Chapter 17

Label-Free, Real-Time Interaction and Adsorption Analysis 1: Surface Plasmon Resonance

Conan J. Fee

Abstract

A key requirement for the development of proteins for use in nanotechnology is an understanding of how individual proteins bind to other molecules as they assemble into larger structures. The introduction of labels to enable the detection of biomolecules brings the inherent risk that the labels themselves will influence the nature of biomolecular interactions. Thus, there is a need for label-free interaction and adsorption analysis. In this and the following chapter, two biosensor techniques are reviewed: surface plasmon resonance (SPR) and the quartz crystal microbalance (QCM). Both allow real-time analysis of biomolecular interactions and both are label-free. The first of these, SPR, is an optical technique that is highly sensitive to the changes in refractive index that occur with protein (or other molecule) accumulation near an illuminated gold surface. Unlike QCM (Chapter 18) SPR is not affected by the water that may be associated with the adsorbed layer nor by conformational changes in the adsorbed species. SPR thus provides unique information about the interaction of a protein with its binding partners.

Key words Surface plasmon resonance, Label-free detection, Biosensors

1 Introduction to Surface Plasmon Resonance

Surface plasmon resonance (SPR) is an optical technique by which one can follow the course of molecular interactions in real time. This and other related optical biosensor techniques have been regularly reviewed by David Myszka and Rebecca Rich, of the University of Utah, e.g., (1–4). SPR instruments detect changes in refractive index that occur through changes in mass within a very fine layer close to the sensor surface, typically using disposable sensors or "chips", which have functionalized surface layers that are prepared for the study of specific interactions. One or more molecular species are immobilized or "tethered" to the surface layer and binding partners in free solution are then presented to the tethered molecule. Flow or stirring is used to minimize mass transfer limitations so that the measured rate of interaction reflects actual binding kinetics rather than mass transfer of solutes between the bulk solution

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and the surface layer. Important information can be determined by SPR, including the association and dissociation kinetic constants, the analyte concentrations, the detection of specific species through biospecific responses, and the gathering of circumstantial evidence in support of proposed binding mechanisms, including epitope mapping or supramolecular complex formation. Measurement of binding kinetics over a range of constant temperatures also allows thermodynamic quantities to be determined. With present technology, SPR can measure association constants, k_a, over the range 100–10^8 M^{-1} s^{-1}; dissociation constants, k_d, over the range 1–10^{-6} s^{-1}; and equilibrium constants, K_D, over the range 100 mM to 1 pM.

The lowest detectable molecular weight is currently claimed to be around 100 Da in the most sensitive instruments but this should not be considered routinely feasible and it depends strongly upon the molecular weight of the tethered species. The maximum SPR response during analyte interaction is directly related to the ratio between the analyte (free solution) and the ligand (tethered) molecular weights. Therefore, one should not expect to be able to routinely detect a single sugar analyte in free solution binding to an antibody ligand tethered to the chip surface, although reversing the analyte/ligand pairing may well yield useful results.

The principles of SPR are described in more detail below but essentially the phenomenon depends on detecting the conversion of energy from light incident on a thin layer of metal that is located between two media of differing refractive indices (5). Normally, for biointeractions, the two media are comprised of a glass prism, onto which a thin layer of gold is coated, and an aqueous flow cell. Light is directed up through the prism from below and the intensity of reflected light from the gold layer is detected by optical detectors on the other side of the prism (Fig. 1). Several different modulations to the light characteristics in response to binding events near the gold surface may be detected, with two important ones being *angular modulation* and *wavelength modulation*. Light intensity and phase changes can also be detected. In *angular modulation*, part of the energy from monochromatic light incident on the metal at a specific angle is converted into an electron wave that travels along the gold surface (the surface plasmon) on the aqueous side and this angle is detected by measuring a localized drop in the reflected light intensity.

Because the specific angle of the incident light that creates the surface plasmon wave depends critically upon the difference in refractive index between the glass and aqueous sides of the gold layer, when a protein binds on the aqueous side of the gold, the change in local refractive index causes a change in the angle of light energy that is coupled to the surface plasmon wave. Thus, the angle of lowest reflected light intensity detected by the optical detectors changes in response to the amount of mass coupled near the gold surface (Fig. 2).

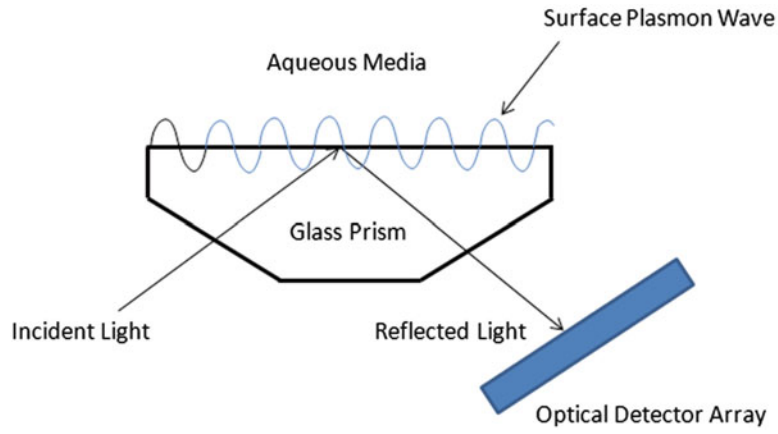

Fig. 1 Surface plasmon resonance configuration for biomolecular interactions

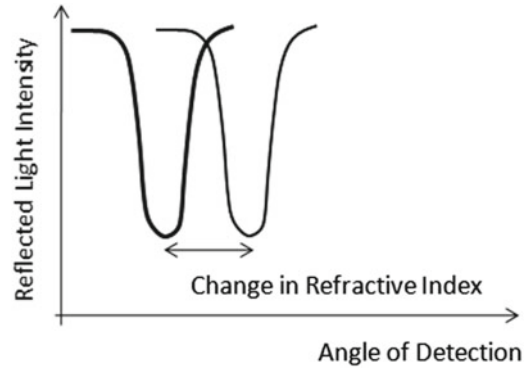

Fig. 2 Change in angle of minimum detected light intensity with change in refractive index

In *wavelength modulation*, different wavelengths of light are used to excite the surface plasmon, while the angle of incidence remains constant. The wavelength of light that has the strongest coupling to the plasmon is detected and this changes in response to changes in refractive index. For simplicity, angular modulation is assumed to be the measured quantity in the subsequent description but analogous phenomena occur for other modes of light modulation.

The actual angles involved and the extent to which they might change in response to binding near the surface will depend upon the particular instrument design (glass refractive index, metal type and thickness, surface coating, frequency and wavelength of incident light, etc.) so normally the change in angle is plotted in terms of "response units" (RU) against time, to give a "sensorgram" (Fig. 3 and Eq. 1). Typically, instruments are calibrated internally

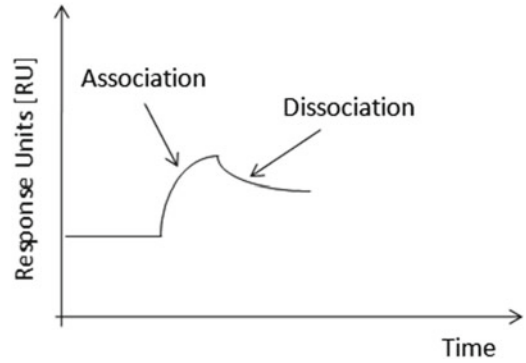

Fig. 3 The SPR "sensorgram"

so that a change of 1,000 RU nominally indicates the addition of 1 ng/mm^2 of bound protein (6). (It is worth noting here that if the surface coating layer on the chip in which the interaction occurs is nominally 100 nm thick, this corresponds to a bound protein concentration of 10 mg/mL per 1,000 RU, so a response of 15,000 RU corresponds to an effective concentration of 150 mg/mL at the chip surface!):

$$\Delta \mathrm{RU} = m\Delta\eta, \tag{1}$$

where m is an instrument-specific constant that relates the "response" of that particular instrument to the change in refractive index, η.

SPR does not require labeling of the molecules involved but, as noted above, at least one partner in the interaction must be immobilized on or near the sensor chip surface. The absence of labeling means that the interactions of the native form of a molecule can be studied, without the potential interference that might arise from a covalently attached moiety such as a fluorophore. However, this is strictly true only for the molecule(s) in free solution, as the tethered molecule must, necessarily, be constrained to the chip surface by a stable linker. Commonly, for proteins, this is achieved through amine coupling, which may result in multiple tethering positions through multiple primary amine groups on lysine residues. Thus, the tethered molecule may not be in its fully native free-solution form and its binding site may be sterically hindered and/or conformationally constrained. For affinity detection or concentration assays, this is not important because either the rate of binding is irrelevant or, in the latter case, is implicitly accounted for by using a calibration curve. However, the possible effects of tethering must be considered when quantifying binding kinetics and, ideally, a single tethering site used that is as far removed as possible from the binding site and that allows full conformational freedom in the tethered molecule.

2 SPR Instruments

Before going through each of the steps involved in SPR assays, it is useful to briefly describe the configurations of several contrasting commercial instruments from three manufacturers: Biacore (GE Healthcare), Bio-Rad, and ForteBio. (Although it should be noted here that while the ForteBio instrument is an optical sensor, it is not actually an SPR device. However, its operation and the data it produces are very similar to an SPR.) The manufacturers chosen are by no means exclusive descriptions and the discussion is deliberately broad, as the technology is evolving and there is little point in providing extensive detail regarding systems that might be superseded. A table summarizing the 2008 peer-reviewed literature lists no fewer than 57 individual SPR instruments from some 25 suppliers and 14 non-SPR optical biosensor instruments from six suppliers (4).

2.1 Biacore

From 1990 until around 2007, it is fair to say that Biacore™ SPR instruments were synonymous with surface plasmon resonance sensing for biomolecular interactions. Indeed, many peer-reviewed papers prior to 2007 used the phrase "Biacore analysis" as a synonym for "SPR analysis". The market dominance of Biacore during that time meant there was little ambiguity in the use of such a term but the availability of alternative commercial SPR systems from other manufacturers means that there is no justification for its use today.

That said, Biacore, acquired by GE Healthcare (Uppsala, Sweden) in 2006, certainly set the standard for SPR analysis during their first 17 years or so of operation and remains the market leader at the time of writing. The company at this time offers no less than eight current commercial models for a range of applications, from a single sample system (the *Biacore J*) through to array systems (e.g., the *Biacore 4000*) that can measure over 2,000 interactions per hour with parallel sample injection.

Although it must be stressed that several configurations are available, a typical automated SPR instrument such as the *Biacore 3000* comprises an autosampler, a microfluidics device, a series of consumable sensor chips, and the optics system.

The autosampler system is essentially a temperature-controlled liquid handler that allows the input of all reagents for sensor chip surface activation, surface immobilization, analyte injection and, in some cases, recovery of eluted analyte, and automated preparation of matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry plates. The sensor surface has independent temperature control for interaction analysis. As described below, activation of the chip surface often involves the mixing of reagents that are prone to rapid hydrolysis, so a feature of the autosampler is the ability to keep these components separate until needed and then mix them in the autosampler system immediately

prior to injection. The system control software is versatile in that it allows step-by-step command level control of the autosampler and injection conditions, etc.

The microfluidic device normally continuously injects running buffer from one of two independent buffer reservoirs, at flow rates between 5 and 100 μL/min. The device in the Biacore 3000 instrument incorporates the optics (prism) and forms four flow channels on the sensor chip by clamping the microfluidics head onto the sensor chip surface. Flow can be independently directed through each channel. Generally, one channel must be used as a noninteracting reference (control) for subtraction of responses due to bulk shift (change in refractive index between the sample solution and the running buffer) and nonspecific adsorption.

A wide range of sensor chips is available, including plain gold, carboxymethyl dextran-coated chips with varying carboxyl group densities and/or dextran layer thicknesses, streptavidin, lipid bilayers, and membranes, and nitrilotriacetic acid (NTA) chips suitable for capture of His-tagged molecules. Although the stability of an immobilized biomolecule will be unique to the particular molecular species involved, the immobilization method used, and the conditions to which it has been subjected, the sensor chips can often be reused many times. The sensor chips themselves are relatively robust and can retain activity after ejection from the system and refrigerated storage in either wet or dry state. An advantage of incorporating the optics, including the prism, within the instrument is that these are unlikely to be contaminated if suitable cleaning and system operation protocols are followed.

2.2 Bio-Rad

Bio-Rad Laboratories (Hercules, CA) currently produce a single multiplex SPR instrument, the ProteOn™ XPR36. Again, the instrument consists of a temperature-controlled autosampler, a microfluidics system, and a series of sensor chips that are docked into the optics/fluidics systems. The sensor surface has independent temperature control. The microfluidic system forms six flow channels when the chip is docked and it rotates 90° so that a 6×6 array is formed at the intersections (spots) formed between the channels in each orientation. A charge-coupled device (CCD) camera views the reflected light from the entire array area so that the instrument response can be followed independently not only at the 36 interaction spots but also between them. These latter "interspots" can be used as controls, eliminating the need to use a channel for subtracting bulk shift and nonspecific adsorptions from the raw signal.

The autosampler consists of six sample needles that move in unison and draw up samples or surface activation reagents from vials or a microtiter plate as a set. Channels can be left unused simply by placing running buffer in the corresponding sample vials/wells. By careful planning of the flow direction and the samples applied, the system allows a single channel to be immobilized with

up to six ligands or replicates, or all six channels to be immobilized with 36 different ligands. Six analyte samples (or any combination of analytes and running buffer blanks) can then be flowed over the array and the interactions measured on any of the 36 spots.

A point of difference for the Bio-Rad system is that the sensor chips themselves incorporate the prism. When docked, the system initializes the optics by normalizing the background signal across the entire CCD array. The initial normalization is carried out in air because the chip is assumed to be completely dry but when re-docked after having been ejected, the user chooses whether to use the original normalization data or to remeasure the background using a glycerol solution to match the refractive index of the glass (assuming that the chip may be wet). The sensor chips may, again depending on the stability of the immobilized biomolecule(s), be reused many times if left docked in the instrument but care must be taken when storing the chips external to the instrument to avoid contaminating the prism surface. Thus, the Bio-Rad sensor chips should be stored dry, in contrast to the Biacore sensor chips, which can be stored either immersed in buffer or dry. Bio-Rad currently supplies chips that are coated with a carboxylated alginate layer with three levels of carboxyl group density, a neutravidin chip for capturing biotinylated molecules and a tris-nitrilotriacetic acid (NTA) chip for his-tagged proteins.

2.3 ForteBio

The ForteBio Octet system is significantly different from the two instruments described above in two major ways. First, it does not use microfluidics but instead uses eight or sixteen parallel, disposable "dipstick" optical tips, immersed in a shaking 96- or 384-well microtiter plate, respectively. One advantage of this approach is that there is no restriction on the use of solids-laden samples because the analyte samples do not pass through microchannels on the sensor tips but the microtiter plates are simply shaken to induce sufficient bulk mass transfer next to the stationary tip surfaces.

Second, although the system exploits a change in the modulated properties of reflected light and is thus an optical sensor, it is not actually a surface plasmon resonance system. Nevertheless, the response of the device to surface binding events is so similar to SPR, that in all practical senses the user can regard it as a comparable system i.e., for real-time, label-free quantification of biomolecular interactions. However, the difference in the underlying physical phenomenon exploited by this system is significant.

The Octet system analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer (Fig. 4). Binding of an analyte to an immobilized ligand will increase the thickness of the surface layer and result in a change in the interference pattern and therefore a change in the wavelength of the lowest light intensity. This is then converted to a sensorgram in a similar way to

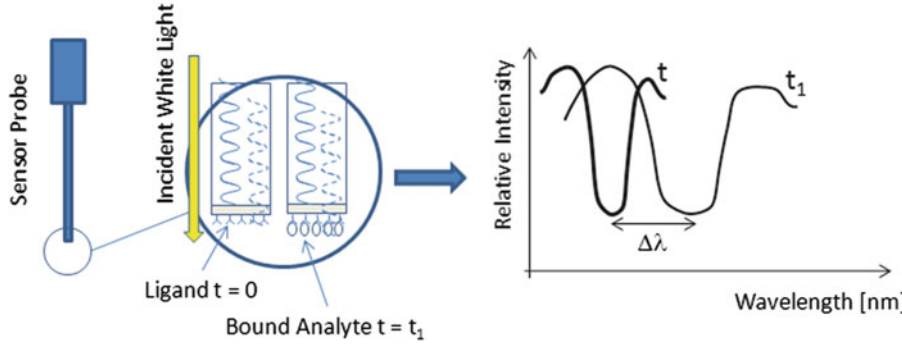

Fig. 4 The ForteBio system

the SPR systems described above. The response is affected only by bound molecules and not by changes in refractive index or bulk solution properties. The advantage is that bulk shift in the signal does not occur. However, a control is still required to account for nonspecific interactions.

The autosampler system moves the sensor probes (8 or 16) in unison between programmed microtiter well locations, so can allow for surface activation, immobilization, washing, analyte binding, regeneration, etc. The microtiter plates are housed in a temperature-controlled compartment.

Because the instrument response depends on a change in *thickness* of the adlayer on the end of the sensor tip, unlike SPR sensor chips, the tips are limited to a monolayer of immobilized ligand and detect a monolayer of bound analyte. The response signal from an SPR sensor chip, on the other hand, can be boosted, at least for concentration analysis if not kinetics analysis, by using a higher density of immobilized ligand within a deep surface layer to increase the level of binding achieved and thereby increasing the total change in refractive index that occurs with binding. A series of preprepared immunoglobulin probes, immunoglobulin-binding probes (e.g., protein A), and probes suitable for amine coupling or His-tagged protein capture and streptavidin and hydrophobic surfaces are available.

3 The SPR Method

The SPR method generally comprises the following steps, designed first to immobilize a ligand to the chip surface, then to measure real-time interactions with analyte(s) in free solution, and, finally, to regenerate the ligand for subsequent experiments and to process the interaction data:

1. Pre-concentration (not required for affinity immobilization techniques such as streptavidin-biotin or His-tag-NTA).

2. Chemical activation of the sensor chip surface.
3. Surface immobilization (tethering) of one or more binding partners.
4. Deactivation of residual activated sites on the sensor chip surface.
5. Surface stabilization to remove weakly bound material.
6. Interaction with the analyte(s). Sequential injection of one or more binding partners and measurement of the response during binding (association), followed by a return to the running buffer to measure the response during dissociation (Fig. 3).
7. Regeneration—removal of residual binding partner(s) from the ligand at the end of the dissociation phase.
8. Repetition of steps 5–7 for subsequent experiments (or steps 3–7 if ligand is to be completely removed during regeneration, as in affinity capture immobilizations).
9. Processing of raw data and appropriate analysis and presentation.

With a new sensor chip, a preconditioning step prior to amine coupling can be carried out with a series of dual injections of 100 mM HCl, 50 mM NaOH, and 0.5% sodium dodecyl sulfate (SDS), for 5 or 6 s each, at a flow rate of 100 μL/min (about 10 μL each injection). This rewets and cleans the sensor chip surface, giving a good, stable response prior to further work.

3.1 Pre-concentration

The objective of pre-concentration experiments is simply to determine the most effective pH and concentration at which to immobilize the target molecule by following the SPR response to electrostatic interactions between the molecule and the sensor chip surface. The usual starting point for interaction analysis is to tether one of the binding partners via carboxyl groups located within a stable polymer coating (e.g., carboxylated dextran or alginate) of about 100 nm thickness on the sensor chip. The carboxyl groups are active in the de-protonated form, i.e., when the pH in the surface layer (which can differ from the bulk phase) is above the pK_a of the carboxyl groups (usually around pH 3.5). Above this pK_a, the carboxyl groups have a single negative charge that is reactive but will repel other negatively charged species, so for instance, if the pH is above a protein's isoelectric point (pI), the protein will not easily penetrate into the surface layer on the sensor chip and binding will be low. To identify the most efficient binding conditions, the molecule of interest (for proteins, 20–200 μg/mL) should first be injected over the chip at a range of pH values to determine the maximum SPR response (Fig. 5). For a protein, the further the pH is below its pI, the more positively charged it will be but the sensor chip surface will also be more protonated, reducing

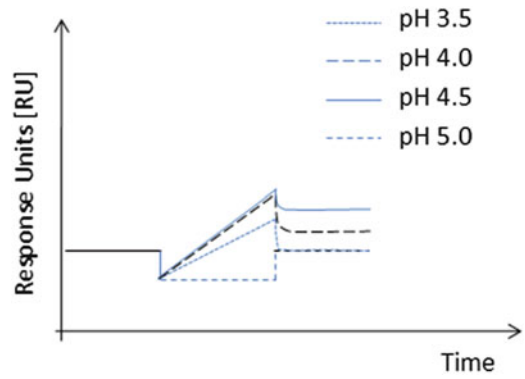

Fig. 5 Pre-concentration runs showing that the optimum immobilization in this hypothetical case would be one carried out at pH 4.5

its reactivity. Also, some proteins are not stable at low pH. Therefore, there is likely to be an optimal pH for binding that is best determined experimentally through pre-concentration experiments. However, immobilization can often be carried out successfully, if not optimally, at a midpoint between pH 3.5 and the protein's pI. The salt concentration of the buffer should be kept below 100 mM (NaCl) during pre-concentration to avoid masking the attractive electrostatic charges. Experiments with a range of protein concentrations should also be carried out at the optimal pH prior to immobilization to obtain a quantitative estimate of the likely response during immobilization. This is important, as the response range should be appropriate for the intended use (a high response (>1,000 RU) to maximize the lower limit of detection for concentration analyses and a low response (<250 RU) to minimize mass transfer effects during kinetic analyses.)

3.2 Chemical Activation of the Sensor Chip Surface

Generally, before the first binding partner is immobilized, the SPR chip surface must be chemically activated to allow coupling. A number of specially prepared surfaces such as streptavidin or metal chelating (NTA) chips may not require this step and are available from some suppliers for capture of biotinylated or His-tagged proteins, respectively. However, the most common approach is to tether one of the binding partners to carboxyl groups within the polymer coating on the sensor chip. One can regard the polymer coating on SPR chips more as a "tangled fishing line" rather than a uniform monolayer so that the carboxyl groups are located in space (Fig. 6a), much as they would be in solution, as opposed to tight orientation on a crowded surface (Fig. 6b).

Activation of the carboxylated surface with *N*-hydroxysuccinimide (NHS) provides a good leaving group on the carboxyl groups suitable for spontaneous formation of various bonds. Such activation can be carried out using a mixture

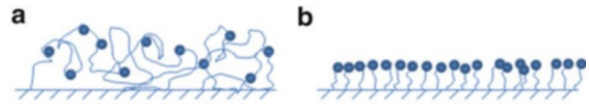

Fig. 6 The polymer coating on an SPR sensor chip is more like the "tangled fishing line" in (**a**) than the uniform monolayer in (**b**)

of *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC or EDAC) and NHS or *N*-hydroxysulfosuccinimide (sulfo-NHS). The simplest approach is to form an amide bond directly with the primary amines on the lysine residues of proteins. However, the chemistry is quite versatile, so after NHS activation, subsequent exposure to a cystamine in the presence of dithiothreitol will produce a thiol group for coupling to amine or carboxyl groups, 2-(2-pyridinyldithio)ethane-amine (PDEA) will produce a reactive disulfide for coupling to free cysteines, and hydrazine will allow aldehyde coupling.

Direct coupling of proteins to EDC/NHS-activated carboxyl groups is straightforward but is nonspecific and may lead to multiple coupling, thereby constraining molecular flexibility. The orientation of coupling is also likely to be somewhat random, so the resulting adlayer will probably comprise a mixture of fully active binding molecules and others wherein the binding sites are partially or fully blocked. Maleimide linkages may be useful in the fortunate case where a single free cysteine is available on the protein of interest and this will almost certainly result in a uniform orientation and single-point tethering. Alternatively, the introduction of a thiol group on the sensor chip surface may be useful if a reactive disulfide group can be introduced onto the target protein first via an amine or carboxyl group. If a free cysteine is not present on the target molecule, disulfide bonds can be broken by a reducing agent under non-denaturing conditions so that these may form a new disulfide bond with reactive disulfide or maleimide but care must be taken that the activity (structure) of the target molecule is not compromised with this approach. Aldehyde coupling may be useful to link to carbohydrate groups (on glycoproteins, for example) if they are prepared first by oxidizing the *cis*-diols of sugar groups with periodate.

The degree of surface activation will affect the amount of ligand immobilized on the surface and can be controlled by the concentration of EDC/NHS and the time of exposure to the reagents. Typically, for concentration analysis, one aims to maximize immobilization so a high activation reagent concentration and a long activation period will be used. For kinetic analysis, the minimum immobilized species that will give a sufficient signal should be immobilized to avoid mass transfer limitations during analyte binding. Thus, in the latter case, a dilute reagent and/or a short activation period is recommended. Surface activation conditions for these

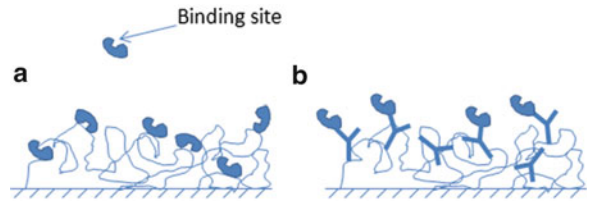

Fig. 7 Orientation of tethered molecules (**a**) random via direct coupling and (**b**) more uniform via indirect coupling

applications should be determined experimentally but good starting points are as follows:

For concentration analysis
A mixture of 200 mM EDAC and 50 mM NHS, injected for about 30 s, using a medium- or high-density sensor chip.

For kinetics analysis
A mixture of 25 mM EDAC and 5 mM NHS, injected for 30 s, using a low-density chip.

A reference channel should be prepared identically, in parallel.

3.3 Surface Immobilization (Tethering) of One or More Binding Partners

For amine coupling, the molecule of interest should be passed over the chip at a concentration in the range 20–200 μg/mL as soon as possible after surface activation to avoid loss of surface reactivity through hydrolysis of the NHS-activated carboxyl groups. The optimal pH should have been determined through preconcentration experiments and the salt concentration should normally be kept below 100 mM (NaCl equivalent) to avoid masking the electrostatic interactions that attract the target species into the surface layer. Typically, a 3–5 min injection at a flow rate of 25–50 μL/min would be used, requiring a total of about 2–50 μg of the target molecule. (A little excess volume (50 μL) in the sample vials/wells might be required to avoid taking up air into the sample needles in most microfluidics-driven systems.) For kinetic analysis, the aim should be to keep the total response of the analyte below about 250 RU to avoid kinetics being complicated by surface crowding. Coupling temperature can be increased to maximize immobilization or lowered to decrease the binding.

Spontaneous coupling via primary amines should then occur. The result of this *direct coupling* will be the immobilized target tethered in essentially random orientations, wherein the direction of the binding site relative to the surface and perhaps even the access to the binding site are variously distributed (Fig. 7a). This heterogeneity can sometimes lead to complex kinetic behaviors, for example if binding sites on some tethered molecules become accessed only at the highest analyte concentrations. A more uniform surface

orientation may be possible through *indirect coupling* by first tethering an antibody to the target molecule to the surface via amine coupling and then capturing the target molecule in a subsequent injection (Fig. 7b). This approach is also likely to result in a highly specific tethering of the target molecule even from a complex mixture so can be useful where prior purification is difficult. Similarly, for affinity surfaces such as streptavidin or NTA, the tethering orientation will be uniform, according to the position of the biotin or His-tag on the ligand, respectively, and direct, selective tethering even from complex mixtures such as lysed cells (after solids removal for systems that use microfluidics) is possible.

The choice of which molecule of an interacting pair should be immobilized and which should be the analyte should be considered carefully. The SPR response during analyte binding will, at least to a first approximation, equal the response during ligand immobilization multiplied by the ratio of analyte to ligand molecular weights. Therefore, one would expect a low molecular weight analyte to produce a very small response if the immobilized ligand has a high molecular weight and is bound sparingly. In this case, one might improve the response by immobilizing the low molecular weight molecule and using the high molecular weight partner as the analyte. If the analyte is multivalent and cross-links the ligand molecules, the surface conformation of the latter may change during interaction and affect the response. In this case the immobilization/analyte choice should be reversed.

The reference channel or sensor should be subjected to running buffer during this step.

3.4 Deactivation of the Sensor Chip Surface

Any unreacted, NHS-activated carboxyl functional groups must be deactivated after immobilizing the target ligand, so as to avoid covalent attachment of the analyte during subsequent interaction analysis. This step is straightforward and can be achieved with a 30-s injection of 1 M ethanolamine to cap the residual NHS-activated carboxyl groups.

The control channel should also be subjected to this step.

3.5 Surface Stabilization

During the above immobilization steps, the surface may have attracted some non-specifically bound species or there may be weakly tethered components, both of which may result in a downward drift in response during subsequent interaction steps. It is best, therefore, to run running buffer over the surface until the response reaches a steady state. In Subheading 3.7, below, regeneration of the sensor chip surface after an interaction is described and it is recommended that at least one regeneration step be carried out to remove any weakly bound ligand prior to following interactions. As described below, particularly when using a new immobilized species, one should always check the stability of the immobilized ligand to the regeneration conditions and adjust these if necessary.

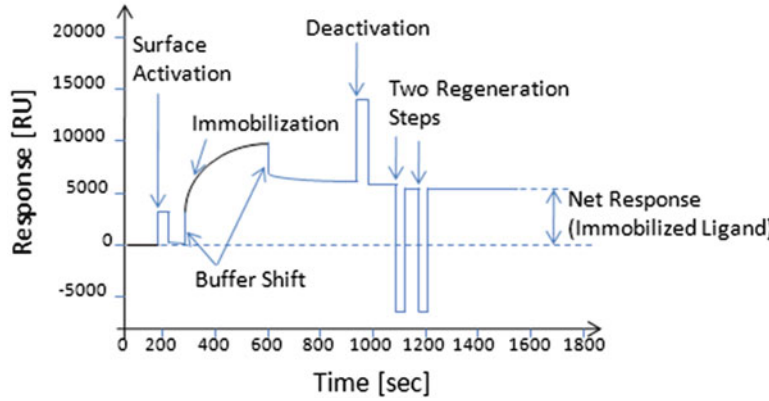

Fig. 8 A typical sensorgram during surface activation, immobilization, deactivation and two regeneration steps, showing a stable net response

At the end of all steps from Subheadings 3.2 to 3.5, the overall sensorgram should have a form similar to that in Fig. 8.

3.6 Interaction with the Analyte(s)

A running buffer should be chosen to stabilize the proteins but not interfere with protein binding. For example, Tris (which contains primary amines) should be avoided when using amine coupling. Generally, any physiologically relevant buffer with a little surfactant added to reduce non-specific interactions will suffice. Two recommended running buffers are HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffered saline (HBS, 10 mM HEPES containing 0.15 M NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 at pH 7.4) or phosphate buffered saline (PBS) containing 0.005% Tween 20, pH 7.4. In some cases, particularly where the analyte comes from a very complex or challenging sample matrix such as whey (7), additional salt can be added to further suppress nonspecific interactions (unlike during covalent immobilization, when high salt will decrease the penetration of the molecule of interest into the surface layer).

Analyte samples and blanks (controls) should be prepared in the running buffer so that nonspecific interactions and bulk shifts can be subtracted and net changes in responses determined.

Prior to studying an interaction, it is recommended that several blank injections of running buffer be made immediately before analyte injection. In theory, as running buffer is constantly being run over the chip surface while docked, there should be no difference between the baseline SPR signal in between steps and the signal during an injection (as long as the same buffer source is used for both). However, SPR instruments are extremely sensitive, and changing the source of running buffer from background flow to injected samples can result in a slightly different response between the immobilized surface and the blank control. Several blank injections can "settle" the surface down, in practice.

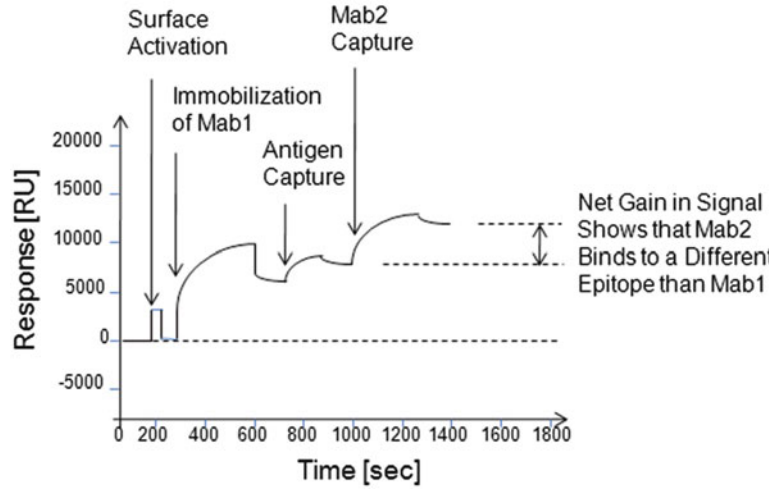

Fig. 9 Epitope mapping by SPR

(As a practical aside, and one which is generally true when working with highly sensitive analytical instruments, one should *never* lean on the instrument or even the bench upon which it is sitting while carrying out an experiment. The optics within these instruments are very sensitive to alignment and a detectable shift in SPR response may result if one leans on the top of an instrument or against the bench upon which it is sitting!)

There are several levels of data one might wish to collect by SPR. The first is a simple "yes/no" answer to either the question of whether or not two proposed binding partners actually interact or the question of whether or not an analyte known to interact with the ligand is present in a given sample solution. Neither of these analyses requires extensive quantification and generally it is only a matter of subtracting the appropriate reference channel data to eliminate nonspecific binding and buffer shift to determine whether there appears to be binding.

Likewise, epitope mapping relies on detecting additive responses to the stepwise creation of complex structures but not necessarily quantitative determination. In epitope mapping by SPR, one can determine, without the use of labels, whether different antibodies bind to different parts of the antigen. The technique involves immobilizing the first monoclonal antibody (Mab1) on the chip surface via amine coupling, binding the antigen to the bound Mab1, and then injecting a second monoclonal antibody (Mab2), also known to bind to the antigen. If the two antibodies bind at the same or a closely located epitope, Mab2 will be blocked and there will be no increase in signal after its injection. If the two Mabs bind to different epitopes, this will be seen by an increase in the signal (Fig. 9).

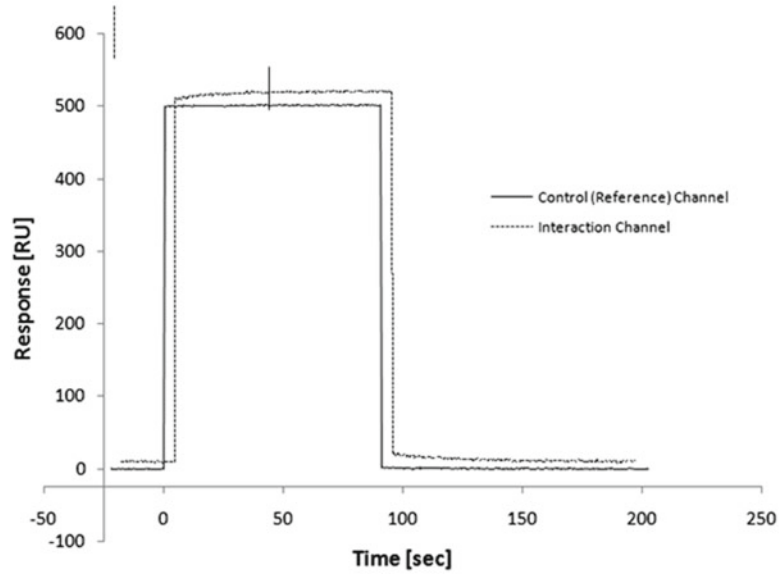

Fig. 10 Typical "raw" interaction and reference channel sensorgrams, requiring data processing before the SPR responses due to specific binding, can be determined (Fig. 10)

Quantitative data are required for concentration analysis. In this case, a calibration series must be prepared using an appropriate serial dilution of standard and the responses of analyte samples compared with this for quantification. This type of analysis requires, above all, consistency of operation to ensure the unknown analyte(s) can be quantitatively compared to the standard curve. The main constraint is to ensure that the data are reproducible and that the surface does not become saturated during binding; otherwise differences between analyte concentrations cannot be distinguished. This, however, should be an obvious constraint and as long as the samples are diluted to bring the SPR responses to within those of the standard curve data, all should be well.

Quantitative analysis of binding kinetics requires the most stringent planning and control of the experiments, careful consideration of the data quality, and elimination of artifacts. Obtaining the correct shapes of the association and dissociation curves is crucial and repeatable sensorgrams should be collected, preferably spanning the range between zero and saturation of the immobilized ligand.

An example of a typical raw interaction sensorgram and the corresponding control or reference channel sensorgram is given in Fig. 10. Note that there is a large step change in the response of both channels, due to the difference in the bulk refractive index of the analyte sample compared to the running buffer; the two curves are slightly offset from one another, which may arise from minor

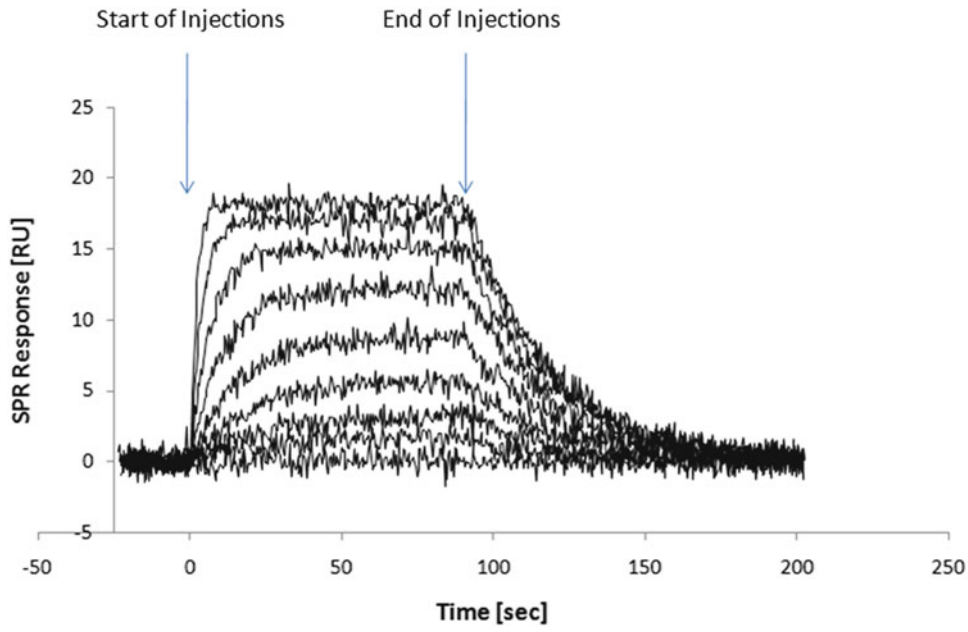

Fig. 11 A series of sensorgrams for an interaction analysis after processing of raw data (simulated using Clamp software)

differences in flow paths and baseline signals between channels; and there are a couple of spikes (artifacts) in the curves. Initial data processing is required to vertically and horizontally align the interaction sensorgrams with the reference channel sensorgram, to delete the spike artifacts, and to then subtract the reference channel from each interaction channel sensorgram. Although the scale of the buffer shift will usually obscure this, the reference channel does not only undergo buffer shift but also undergo some association and dissociation due to nonspecific interactions. The scale also obscures the contribution of noise to the signal. The result, including a number of interaction sensorgrams, rather than just the single one shown in Fig. 10 for clarity, is shown in Fig. 11. Note that the noise in the sensorgrams is much more evident. The data for these two figures were generated using Clamp, a data fitting, and modeling software package obtained from the Center for Interaction Analysis at the University of Utah (http://www.cores.utah.edu/interaction/software.html).

The curves in Fig. 11 represent a good set of data for kinetic analysis. The shapes of the association and dissociation curves indicate simple binding; the sensorgrams cover the range from zero response (a blank analyte sample) through to a well-saturated equilibrium condition, with an even distribution over that range; the responses are small but clearly distinguishable (ideal for kinetic analysis, whereas large responses may be subject to mass transfer effects); and the dissociation curves have clear curvature. Ideally,

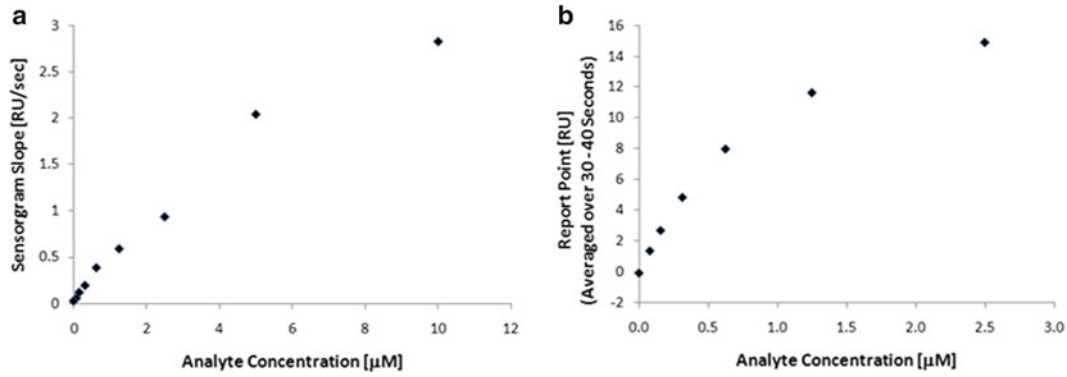

Fig. 12 Standard concentration curves derived from (**a**) initial slopes and (**b**) report points of average responses between 30 and 40 s from the data in Fig. 11

one should obtain at least triplicate data sets for the sensorgrams at each concentration. Somewhat unusually, in this case, the sensorgrams all appear to return to the baseline during the rather brief dissociation phase—this is not typical of all interactions, by any means, and usually a regeneration step is required, as described below, to remove the analyte completely between interactions.

For concentration analysis, there are two methods of quantifying the analyte concentration. In the first method, the initial slopes of the sensorgrams during association are determined and used to determine concentration by comparing against a standard curve of initial sensorgram slope versus concentration (8). In the second method, a "report point" is used, in which the SPR response is taken during the association at a set time after injection—the higher the analyte concentration, the higher the response should be at the report point, provided that the surface has not become saturated (7). Usually an average of the SPR response over a short time interval around the report point is used to smooth out noise. Fig. 12 shows the standard curves generated from the data in Fig. 11, using (a) initial slopes of the sensorgrams and (b) the average responses over the period 30–40 s as report points. Note that the initial slopes give a reasonable standard curve over a greater concentration range than the report points in this case, where the data are not shown for the latter above 3 μM because the responses flatten out at higher concentrations. This should not present a limitation if more concentrated analyte samples can be diluted to give report point values within the standard curve range.

In the simplest case, we can consider simple one to one binding to be of the form

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB \tag{2}$$

and the kinetics to follow the form

$$\frac{\mathrm{d}[AB]}{\mathrm{d}t} = k_a[A][B] - k_d[AB] \tag{3}$$

where (A) is the concentration of free, immobilized ligand, A; (B) is the concentration of free analyte, B; (AB) is the concentration of the analyte-ligand complex, AB; k_a is the association constant; and k_d is the dissociation constant.

Initially, when the analyte encounters the clean ligand surface, (AB) is zero, while there are plenty of ligand sites available so (A) may be assumed to be essentially constant for a short time. Thus, according to Eq. 3, the initial rate of change in (AB) (the slope of the sensorgram) is approximately directly proportional to (B), as shown in Fig. 12a. On the other hand, at the report point between 30 and 40 s, the ligand approaches saturation at the higher concentrations (i.e., (AB) is appreciable, while the free ligand sites available, (A), has decreased), so the SPR response is not as sensitive to differences in analyte concentration above 3 μM. Note that the upper useful concentration is given here only by way of an example and is unique to the hypothetical interaction simulated in Figs. 10 and 11—the actual analyte concentrations over which sufficient resolution can be obtained in any particular case will depend upon the particular binding pair under consideration, as well as the density of the immobilized ligand on the surface. For concentration analysis by either method, one would wish to use data far below ligand saturation, so maximizing the ligand density during immobilization is desirable.

At the end of the association period, the analyte sample is replaced by running buffer and the interaction monitoring continued for a given time. Accurate determination of k_d requires some curvature in the dissociation curve, which may require the extension of dissociation for many hours if k_d is very low. The limiting factor for measuring very slow dissociation ($k_d < 10^{-6}$ s^{-1}) is signal drift, whereby the drift in baseline signal becomes significant compared to the change in response due to dissociation.

Early analyses of binding kinetics were carried out using linearization but this is unnecessary when computers are easily able to fit data by iterative means. Instruments usually come with proprietary software packages that not only drive the instrument during data collection but analyze and report association/dissociation kinetics. Two analysis software packages, Clamp and Scrubber 2, are available (both via http://www.cores.utah.edu/interaction/software.html), the latter being a particularly powerful package for manipulation and analysis of raw SPR data.

A system of differential equations can be derived from Eq. 2. Because the amount of free analyte in the injection is large compared to the number of ligand sites available for binding on the

chip surface and we can assume that the supply of A to the chip surface is not limited by mass transfer, (A) is constant:

$$\frac{\mathrm{d}[A]}{\mathrm{d}t} = 0 \tag{4}$$

The change in immobilized ligand concentration is then

$$\frac{\mathrm{d}[B]}{\mathrm{d}t} = -k_{\mathrm{a}}[A][B] + k_{\mathrm{d}}[AB], \tag{5}$$

and the change in the analyte-ligand complex is given by Eq. 3, repeated below for convenience:

$$\frac{\mathrm{d}[AB]}{\mathrm{d}t} = k_{\mathrm{a}}[A][B] - k_{\mathrm{d}}[AB] \tag{3}$$

For equilibrium analysis, we can say that $\frac{\mathrm{d}[AB]}{\mathrm{d}t} = \frac{\mathrm{d}[B]}{\mathrm{d}t} = 0$, and therefore

$$k_{\mathrm{a}}[A][B] = k_{\mathrm{d}}[AB] \tag{6}$$

Amongst the variables in Eq. 6, (A) is known, as it is the concentration of the analyte injections we control, and (AB) is the measured (dependent) variable, while (B), k_{a}, and k_{d} are all unknown. Note that (A), (B), and (AB) are all functions of time. We may take a number of approaches to the determination of k_{a} and k_{d}. First, we recognize that (AB) is proportional to R, the response of the instrument to bound analyte at any time, so

$$[AB] = \omega R \tag{7}$$

where ω is a constant of proportionality. Also, $(B)_0$ is the total initial concentration of ligand sites available and because $(AB)_{\max} = (B)_0$, then $(B)_0 = \omega R_{\max}$. As (AB) goes from 0 to $(AB)_{\max}$, (B) goes from $(B)_0$ to 0. Therefore

$$[B] = [B]_0 - [AB] = \omega(R_{\max} - R) \tag{8}$$

We can then write the kinetics in terms of R as

$$\frac{\mathrm{d}(\omega R)}{\mathrm{d}t} = k_{\mathrm{a}}[A]\omega(R_{\max} - R) - k_{\mathrm{d}}\omega R \tag{9}$$

Finally, we can eliminate the constant ω to express the kinetics entirely in terms of the two known variables (A) and R, the constant $R_{\max}$ estimated from the sensorgram curves and the two unknowns k_{a} and k_{d}:

$$\frac{\mathrm{d}R}{\mathrm{d}t} = k_{\mathrm{a}}[A](R_{\max} - R) - k_{\mathrm{d}}R \tag{10}$$

We can now make initial guesses at R_{max}, k_a, and k_d and then have the software iterate to fit the model in Eq. 10 to the experimental data by minimizing the sum of squares of the residuals between fitted and experimental data.

At equilibrium, $\frac{dR}{dt} = 0$, so solving for R_{eq} gives

$$R_{eq} = \frac{k_a[A]R_{max}}{k_d + k_a[A]} = \frac{[A]R_{max}}{K_D + [A]} \tag{11}$$

where K_D is the dissociation equilibrium constant $K_D = \frac{k_d}{k_a}$.

Integrating Eq. 10 with respect to time gives, during association,

$$R = \frac{[A]R_{max}}{K_D + [A]}\left[1 - e^{-(k_a[A]+k_d)t}\right] \tag{12}$$

Equations 10 or 12 can be used to fit sensorgrams taken over a range of (A) and the fit should be shown in published results to visually demonstrate that the fitted lines pass properly through the curves and that there are no artifacts or deviations from expected behavior. Equilibrium analysis determines K_D from a series of injections over a range of (A), where each curve should give the same value of K_D as long as they have reached a true plateau at the point for which the analysis is undertaken. Attaining a stable response at equilibrium is critical—equilibrium analysis *cannot* be applied if the association response is still increasing at the end of injection! The K_D values should also be consistent with the individual k_a and k_d values calculated from fitting Eq. 10 or 12.

During the dissociation phase, $(A) = 0$, so

$$\frac{d(R)}{dt} = -k_d R \tag{13}$$

Equation 13 is a typical first-order decay equation, characterized by a constant half-life. This means that the dissociation response curve is independent of (A) and will look identical around any given response level, R_0,

$$R = R_0 e^{-k_d t} \tag{14}$$

Curvature must be evident in the decay curve or else the decay is indistinguishable from a zeroth-order decay, $\frac{dR}{dt} = -k'$, where k' is a constant. Fitting of the dissociation curves by Eq. 14 to determine k_d is straightforward.

Figure 13 shows processed sensorgrams and fitted curves for all three systems described in Subheading 2 (9). It is always important that such curves and fitted lines are shown, rather than simply

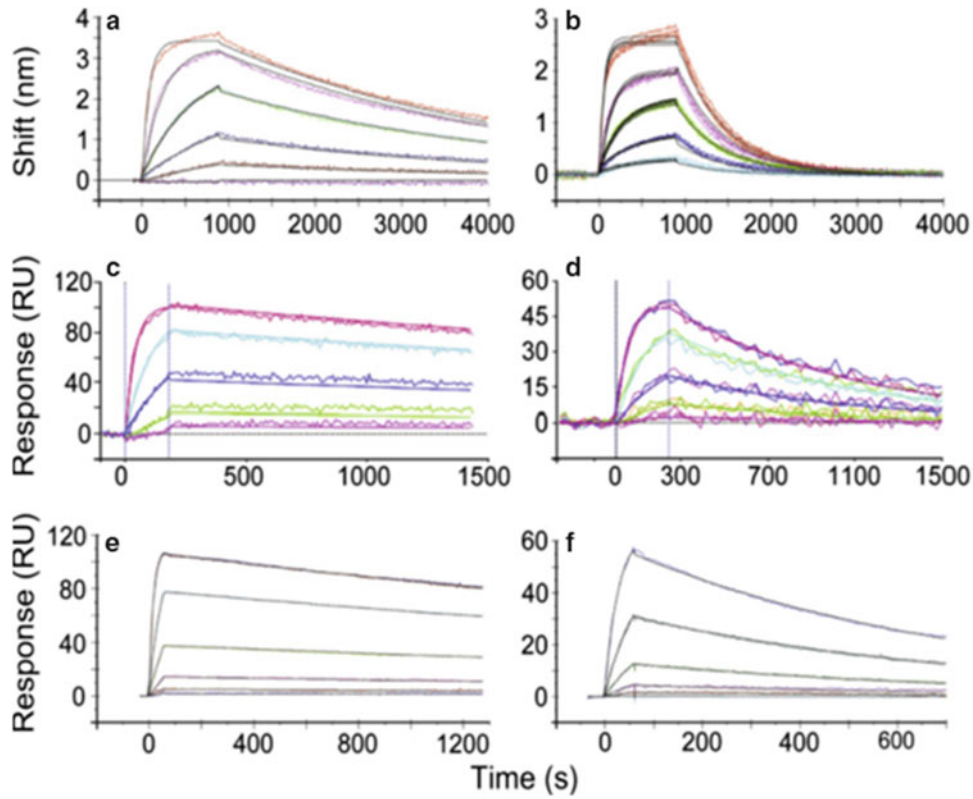

Fig. 13 Sensorgrams with fitted curves for interactions of an antibody fragment with rat (**a**, **c**, **e**) and human (**b**, **d**, **f**) calcitonin gene-related peptide alpha, measured on ForteBio Octet (**a**, **b**), Bio-Rad ProteOn (**c**, **d**), and Biacore 3000 (**e**, **f**) systems. Noisy and smooth lines distinguish measured data from simulated fits (reproduced from ref. 9)

a table of calculated association/dissociation of equilibrium constants. Ideally, a plot of residuals should also be shown but from Fig. 13, at least the reader can judge the goodness of fit for each sensorgram.

3.7 Regeneration

Regeneration is described here after the interaction step because it is routinely used between interaction cycles. Nevertheless, regeneration conditions must be determined prior to carrying out interaction analyses because they will critically affect surface stability and therefore repeatability. Usually, the analyte will not dissociate quickly enough to be completely dissociated by the end of an experimental run but it can be quickly removed by injecting an appropriate regeneration buffer to disrupt the intermolecular forces (Fig. 14). In the ideal case, regeneration removes all residual analyte but maintains analyte binding capacity of the surface from one interaction cycle to the next. In practice, a compromise is normally necessary between attaining sufficiently rigorous conditions for the effective removal of all residual analyte and nonspecifically bound materials from the ligand/surface and avoiding overly harsh

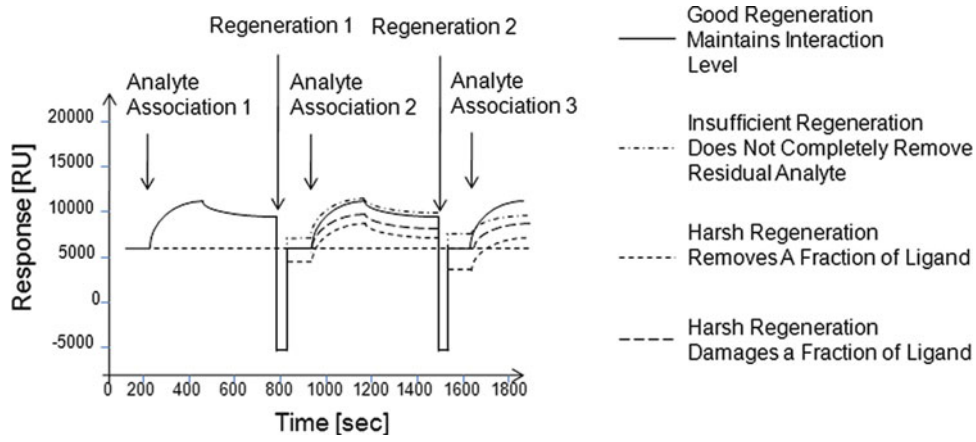

Fig. 14 Post-interaction regeneration of sensor chip surface

conditions that can remove ligand from the surface or damage the ligand's binding functionality. Figure 14 shows a number of scenarios where either too gentle a regeneration buffer results in insufficient removal of analyte, leading to an increase in baseline and gradual decline in analyte binding capacity, or too harsh a regeneration buffer results in either removal of ligand, causing a decrease in baseline and reduced analyte binding capacity or damage to the ligand binding functionality (without removal of mass), again reducing analyte binding capacity. It is important to establish the stability of the immobilized surface from one run to the next and the best conditions for regeneration must be determined experimentally by scouting a range of regeneration buffers. Start with gentle conditions and move to more stringent conditions if insufficient analyte/nonspecifically bound material is removed. Generally, the most significant variables are the regeneration buffer pH, salt concentration, presence of detergents or chaotropes, and the length of the regeneration buffer injection.

Table 1 lists a number of commonly used regeneration buffers for various strengths and types of interaction. A good starting point for regeneration for antibody-antigen interactions with immobilization by amine coupling of the ligand and providing that the ligand is stable at acid pH is to scout a range of 10 mM glycine-HCl buffers at pH values between 1.75 and 3, with 10 s injections. Regeneration of non-covalently immobilized ligands may require preparation of the immobilized surface between interaction cycles and care must be taken to attain as close as possible to identical surfaces for each run to ensure repeatability and comparison between interactions. As mentioned at the beginning of this section, regeneration conditions must be thoroughly scouted and a robust protocol determined before interaction studies begin. For concentration analyses, preparation of a new standard curve is recommended every ten cycles or so, depending on the stability of the surface under repeated interaction/regeneration runs. Running a

Table 1
Common regeneration buffers

Type of interaction	Acidic	Basic	Hydrophobic	Ionic
Weak	pH > 2.5 Formic acid HCl 10 mM Glycine–HCl	pH < 9 10 mM NaOH in HEPES or PBS	pH < 9 50% Ethylene glycol	1 M NaCl
Intermediate	pH 2–2.5 Formic acid HCl 10 mM Glycine–HCl H_3PO_4	pH 9–10 NaOH 10 mM Glycine/NaOH	pH 9–10 50% Ethylene glycol	2 M $MgCl_2$
Strong	pH < 2 Formic acid HCl 10 mM Glycine–HCl H_3PO_4	pH > 10 NaOH	pH > 10 25–50% Ethylene glycol	4 M $MgCl_2$ 6 M Guanidine chloride

few interaction/regeneration cycles before beginning to record critical data is worthwhile, to attain a steady state condition of the sensor chip surface whereby weakly bound ligand molecules are removed, labile ligands are destroyed, and a balance is reached between removal and binding of analyte and nonspecifically bound species with successive cycles.

3.8 Reproducibility of Experiments

Clearly, for a robust analysis, a statistically significant number of repeated measurements must be made to determine the certainty of results. There is no reason why a well-designed and executed sensor analysis should not produce excellent repeatability within an acceptable level of experimental error. One should *never* attempt to publish interaction behaviors that have not been verified by repeated observations because without this, it is not possible to tell whether there are systematic errors between injections of analyte, such as incomplete removal of analyte, loss of binding activity, or loss of ligand from the chip surface. One should also be very cautious about reporting "strange" kinetic behaviors in sensorgrams or over-interpreting unexpected deviations from the standard "sawtooth" shape of the association/dissociation curves shown here in Figs. 3, 9, 10, 11, and 13. Such deviations may well offer interesting engineering or scientific insights into mass transfer, nonspecific interactions, electrostatics, and so on but they are highly unlikely to be relevant to physiological binding events. For kinetic analysis of physiologically relevant biomolecular interactions, or detailed study of supramolecular assembly of proteins, the SPR should reliably allow a careful user to quantify k_a, k_d, and K_D.

3.9 Processing of Raw Data and Appropriate Analysis and Presentation of Data

Subheading 3.6 (Figs. 10 and 11) introduced the idea of processing of raw data. For kinetic analysis, each sensorgram in an analyte concentration interaction series must be aligned in both the "*y*" and "*x*" directions to ensure all interaction response curves start from the same point. Slight misalignments of raw data will inevitably occur, due to variations in injection times or tiny differences in flow path lengths between sample needles and the chip surface. Flow channels may undergo a slightly different degree of drift between injections, while small differences in the chip surfaces, extent of regeneration, and so on will mean each interaction starts from a slightly different response baseline. These must all be brought together to properly compare binding rates. Spikes, caused by transient air bubbles or electrical interference, must then be removed so that data can be fitted without distortions due to artifacts that do not represent the association/dissociation events. Most systems and independent analysis software packages have features that allow this to be done either automatically or under manual control.

3.10 Thermodynamics

Some information on binding, the water structure around proteins before and after association, and various relationships between enthalpic and entropic contributions to interactions and conformational changes in the proteins can be inferred from thermodynamic measurements. A discussion of these effects is beyond the scope of this chapter but the following relationships can be explored with a number of instruments in which sensor chip temperature can be systematically altered between runs.

Association and dissociation constants are functions of temperature, with both typically increasing with temperature according to an Arrhenius relation as follows:

$$\ln k = \ln A - \frac{E_A}{RT} \tag{15}$$

where k is the rate constant in question, A is the pre-exponential factor (as distinct from analyte A), E_A is the activation energy, and R is the universal gas constant. By varying the temperature between runs and plotting $\ln k$ versus $1/T$, one can determine both A and E_A. Also

$$\Delta G^\circ = RT \ln \frac{K_D}{C^\circ} \tag{16}$$

where ΔG° is the Gibbs free energy change associated with binding and C° is the standard state concentration (usually 1 M). The binding affinity includes both enthalpic (ΔH) and entropic (ΔS) contributions

$$\Delta G = \Delta H - T\Delta S \tag{17}$$

so combining Eqs. 16 and 17 and assuming that ΔH and ΔS are constant with temperature, one can use Eq. 18 below to plot $\ln \frac{K_D}{C^\circ}$ versus $1/T$ for runs made over a range of temperatures to determine both thermodynamic quantities:

$$\ln \frac{K_D}{C^\circ} = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \tag{18}$$

However, unless the temperature range is quite narrow, neither ΔH nor ΔS will be constant so that the plot suggested by Eq. 18 will not be truly linear. A more accurate approach in that case is to fit the integrated form of the van't Hoff equation, which requires information regarding the change in heat capacity with binding (10).

4 Summary

Optical biosensors such as SPR offer the ability to follow association and dissociation kinetics in real time with label-free molecules. A wide variety of systems is available from a number of suppliers, with various immobilization chemistries. The most common starting point for immobilization is through amine coupling to carboxy groups, while other chemistries such as streptavidin-biotin or NTA-His-tag can offer biospecific tethering with the advantage of uniform orientation of the ligand. Interaction analysis requires exacting control of experimental conditions and careful attention to detail, together with the demonstration of reproducible results. The most important aspect of biointeraction kinetic analysis is that the fitted curves are shown to closely and reproducibly fit the experimental data across an appropriate range of analyte concentrations.

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