concentration of ethanolamine also helps to elute any non-covalent bound material. The final regeneration step is optional. When a protein is coupled for the first time it is advisable not to include any regeneration step. If it is included and the protein has poor activity it will not be clear whether covalent coupling or regeneration was responsible for disrupting the protein. The structural integrity of the protein should be evaluated (Section 4.2.6) before regeneration is attempted. Once regeneration conditions have been established these can be added on to the coupling protocol.

Protocol 4. Amine coupling

Reagents

120 μL of protein at 20-50 μg/ml in suitable pre-concentration buffer (Protocol 3)
120 μL of EDC (0.4 M) mixed 1:1 with NHS (0.1 M). Make up just before coupling
120 μL of ethanolamine/HCl 1 M, pH 8.0
120 μL of regeneration solution (if regeneration conditions known)

Procedure

1. Establish pre-concentration conditions (Protocol 3)
2. Set flow-rate to 10 μL/min
3. Activation: inject 70 μL of EDC/NHS
4. Coupling: inject 70 μL of protein
5. Blocking: inject 70 μL of ethanolamine
6. Regeneration: inject 30 μL of regeneration solution (if regeneration conditions known, see Protocol 5).
7. View the 'dips' (Protocol 1) to confirm that the immobilisation is homogeneous.

4.2.6. Regeneration

Once a covalently immobilised protein has been shown to be active with respect to binding its natural ligand or a monoclonal antibody (Section 4.4), regeneration can be attempted. A general approach to establishing regeneration conditions can be used (Protocol 5). The goal here is to elute any non-

covalently bound analyte without disrupting the activity of the ligand. Regeneration allows surfaces to be re-used many times, saving both time and money. However establishing ideal regeneration conditions can be a very time-consuming, and in many cases impossible, task. Thus it may be more cost-effective to opt for imperfect or no regeneration, using new sensor surfaces instead.

Protocol 5. Establishing regeneration conditions

Regeneration solutions

- These fall into four main classes: divalent cation chelator, high ionic strength, low pH, high pH (Section 9.2.2).
- If the interaction is likely to be dependent on divalent cations try buffers with EDTA.
- If the monomeric interaction is weak, try high ionic strength buffers.
- Otherwise start with a low pH buffer.
- If this is without effect, try high pH buffer.

Approach

1. Covalently couple ligand to the surface.
2. Make up enough analyte for several injections.
3. Inject analyte over the immobilised ligand and measure the amount of binding.
4. Inject selected regeneration buffer for 3 min, and measure the amount of analyte that remains bound after this.
5. If the decrease is <30% switch to a different class of regeneration buffer and return to step 4.
6. If the decrease is >30% but <90%, try repeated injections. If this fails to elute >90% select a stronger regeneration buffer of the same type and return to step 4.
7. If this fails, try a different class of buffer and return to step 4.
8. When >90% of bound analyte is eluted, return to step 3, using identical analyte and injection conditions. If binding post-regeneration remains >90% of binding before regeneration, the regeneration conditions may be adequate.
9. If residual activity is <90%, select a different class of regeneration buffer. If residual binding is very low it may be necessary to return to step 1, starting with a new surface.
10. If two different types of buffer give partial elution, try using both sequentially.

4.2.7. Adjusting the immobilisation conditions.

Since the level of ligand coupling achieved is unpredictable it is usually necessary to modify the initial protocol in order to achieve the desired immobilization level. It is also often necessary to create several surfaces on the same chip with different levels of coupling. The best way to achieve different levels of coupling is to change the duration of the activation step, by varying the volume of NHS/EDC injected. The level of coupled ligand varies in proportion with the duration of the activation step. Thus, a two-fold reduction in activation period will usually lead to a two-fold reduction in coupling. It is possible using an option in the inject command to couple ligand simultaneously in multiple flow cells, varying only the length of activation step.

4.3. Non-covalent immobilisation (ligand capture)

There are two requirements for non-covalent or indirect immobilization of a ligand (henceforth called 'ligand capture'). First, it must be possible to obtain or create a sensor surface that can capture a ligand. Ideally, it should be possible to regenerate this surface so that repeated capture is possible. Second, the ligand needs to have a suitable binding site or modification that allows it to be captured.

4.3.1. Using an existing strategy

A number of established techniques are available for ligand capture (Table 4). When expressing a recombinant protein to be used in SPR studies it is advisable to consider adding a suitable domain or peptide motif so that one of these techniques can be used. The precise choice of tag will depend on whether the tag is needed for purification and what other uses are envisaged for the ligand.

- If it is envisaged that the ligand is to be used as an analyte in SPR studies it should be monovalent or, if multivalent (Fc- and GST-chimeras), the tag should be readily removable.
- If the ligand is to be used for structural studies, the tag should also be removable.

- If the ligand is to be used to probe for binding partners on cells or tissues it should be multivalent, or it should be possible to make it multivalent.
- If the ligand is to be captured from crude mixtures (i.e. after expression without purification) the capturing agent needs to have a high affinity and to be highly specific. For example, CD4 (14) and anti-human IgG_1 (13) mAbs have been shown to be suitable for this purpose.

4.3.2. Developing a new strategy

The widespread availability of purified monoclonal or polyclonal mAbs, and their ability to tolerate harsh regeneration conditions, make them suitable reagents for non-covalent immobilization. Typically there is a need to immobilize a number of related ligands, with an invariant and a variant portion. If several antibodies against the invariant portion are available, it is likely that at least one of these will be suitable for indirect coupling. A basic approach to developing such a method is described in Protocol 6.

Protocol 6. Developing a new antibody-mediated indirect coupling method

1. Obtain a panel of antibodies that can bind a suitable ligand and are available in pure form.
2. Analyse the binding properties and select a high affinity antibody with a slow k_{off}.
3. Covalently couple the antibody by amine coupling (Protocol 4) and check for activity. If inactive, try a different antibody.
4. Establish regeneration conditions (Protocol 5).
5. If suitable regeneration conditions cannot be found, try a different antibody.

4.4. Activity of immobilised ligand

It is important to evaluate the functional integrity of the immobilised protein. This is best achieved by using a protein which binds to correctly-folded ligand. An ability to bind its natural ligand is reassuring evidence that an immobilised ligand is functionally intact. Since monoclonal antibodies

(mAbs) usually bind to 'discontinuous epitopes' on a protein, they are excellent probes of protein structure. It is preferable to check the binding of several mAbs, including ones that bind to the same binding sites or, failing this, the same domain of the natural ligand. MAbs are particularly useful if the natural binding partner has not been identified and/or a candidate binding partner being assessed for an interaction with the ligand. In is important to know not just whether immobilised ligand is 'active' but also what proportion is active (Protocol 7).

Protocol 7. Quantitating binding levels

The ligand activity (*ActL*) , stoichiometry (*S*), molecular mass of ligand (M_L) and analyte (M_A), and the analyte binding level at saturation (A) are related as follows:

$$ActL * S = \left(\frac{M_L}{M_A} \right) * \frac{A}{L}$$

Where L is the level of ligand immobilised

S is the molar ratio of analyte to ligand in analyte/ligand complex

The product ActL*S is readily calculated once A and L have been measured. Either ActL or S need to be independently determined in order to calculate the other. A convenient way to measure ActL by SPR is to use Fab fragments of mAbs specific for the ligand. Intact mAbs are less useful because of the uncertaintly as to their binding stoichiometry.

4.5. Control surfaces

A control surface should be generated which is as similar as possible to the ligand surface, including similar levels of immobilisation. This is to measure non-specific binding and to record the background response. The immobilisation levels need to be similar because this affects the background response measured with analytes that have a high refractive index (Section 5.3).

4.6. Re-using sensor chips

Because each sensor chip has several flow cells, it is common to have unused flow cells at the end of an experiment. In addition many covalently coupled ligands are very stable, enabling surfaces to be re-used over several days. It is therefore convenient to be able to remove and reinsert sensor chips in the BIAcore (Protocol 8).

Protocol 8. Reusing sensor chips

- Undock the sensor chip, choosing the empty flow cell option in the dialogue box.
- Store the sensor chip in its cassette at 4ºC.
- When it is to be re-used, re-insert and dock the sensor chip.
- After priming the system check the 'dips' (Section 3.3).

5. Analyte

The extent to which the analyte needs to be characterised depends on the nature of the experiment. Quantitative measurements (Section 7) require that the analyte is very well characterised and of the highest quality. In contrast, this is less important for qualitative measurements (Section 6).

5.1. Purity, activity and concentration

In order to determine affinity and association rate constants it is essential that the *concentration* of the injected material is known with great precision. The only sufficiently accurate means of measuring concentration is to use a spectrophometer to measure the optical density of a solution of *pure* protein, usually at 280 nm (OD_{280}). In order to calculate the concentration from the absorption at $OD_{280,}$ two additional measurements are required. Firstly, it is necessary to determine the extinction coefficient. Although this can be calculated from the primary sequence it is best determined directly by amino acid analysis of a sample of the protein with a known OD_{280}. Secondly, it is important to assess what proportion of the purified protein is 'active', i.e. able to bind to the

ligand. This can be done by depletion experiments in which the ligand-coated sepharose beads are used to deplete the analyte from solution (16). mAb-coated sepharose beads can be used instead if ligand-coated beads are impractical. If all the analyte can be depleted in this way it is 100% active.

5.2. Valency

In general, affinity and kinetic measurement require that each analyte molecule has a single binding site, i.e. is monovalent. If the protein has a single binding site it is only necessary to show that it exists as a monomer in solution. This is most readily achieved by size-exclusion chromatography (Chapter 3) or analytical ultracentrifugation (Chapters 4 and 5). It is important to emphasise that these analytical techniques will not detect the presence of very low concentrations of multivalent aggregated material (17,18). In order to ensure that this material does not contribute to the binding it is ESSENTIAL to purify the monomeric peak by size exclusion chromatography immediately before BIAcore experiments and to analyse it before concentrating or freezing it. Only when it has been shown that concentration, storage or freezing do not affect the measured affinity and kinetic constants is it wise to deviate from this strict principle. Fortunately the presence of multivalent aggregates is readily excluded by analysis of the binding kinetics (Section 7.2). If the dissociation of bound analyte is monophasic (mono-exponential) multivalent binding can be ruled out. If dissociation is bi-exponential with >10 fold difference in the two k_{off} values, multivalent binding is likely. Bi-exponential dissociation with smaller differences in the two k_{off} values could have several explanations (see Section 7.2).

5.3. Refractive index effect and control analytes

When an analyte is injected over a surface it is important, for two reasons, to perform a second injection with a control analyte. Firstly, this helps rule out non-specific binding. Secondly, it controls for any refractive index artefacts. These occur when the background signal measured during the

injection of an analyte sample differs between flow cells. Clearly such an artefact will create problems for affinity measurements. It can occur whenever there is a substantial difference between surfaces. For example, if very different levels of material are immobilised on each surface. In this case, because the immobilised material displaces volume, the volume accessible to the injected analyte sample will differ between flow-cells. If the analyte sample has a higher refractive index than the running buffer, a larger background signal will be seen from the surface with less immobilised material. This artefact is greater when (i) there are big differences in the levels of immobilised material (e.g. >2000 RUs), (ii) the background signal is very large (e.g. >1000 RUs), and (iii) the binding response is much smaller (<10%) than the background response. These conditions are common when measuring very weak interactions, because high concentrations of analyte are injected, and with low molecular weight analytes, which give a very small response. A refractive index artefact can be detected by injecting a control solution with a similar refractive index to the analyte sample. If the control solution gives the same response in both flow-cells a refractive index artefact can be excluded. Refractive index artefacts are most easily avoided by taking care to immobilise the same amount of total material on both the control and ligand sensor surfaces.

5.4. Low molecular weight analytes

Recent improvements in the signal to noise ratios of BIAcore instruments have enabled binding to be detected of analytes with M_r as low as 180 (19). There are two major problems associated with such studies. Firstly, very high densities of ligand must be immobilised in order to detect binding. The levels can be calculated using equation in Protocol 7. For an analyte of M_r ~200 that binds a ligand with an M_r of ~40000, approximately 10000 RU of active ligand needs to be immobilised to see 50 RU of analyte binding. Achieving this level of immobilization is very difficult. A second problem is that with such small binding responses refractive index effects become significant. The latter can be

avoided by dissolving and/or diluting the analyte in the running buffer and using a control flow cell with very similar levels of immobilization.

6. Qualititative analysis; do they interact?

6.1. Positive and negative controls

The main purpose of a qualitative analysis is to establish whether or not there is an interaction between a given analyte and ligand. If binding is detected it is necessary to test negative controls to exclude a false positive. These include negative ligand controls and negative analyte controls. Blocking experiments, using molecules known to block the interaction, are also useful negative controls. In contrast, if no binding is detected it becomes necessary to run positive controls to establish whether this reflects the absence of an interaction, a very low affinity, or an artefact resulting from defective ligand or analyte. The ligand is readily assessed by showing that it can bind to one or more mAbs or additional analytes should they exist. The analyte can be tested by injecting it over a surface with either a known interacting ligand or or mAb immobilised to the surface.

6.2. Qualitative comparisons using a multivalent analyte

The binding of a multivalent analyte can be heavily influenced by the level of immobilised ligand, since the latter will influence the valency of binding. Thus when comparing the binding of a multivalent analyte to different ligands it is important that these ligands are immobilised at comparable surface densities.

7. Quantitative measurements

Quantitative measurements are far more demanding than qualitative measurements because of the quality and amount of materials required and the difficulties associated with designing the experiments and analysing the data. When undertaking these measurements it is particularly important to understand the various pitfalls and how these can be avoided (4,5,8). *Any quantitative*

analysis on the BIAcore requires that the analyte binds in a monovalent manner. Since many binding parameters are temperature dependent *it is important to perform key measurements at physiological temperatures (i.e. 37°C in mammals)*. Considering how easy it is to regulate the temperature on the BIAcore it is surprising how seldom this is done.

7.1. Affinity

7.1.1. Concepts

As has been described earlier in this volume (see, e.g. Chapters 1, 3-5), there are a number of ways to represent the affinity of an interaction.

- The 'association constant' (K_A) or affinity constant is simply the ratio at equilibrium of the 'product' and 'reactant' concentrations. Thus for the interaction A + B $\leftrightarrow$ AB

$$K_A = \frac{C_{AB}}{C_A * C_B}$$

  Note that K_A has units M^{-1} (i.e. $L.mol^{-1}$)

- Many prefer to express affinity as the 'dissociation constant' or K_D, which is simply the inverse of the K_A, and therefore has the units M.
- Affinity can also be expressed as the binding energy or, more correctly, the standard state molar free energy ($\Delta G°$). This can be calculated from the dissociation constant as follows.

$$\Delta G° = RT \ln \frac{K_D}{C^o}$$

where T is the absolute temperature in Kelvin (298.15 K = 25 °C)

R is the Universal Gas Constant (1.987 $cal.K^{-1}.mol^{-1}$)

C^o is the standard state concentration (i.e. 1 M)

7.1.2. Experimental design

In principle the affinity constant can be measured directly by equilibrium binding analysis, or calculated from the k_{on} and k_{off}. However, because of the difficulties associated with obtaining definitive kinetic data on the BIAcore, equilibrium binding analysis is more reliable. It involves injecting a series of analyte concentrations and measuring the level of binding at equilibrium. The relationship between the binding level and analyte concentration enables the affinity constant to be calculated (7). A basic approach to such measurements is outlined on Protocol 9.

7.1.2.1. Preliminary steps

Because equilibrium measurements are unaffected by mass-transport or re-binding artefacts, high levels of immobilisation can be used to increase the binding response. This is particularly useful when the background signal is high or the analyte very small.

Ideally, equilibrium affinity measurements require that the level of active ligand on the surface is the same for each concentration of analyte injected. This is usually straightforward when the ligand is covalently coupled (and so does not dissociate) and the analyte dissociates spontaneously within a few minutes (so that regeneration is not required). In cases where regeneration *is* required it must be shown that ligand activity is unaffected by repeated regeneration. Where captured ligands dissociate spontaneously or require regeneration, it may be difficult to maintain the level of the ligand constant. It is possible to correct for this if the level of active ligand can be accurately monitored (20).

It is important to ensure that the analyte injections reach equilibrium. While the approximate time it takes to reach equilibrium can be calculated (see footnote to Protocol 9), it is advisable to measure this directly in preliminary experiments under the same conditions (flow rate, analyte concentration, ligand density) as those to be used for the affinity measurements. Enough time must be allowed following the injection for the bound analyte to dissociate completely from the sensor surface (see footnot to Protocol 9). If dissociation is incomplete, or takes too long, it may be necessary to enhance dissociation by injecting regeneration solution.

7.1.2.2. The experiment

Ideally the analyte concentration should be varied over four orders of magnitude, from 0.01* K_D to 100 * K_D. However it is often only practical to vary the concentration over 2-3 orders of magnitude. This can be achieved with 10 two-fold dilutions starting at between 10 * K_D and 100 * K_D.

An assumption in these affinity measurements is that the level of active immobilised ligand remains constant. This should be checked by showing that a reference analyte binds to the same level at the beginning and end of the experiment. A second internal control is to reverse the order of injections. An efficient way of doing this is to work up from the lowest concentration of analyte, give one injection at the highest concentration, and then work back down to the lowest concentration. The same affinity should be obtained irrespective of the order of injections.

7.1.3. Data analysis

In order to derive an affinity constant from the data a particular binding model must be used. The simplest (Langmuir) model (A+L↔AL) is applicable in the vast majority of cases. It assumes that the analyte (A) is both monovalent and homogenous, that the ligand (L) is homogeneous, and that all binding events are independent. Under these conditions data should conform to the Langmuir binding isotherm,

$$Bound = \frac{C^A * Max}{C^A + K_D}$$

where "Bound" is measured in RUs and "Max" is the maximum response (RUs).

C^A is the concentration of injected analyte and K_D is in the same units as C^A (normally M)

The K_D and *Max* values are best obtained by non-linear curve fitting of the equation to the data using a suitable computer software such as Origin (MicroCal) or Sigmaplot.

A Scatchard plot of the same data (see Chapter 3), obtained by plotting Bound/C^A against Bound, is useful for visualising the extent to which the data conform to the Langmuir model. A linear Scatchard plot is consistent with the model. *Scatchard plots alone should not be used to estimate affinity constants* since they place inappropriate weighting on the data obtained with the lowest concentrations of analyte, which are generally the least reliable.

Non-linear Scatchard plots indicate that the data do not fit the Langmuir model. Before considering models that are more complex it is important to exclude trivial explanations (Section 7.1.5).

7.1.4. Controls

Several artefacts can result in erroneous affinity constants. These include an effect of ligand immobilisation on the binding, an error estimating the active concentration of analyte, and an incorrect assumption that the analyte is monovalent. The most rigorous control is to confirm the affinity constant in the reverse or 'upside down' orientation since this excludes all three artefacts. If this is not possible the experiment should be repeated with the ligand immobilised in a different way, which addresses the possible effects of immobilization on binding. The affinity should be also be confirmed with two independently-produced batches of protein and with different recombinant forms of the same proteins.

7.1.5. Non-linear Scatchard Plots

A non-linear Scatchard plot indicates that binding does not conform to the Langmuir model. Many binding models can be invoked to explain non-linear Scatchard plots. Distinguishing between these models can be very difficult and is beyond the scope of this Chapter. The priority should be to exclude trivial explanations for non-linear Scatchard plots. A 'concave up' Scatchard plot is the most common deviation from linearity. It may be a consequence of heterogeneous ligand, multivalent analyte, or (rarely) negative cooperativity between binding sites. A trivial cause of analyte

heterogeneity is the presence of multivalent analyte. Ligand heterogeneity may be a consequence of immobilisation. A 'concave down' Scatchard plot is unusual and indicates either positive cooperativity between binding sites or self-association of the analyte, either in solution or on the sensor surface.

The control experiments outlined in 6.1.2.4 will help to eliminate some trivial explanations. For example, if the analyte has a multivalent component the non-linear Scatchard plot will not be evident in the reverse orientation. The shape of the Scatchard plot will also depend on the surface density of ligand. If the ligand immobilization is responsible for heterogeneity, this should be eliminated in the reverse orientation and if the ligand is immobilised indirectly.

Protocol 9. Affinity measurements

Preliminary steps

1. Immobilise the ligand and a control. High levels of immobilisation are acceptable.
2. Ensure that the analyte is monomeric and binds monovalently. Determine accurately the concentration of the analyte and the proportion that is active.
3. Determine the time it takes to reach equilibrium and the time it takes for the bound analyte to dissociate completely from the sensor surface. While this should be done empirically, under the conditions to be used for the equilibrium measurements, approximate times can be calculated from the k_{off} [a].
4. If necessary, establish regeneration conditions (Protocol 5).
5. Obtain a rough estimate of the K_D by injecting a series of five-fold dilutions.

Measurements

1. Prepare a dilution series of analyte starting at 10-100 times the K_D with at least 9 two-fold dilutions thereof. There should be enough for two injections at each concentration except the highest concentration, where only enough for one injection is required. A minimum of 17 µL of sample is required per injection[b].
2. Make up separate control sample of analyte (at concentration ~ K_D) with enough for 2 injections.

3. Set the flow rate. To conserve sample this can be as slow as 1 μL.min^{-1}.
4. Inject the control sample.
5. Inject the dilution series starting from low and moving up to the highest concentration (low-to-high) and then moving back down to the low concentrations (high-to-low). Inject the highest concentration only once. It is important to inject for a period sufficiently long to reach equilibrium. Either enough time must be allowed for spontaneous dissociation or the analyte must be eluted with regeneration buffer.
6. Repeat the injection of the control sample.
7. For all injections measure the equilibrium response levels in the ligand and control flow cells. The difference between these two is the amount of binding at each concentration.

Data analysis

1. Plot the binding versus the concentration for both the low-to-high and the high-to-low series (20).
2. Fit the Langmuir (1:1) binding isotherm to the data by non-linear curve fitting. Use this to determine the K_D and maximal level of binding.
3. Do a Scatchard plot and check if it is linear. The points on the plot where binding is less than 5% of maximum are highly inaccurate and should be ignored.
4. If the Scatchard plot is not linear do further experiments to establish cause (Section 7.1.5).

Controls

1. The affinity constant should be confirmed in the reverse orientation.
2. Should this be impossible (e.g. because the ligand is multivalent) the affinity constant should be confirmed with the ligand immobilised by a different mechanism, preferably by ligand capture.
3. Use at least two independently produced batches of protein.
4. Use different recombinant forms of the same proteins.

[a]Both the time taken to reach equilibrium and the time it takes for the bound analyte to dissociate are governed primarily by the k_{off}. For the simple 1:1 model binding will reach 99% of the equilibrium level within 4.6/k_{off} seconds. Similarly, it will take 4.6/k_{off} seconds for 99% of the analyte to dissociate. Thus for k_{off} ~0.02 s^{-1}, equilibrium will be reached within ~ 230 s and the bound analyte will take ~230 s to dissociate.
[b]If the Quickinject command is used as little as 15 μL of analyte sample is used.

7.2. Kinetics

7.2.1. Concepts

The period during which analyte is being injected is termed the 'association phase' whereas the period following the end of the injection is termed the 'dissociation phase'. During the association phase there is simultaneous association and dissociation. Equilibrium is reached when the association rate equals the dissociation rate. Under ideal experimental conditions only dissociation should take place during the dissociation phase. In reality some re-binding (see below) often occurs. The main factors affecting the association rate are the concentration of analyte near the ligand (C^A), the concentration of ligand (C^L), and the association rate constant (k_{on}). Because of the high surface density of ligand on the sensor surface, the rate at which analyte binds ligand can exceed the rate at which it is delivered to the surface (referred to as mass transport). In this situation binding is said to be mass transport limited. Analysis of association rate under mass transport limited conditions will yield an *apparent* k_{on} that is slower than the true k_{on}. It is difficult to determine the k_{on} under these circumstances, and so experimental conditions must be sought in which mass-transport is not limiting. Analyte is transported to the surface by both convection and diffusion. Convection transport can be increased simply by increasing the flow rate. However even at the maximal flow-rates permissible mass transport can still be limiting (10,16) because there is an unstirred 'diffusion' layer near the sensor surface through which transport is solely by diffusion (5). In this case mass transport limits can only be avoided by decreasing the surface density of immobilised ligand.

The main factors affecting the analyte dissociation rate are the surface density of bound analyte, the dissociation rate constant (k_{off}), and the extent to which dissociated analyte rebinds to ligand before leaving the sensor surface (termed 're-binding'). The latter is also a consequence of mass transport deficiency but here it is transport away from the surface that is limiting. Convection transport can be increased by increasing the flow rate. However re-binding will still occur because diffusion out of

the unstirred layer is little affected by convection transport. Re-binding is most easily avoided by decreasing the level of ligand immobilised on the surface. An alternative method is to inject during the dissociation phase a competing molecule that can rapidly bind to free analyte or ligand and block re-binding (21). If it binds ligand the competing molecule needs to be small so that it does not influence the SPR signal. Finally, when the ligand is saturated the initial part of the dissociation phase will not be affected by re-binding, since no free ligand is available for re-binding. However such selective analysis of a part of the dissociation phases should be avoided; it provides no indication as to whether the data conforms to any particular binding model and can give highly misleading results.

In summary, mass transport limitations, which lead to an underestimation of the intrinsic kinetics, are aggravated by low flow rates, high levels of immobilised ligand, and high intrinsic association rate constants. They can be reduced by increasing the flow rate and, most importantly, lowering the level of immobilised ligand.

7.2.2. Experimental design

Because mass transport may limit binding it is essential to use the lowest density of ligand that gives an adequate level of analyte binding. Depending on the background response 100 RU of binding should be adequate. In order to determine whether binding is limited by mass transport the kinetics should be measured in several flow cells with different levels of immobilised ligand. The immobilization level should vary at least two-fold.

Protocol 10. Kinetic measurements

Preliminary steps

1. Immobilise the ligand in three flow cells at different levels (e.g. 500, 1000, and 2000 RU).

2. Immobilise a control ligand in the remaining flow cell at a level midway between the range of immobilisation levels in the other three flow cells.
3. Ensure that the analyte is monomeric and binds monovalently. Determine accurately the concentration of the analyte.
4. Determine the time it takes reach equilibrium and for the bound analyte to dissociate completely from the sensor surface (see footnote in Protocol 9). If less than 4-5 seconds (fast) it will be necessary to collect at the maximal rate possible (10 Hz). This is only possible if data is collected from one flow cell at a time. Because the sample needs to be injected once for each flow cell studied, more sample is needed.
5. Establish regeneration conditions if necessary. With kinetic determinations it is not as important to maintain the same level of active ligand for each injection.

Measurements

1. Prepare a two-fold dilution series of the analyte ranging from concentrations of $8*K_D$ to approximately $0.25*K_D$. Take care to prepare enough sample for the special kinetic injection command (KINJECT), which utilizes more material. If kinetics are fast and a high data collection rate is needed, enough analyte needs to be prepared for separate injections in each flow-cell.
2. Set the flow rate to 40-100 µL/min in order to maximize analyte mass transport. The duration of the injection is not critical since binding does not need to reach equilibrium. However, equilibrium should be approached at the higher analyte concentrations.
3. Inject the dilution series in any particular order. It is usual to start from lower concentrations.

Data analysis

1. Use the BIAevaluation software supplied by BIAcore.
2. Subtract the response in the control flow cell from the responses in each of the ligand flow cells.
3. Group and analyse together the binding curves obtained with each dilution series, one flow cell at a time (with control responses subtracted).
4. If equilibrium is reached within 1 second the association phase will not produce useful data. In this case only the dissociation phase should be analysed.
5. Attempt a global fit of the simple 1:1 binding model to the entire series of curves. Include in the fit as much of the association and dissociation phase as possible.
6. Repeat the analysis with data obtained at the other levels of ligand immobilisation. In order to prove that binding is not limited by mass transport it is necessary to show that the same rate constants are obtained at two different ligand immobilization levels. *If this is not possible the measured rate constants should be considered to be lower limits of the true rate constants*

(16,18).

7. If poor fits are obtained using the simple 1:1 binding model, the binding is considered complex. After excluding trivial explanations (Table 5) an attempt should be made to establish the cause (Table 6).

Controls

1. The kinetics constants should always be confirmed in the reverse orientation and/or with the ligand immobilised in a completely different manner.
2. Always confirm the results using separate batches of recombinant protein.
3. If possible, confirm the results using different recombinant forms of the same protein.

A second important point is that more of the analyte is needed for kinetic analysis. This is because the experiments are performed at a high flow-rate, the KINJECT command wastes more material, and separate injections may be required for each flow-cell.

7.2.3. Data analysis

Analysis of kinetic data is best performed using the BIAevaluation software supplied with the instruments as this has been designed especially for the purpose (9). Another programme CLAMP (available at http://www.hci.utah.edu/cores/biacore/) has also been designed specifically for analysis of kinetic data generated on the BIAcore (22). While a complete discussion of kinetic theory is beyond the scope of this review a basic approach to kinetic analysis is provided instead. After subtracting the background responses (obtained in the control flow-cells) an attempt should be made to fit the simple 1:1 Langmuir binding model to the data. For any particular sensorgram as much of the data as possible should be included in the fit. This normally includes the entire association and dissociation phases, omitting only the 'noisy' few seconds at the beginning and end of the analyte injection. Noise in the dissociation phase is reduced by using the KINJECT command. It is good practise to fit both the association and dissociation phases simultaneously rather than separately. However the association phase cannot be analysed if equilibrium is attained within 2-4 s, which is

usually the case if the k_{off} is > 1 s^{-1}. In contrast, the dissociation phase can analysed even if the k_{off} is >1 s^{-1} (11,20). A rigorous test of the binding model is to fit it simultaneously to multiple binding curves obtained with different analyte concentrations. This *global fitting* (8,9) establishes whether a single 'global' k_{on} and k_{off} provide a good fit to all the data. An important internal test of the validity of the kinetic constants is to determine whether the calculated K_D (K_{Dcalc}= k_{off}/k_{on}) is equal to the K_D determined by equilibrium analysis.

When a poor fit is obtained to the data using the simple 1:1 binding model the binding kinetics are considered *complex*. Since the most likely explanation for this is experimental artefact, initial efforts should be directed at excluding trivial causes (Table 5). Only when trivial explanations have been excluded should any effort be expended on trying to establish what complex binding model explains the kinetics. This is a difficult and often impossible task (8,9). The simple 1:1 binding model predicts that both the association and dissociation phases are monoexponential, i.e. described by an equation with a single exponential term. When a poor fit is obtained excellent fits can usually be obtained using equations with two exponential terms (a bi-exponential fit). Because all complex binding models generate equations with two or more exponential terms, it is usually impossible to distinguish between different models by curve fitting alone. Instead further experiments need to be performed (Table 6).

7.2.4. Controls

The same controls should be performed as for the affinity measurements (see Section 7.1.4 and Protocol 10)

7.3. Stoichiometry

The binding stoichiometry can be determined if the molecular mass of ligand and analyte are known and the activity of the ligand is known. The basic approach is to immobilise a defined amount of

ligand and then saturate this with analyte. The stoichiometry can then be calculated according to Protocol 7. Because it is very difficult to saturate with analyte the maximum level of analyte binding is best obtained by doing a standard equilibrium affinity determination. A fit of the simple 1:1 binding model to this data yields the maximum level of analyte binding as well as a K_D. The key problem is establishing the activity of the immobilised ligand. If the ligand has 100% activity in solution and is immobilised by ligand capture, it is reasonable to assume that it is all active. Activity levels can also be determined using an Fab fragment of a mAb specific for the ligand. Finally, the stoichiometry should be identical when measured in the reverse orientation.

7.4. Thermodynamics

The binding energy or affinity includes contributions from changes in enthalpy (heat absorbed or ΔH) and entropy (increased disorder or ΔS).

$$\Delta G = \Delta H - T*\Delta S$$

While ΔS cannot be measured, ΔH (or the heat absorbed upon binding) can be measured directly, by microcalorimetry (ΔH_{cal}), or indirectly, by van't Hoff analysis (ΔH_{vH}). If it is assumed that ΔH and $\Delta S°$ are temperature-independent, the linear form of the van't Hoff equation can be used.

$$\ln \frac{K_D}{C^o} = \frac{\Delta H_{vH}}{R * T} - \frac{\Delta S°}{R}$$

where C^o is the standard state concentration (1 M) and $\Delta S°$ is the change in entropy in the standard state. K_D is measured over a range of temperatures and $\ln(K_D/C^o)$ plotted against 1/T. If linear, the slope of this plot equals $\Delta H_{vH}/R$. A drawback of this approach is that ΔH varies with temperature for protein/ligand interactions and so the plot is not linear. Consequently, K_D needs to be measured over a small range around the temperature of interest, and the slope determined within this range. This is technically difficult and likely to be inaccurate. A more rigorous approach is to

measure the affinity (ΔG^o, see section 7.1.1) over a wider range of temperatures and then fit an integrated (non-linear) form of the van't Hoff equation to the data (23).

$$\Delta G^\circ = \Delta H_{To} - T * \Delta S^\circ_{To} + \Delta C_p (T - To) + \; T * \Delta \mathbf{C_p} * \ln\left(\frac{T}{T_o}\right)$$

where T is the temperature in Kelvin (K)

To is an arbitrary reference temperature (e.g. 298.15 K)

ΔH_{To} is the enthalpy change upon binding at To (kcal.mol^{-1})

ΔS^o_{To} is the standard state entropy change upon binding at To (kcal.mol^{-1})

and $\Delta \mathbf{C}_p$ is the specific heat capacity (kcal.mol^{-1}.K^{-1}), and is assumed to be temperature-independent.

The $\Delta \mathbf{C}_p$ is a measure of the dependence of ΔH (and ΔS) on temperature. It is almost invariably negative for protein/protein interactions (24), indicating that enthalpic effects become more favourable and entropic effects less favourable as temperature increases. This negative heat capacity is believed to be the result of the disruption at high temperatures of the ordered 'shell' of water that forms over the non-polar surfaces of a macromolecule. Consequently, the favourable entropic effect of displacing the shell upon binding is reduced. And because fewer solvent bonds are disrupted at the higher temperature the net enthalpy change becomes more favourable. $\Delta \mathbf{C}_p$ is a useful measure of the extent of non-polar surface that is buried upon binding (25). However determining $\Delta \mathbf{C}_p$ by van't Hoff analysis is likely to be inaccurate. A second drawback of van't Hoff analysis is that changes in temperature may also affect the interactions between the proteins and the solution components, including water (26). If these equilibria are coupled to the protein/protein interaction they will contribute to the ΔH_{vH}, which will therefore differ from the ΔH determined by calorimetry.

Because of these drawbacks, it is advisable to confirm ΔH and ΔC_p determinations by calorimetry. Unfortunately even recently developed microcalorimeters require about one hundred fold more protein than the BIAcore. Thus the BIAcore may be the only means of obtaining enthalpy and heat capacity data when limited amounts of material are available.

7.5. Activation energy

The k_{on} and k_{off} will generally increase with temperature. The extent of this increase is a measure of the amount of thermal energy required for binding or dissociation, and is referred to as the activation energy of association (E_a^{ass}) or dissociation (E_a^{diss}). E_a can be determined using the Arrhenius equation. Assuming that E_a is constant over the temperature range examined, then

$$\ln k = \ln A - \frac{E_a}{R * T}$$

where k is the relevant rate constant (e.g. k_{on} and k_{off}), R is the gas constant, and A is a constant known as the pre-exponential factor. E_a is determined from the slope of a plot of ln*k* versus *1/T*. Importantly, because E_a^{ass} and E_a^{diss} can be considered activation enthalpies, the reaction enthalpy can be calculated from the relationship

$$\Delta H = E_a^{ass} - E_a^{diss}$$

An unusually high E_a value indicates that binding and/or dissociation require the surmounting of high potential energy barriers, suggesting that conformational rearrangements are required.

8. Conclusion

SPR provides a powerful tool for the analysis of protein/protein interactions. This is particularly true for low affinity interactions, which are difficult to study using any other technique. One of the most

useful features of SPR is that it provides, through binding analysis, a quick way of checking the structural integrity of recombinant molecules. SPR is also useful for measuring the affinity, enthalpy, stoichiometry, kinetics and activation energy of an interaction. A major advantage of SPR over other techniques such as calorimetry is that much smaller amounts of protein are required. The pitfalls associated with SPR are easily avoided once they are understood. SPR is not well-suited to high-throughput assays, or the analysis of small molecules ($M_r < 1000$).

9. Appendix

9.1. Physical basis of SPR

When a beam of light passes from material with a high refractive index (e.g. glass) into material with a low refractive index (e.g. water) some light is reflected from the interface. When the angle at which the light strikes the interface (the angle of incidence or θ) is greater than the critical angle (θ_c), the light is completely reflected (total internal reflection). If the surface of the glass is coated with a thin film of a noble metal (e.g. gold), this reflection is not total; some of the light is 'lost' into the metallic film. There then exists a second angle greater than the critical angle at which this loss is greatest and at which the intensity of reflected light reaches a minimum or 'dip'. This angle is called the surface plasmon resonance angle (θ_{spr}). It is a consequence of the oscillation of mobile electrons (or 'plasma') at the surface of the metal film. These oscillating plasma waves are called surface plasmons. When the wave vector of the incident light matches the wavelength of the surface plasmons, the electrons 'resonate', hence the term surface plasmon resonance. The 'coupling' of the incident light to the surface plasmons results in a loss of energy and therefore a reduction in the intensity of the reflected light. It is because the amplitude of the wave vector in the plane of the metallic film depends on the angle at which it strikes the interface that an θ_{spr} is observed. An evanescent (decaying) electrical field associated with the plasma wave travels for a short distance (~300 nm) into the medium from

the metallic film. Because of this, the resonant frequency of the surface plasma wave (and thus θ_{spr}) depends on the refractive index of this medium. If the surface is immersed in an aqueous buffer (refractive index or μ ~1.0) and protein (μ ~1.33) binds to the surface, this results in an increase in refractive index which is detected by a shift in the θ_{spr}. The instrument uses a photo-detector array to measure very small changes in θ_{spr}. The readout from this array can be viewed on the BIAcore as 'dips' (Section 3.3). The change is quantified in resonance units or response units (RUs) with 1 RU equivalent to a shift of 10^{-4} degrees. Empirical measurements have shown that the binding of 1 ng/mm^2 of protein to the sensor surface leads to a response of ~1000 RU. Since the matrix is ~100 nm thick, this represents a protein concentration within the matrix of 10 mg/mL. Apart from the refractive index, the other physical parameter which affects θ_{spr} is temperature. Thus a crucial feature of any SPR instrument is precise temperature control.

9.2. Samples and buffers

9.2.1. Guidelines for preparing samples and buffers for use on the BIAcore

- All buffers should be filtered through 0.2 μm filters and degassed at room temperature. The latter can be achieved by filtering under vacuum or by using a vacuum chamber.
- All samples >3 mL should be filtered and degassed in the same way.
- If samples <3 mL should be spun at high speed in a microcentrifuge for 10-20 min at 4 C and then degassed in a vacuum chamber.
- Before samples are placed in the sample rack they should be pulsed briefly in a microcentrifuge. This dislodges air-bubbles from the bottom of the container, helps ensure that the meniscus is horizontal.
- Vials should be capped to prevent sample evaporation.
- When running long experiments consider cooling the sample rack base using a thermostatic re-

circulator.

9.2.2. Standard buffers

9.2.2.1. Running buffers

HBS or HBS-EP: 10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% P20

HBS-P: 10 mM Hepes pH 7.4, 150 mM NaCl, 0.005% P20

HBS-N: 10 mM Hepes pH 7.4, 150 mM NaCl

9.2.2.2. Pre-concentration buffers

pH 3.0-4.5 10 mM formate

pH 4.0-5.5 10 mM acetate

pH 5.5-6.0 5 mM maleate

9.2.2.3. Regeneration buffers

Grouped according to chemical properties. They increase in strength from left to right.

Cation chelator: HBS with 20 mM EDTA pH 7.5

High ionic strength: NaCl 1 M; KCl 4 M; $MgCl_2$ 2 M

Low pH: Glycine/HCl 100 mM pH 2.5; HCl 10 mM; HCl 100 mM; H_3PO_4 100 mM

High pH: NaOH 5 mM; NaOH 50 mM

9.3. Additional information

BIAcore maintains a useful **web site** [http://www.biacore.com/]. This announces new product developments and includes a continuously updated list of SPR publications and an electronic version of the BIAjournal.

The **handbooks** that come with the instrument are an essential resource. These can also be purchased from BIAcore along with the following books, which describe the technology and its applications in

more detail.

BIAtechnology Handbook

BIAapplication Handbook

BIAevaluation software Handbook

10. References

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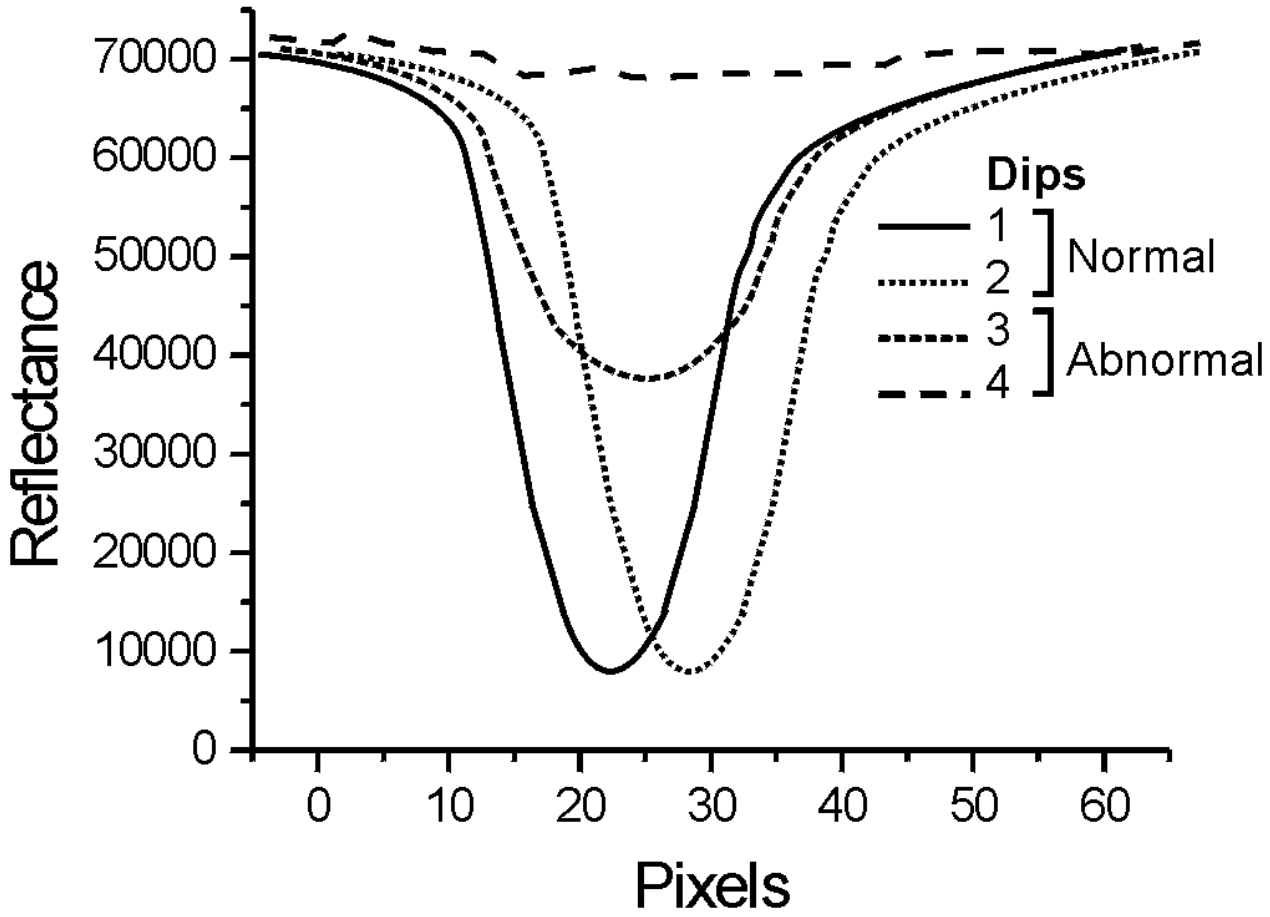
12. GLOSSARY

K_D	dissociation constant (M)
k_{on}	association rate constant ($M^{-1}.s^{-1}$)
k_{off}	dissociation rate constant (s^{-1})
mAb	monoclonal antibody
RU	response units or resonance units
SPR	surface plasmon resonance

13. Figure 1. Analysis of dips.

Several examples are shown of normal (1 and 2) and abnormal (3 and 4) dips. These are discussed in Protocol 1 .



14. Tables

14.1. Table 1. BIAcore instruments currently available (January 1999)

	BIAcore X	**BIAcore2000**	**BIAcore3000**
Automated	No	Yes	Yes
Temperature control (°C)	10 below ambient to 40	4 to 40	4 to 40
Flow-cell number	2	4	4
Flow-cell volume (nL)	60	60	20
Time resolution (Hz)			
single flow cell	10	10	10
two flow cells	5	2.5	5
Refractive index range	1.33-1.40	1.33-1.36	1.33-1.40
Sample recovery	Manual	Automatic	Collects surface bound material only, but in very small volume (3-7 μL)
Fraction collection	Manual	Automatic	
Baseline noise (RU, Root Mean Square)	0.3	0.3	0.1
Online subtraction of background response	Yes	Yes (flow cells 2-1, 3-1, 4-1)	Yes (flow cells 2-1, 3-1, 4-1, 3-4)

14.2. Table 2. Sensor chips available for use on the BIAcore

	Surface chemistry	Free carboxymethyl groups	Comments
Sensor Chip CM5 (research grade)	carboxymethylated dextran matrix	Yes	Most widely used sensor chip. Suitable for most applications.
Sensor Chip CM5 (certified grade)	as above	Yes	Less chip-to-chip variation than research grade chip but much more expensive.
Sensor chip SA	as above with streptavidin pre-coupled	No	Quite expensive, especially since it cannot be re-used. It is cheaper to couple streptavidin to CM5 research grade chip (11).
Sensor chip NTA	As above with NTA pre-coupled	No	Comes with nitrilotriacetic acid (NTA) coupled. For capturing proteins with oligo-histidine tags. Expensive but can be re-used many times.
Sensor chip HPA	Flat hydrophobic surface	No	For immobilising lipid monolayers. Problem with non-specific binding.
Pioneer Chip C1	No dextran matrix. Carboxymethylated	Yes	For binding particles (eg cells) too large to enter dextran matrix. Also helps eliminate potential matrix-related artefacts.
Pioneer Chip B1	Low level of carboxymethylation	Yes	Its lower charge density makes it useful reducing non-specific interactions with charged analytes.
Pioneer Chip F1	Thin dextran matrix	Yes	For binding to bulky particles. Reduces matrix-related artefacts.
Pioneer Chip J1	Unmodified gold surface	No	Can be used to design novel coupling chemistry
Pioneer Chip L1	Dextran derivatized with lipophillic compounds	No	For immobilizing liposomes via interactions with lipophilic compounds. Apparently lower levels of non-specific binding than HPA chip.

14.3. Table 3. Covalent coupling chemistry

	Surface activation[a]	Group required on protein	Protein preparation	Comments
Amine	EDC/NHS[b]	Amine groups (lysine and unblocked N-termini)	No preparation needed.	Can be used with most proteins. Risk of multiple coupling.
Surface-thiol	EDC/NHS followed by cystamine/DTT to introduce a thiol group	Amine or carboxyl groups.	Need to introduce reactive disulphide using heterobifunctional reagent such as PDEA (carboxyl) or SPDP (amine).	Not widely used
Ligand-thiol	EDC/NHS followed by PDEA to introduce a reactive disulphide group	Surface exposed free cysteine or disulphide.	Reduce disulphides under non-denaturing conditions to generate free cysteine.	Useful for proteins with exposed disulphides or free cysteines. Multiple coupling unlikely.
Aldehyde coupling	EDC/NHS followed by hydrazine	Aldehyde groups.	Create aldehydes by oxidising *cis*-diols (in sugars) with periodate. Works especially well with sialic acid.	Useful for polysaccharides and glycoproteins.

[a] All coupling reactions utilise carboxymethyl groups on the sensor surface and can be used with any sensor chip which has free carboxymethyl groups (Table 2). The first step is to activate these groups with N-hydroxysuccinimide, thus creating a highly reactive succinimide ester which reacts with amine groups on protein, or can be further modified.

[b] Abbreviations: EDC, N-ethyl-N'-(dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; DTT, dithiothreitol; PDEA, 2-(2-pyridinyldithio)ethane-amine.

14.4. Table 4. Techniques for ligand capture.

	Covalently coupled molecule	Captured ligand	Valency of ligand	Removal of Tag	Comments
Antibodies	Rabbit-anti mouse Fc polyclonal (26)	Any mouse IgG monoclonal antibody	divalent	no	Antibody available from BIAcore. Because it is polyclonal very high levels of binding to a particular isotype are difficult to achieve
	Mouse anti-human Fc, monoclonal (R10Z8E9) (13,27)	Any protein fused to the Fc portion of human IgG_1. This includes most Fc chimeras.	divalent	not routinely. However new vectors incorporate	Antibody available from Recognition Systems, University of Birmingham Science Park, Birmingham B15 2SQ, U.K.
	Mouse anti rat CD4, monoclonal (OX68) (17)	Any protein fused to rat CD4 domains 3 and 4.	monovalent	no	Antibody available from Serotec.
	Anti-GST[b]	Any protein expressed as a GST chimera	divalent	yes	Antibody available from BIAcore. Because GST dimerizes, the fusion proteins are dimers.
Other	Streptavidin	Any biotinylated molecule. Biotinylation can be indiscriminate or (preferably) targeted[a.]	monovalent, can be made tetravalent with streptavidin	no	Steptavidin sensor chips are available from BIAcore. However streptavidin can be coupled to standard CM5 chips at lower cost (11). Note that streptavidin surfaces cannot be regenerated.
	Ni-NTA	Any protein with oligo histidine tag	monovalent	yes	Sensor chips can be purchased from BIAcore with Ni-NTA already coupled.

[a]Targeted biotinylation involves introducing a single biotin group on the molecule in a position in which it will not interfere with binding to analyte. This can be achieved either by chemical synthesis (e.g. oligonucleotides), or by enzymatic biotinylation. An established method uses the enzyme BirA to biotinylated a specific peptide which has been added on to the N or C terminus of a recombinant protein (11).
[b]Abbreviations: GST, glutathione-S-transferase; Ni-NTA, nickel-nitrilotriacetic acid

14.5. Table 5. Trivial causes of complex binding kinetics

Cause	How to detect and/or eliminate
Mass-transport limitations	• Increase the flow-rate and lower the density of immobilised ligand • Alternatively, include mass-transport term in fitting equation.
Drifting baseline	• Should be evident in the control sensorgrams. • Try to eliminate this by subtracting the control sensorgram. • Alternatively, the simple 1:1 binding model can be modified to include a drifting baseline.
Bulk refractive index artefacts	• Should be evident in the control sensorgrams. • Try to eliminate this by subtracting the control sensorgram. • Alternatively, the simple 1:1 binding model can be modified to include bulk refractive index artefacts.
Rebinding	• The fit gwill be worse at higher levels of immobilization. • Increase the flow-rate and lower the density of immobilised ligand. • Inject a competing small analyte during the dissociation phase (21).
Heterogeneous immobilisation	• Immobilise ligand in a different way (preferably indirectly, by ligand capture).
Analyte is multimeric	• Perform size-exclusion chromatography and/or analytical ultracentrifugation to ensure that analyte is monomeric. • If the ligand is monomeric, perform kinetic analysis in reverse orientation.
Analyte is monomeric but contaminated by multivalent aggregates	• Repeat the experiment using the monomeric fraction immediately after size-exclusion chromatography, avoiding concentrating or freezing the sample.

14.6. Table 6. Distinguishing some non-trivial causes of complex kinetics

Binding mechanism	**Description**	**Distinguishing features**
Heterogenous analyte	More than one form of the analyte binds to a single ligand.	Dissociation will slow as the duration of the injection is increased. This effect will not be seen in the reverse orientation.
Heterogenous ligand	One analyte binds to more than one ligand	Dissociation will not be affected by the duration of the injection. However, in the reverse orientation dissociation will slow as the duration of the injection is increased.
Two-state binding (conformational change)	After forming, the ligand/analyte complex interconverts between two or more forms with different kinetic properties.	The dissociation phase will slow as the duration of the injection is increased. This affect will also be seen in the reverse orientation.