Measurement of the Kinetics of Biomolecular Interactions Using the IAsys Resonant Mirror Biosensor

The development of biosensor technology has made the measurement of the kinetics of biomolecular interactions accessible to most immunologists. *UNIT 18.6* describes the use of the BIACORE series of plasmon-resonance biosensors. In addition, other more traditional techniques can be used to determine the affinity constants of an interaction (e.g., *UNITS 6.1, 18.3, & 18.4*). In this chapter the use of an alternate form of biosensor, the resonant mirror biosensor or IAsys, produced by Affinity Sensors, will be discussed.

All biosensors operate using a common principle—that binding events occurring on the sensing surface of the biosensor are converted by a transducer into an electronic signal which can then be processed by a computer to yield information on the amount of binding (Fig. 18.5.1). Where biosensors vary is in their configuration and the nature of the transducer. Both the IAsys and BIACORE series of machines use optical techniques to detect the binding event, but the IAsys uses a resonant mirror device as the transducer, while the BIACORE uses surface plasmon resonance.

When using biosensors the experiment can be divided into various phases, which will, for simplicity, be described separately. These are shown diagrammatically in Figure 18.5.2 and involve (1) the immobilization of the ligand onto the sensing surface (see Basic Protocol and Alternate Protocol); (2) addition of the ligate to the sensing surface, and

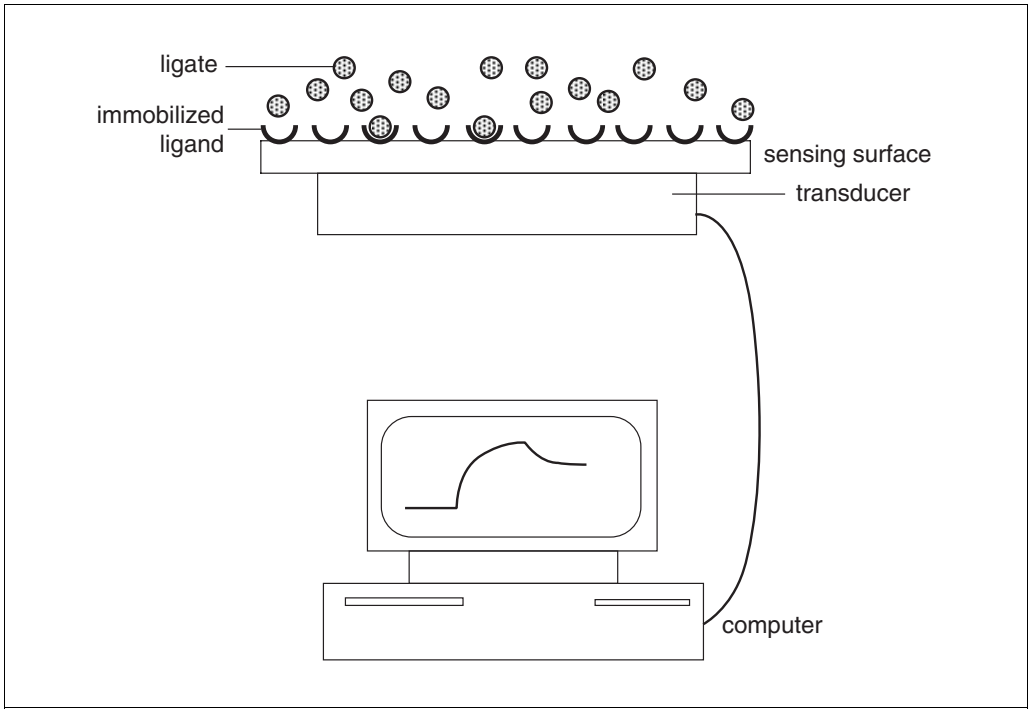

Figure 18.5.1 Principle of the biosensor. The principle of both the resonant mirror and plasmon resonance biosensor is shown in this figure. Both types of machine use a sensing surface in which one of the interacting molecules in the pair is immobilized (the immobilized molecule is referred to as the ligand). A soluble ligate is then allowed to interact with the ligand. These interactions are monitored by a transducer, which then sends data to the computer for analysis. The resonant mirror and plasmon resonance biosensors differ in details of their configuration, and also in the type of transducer used.

Contributed by Andrew J.T. George
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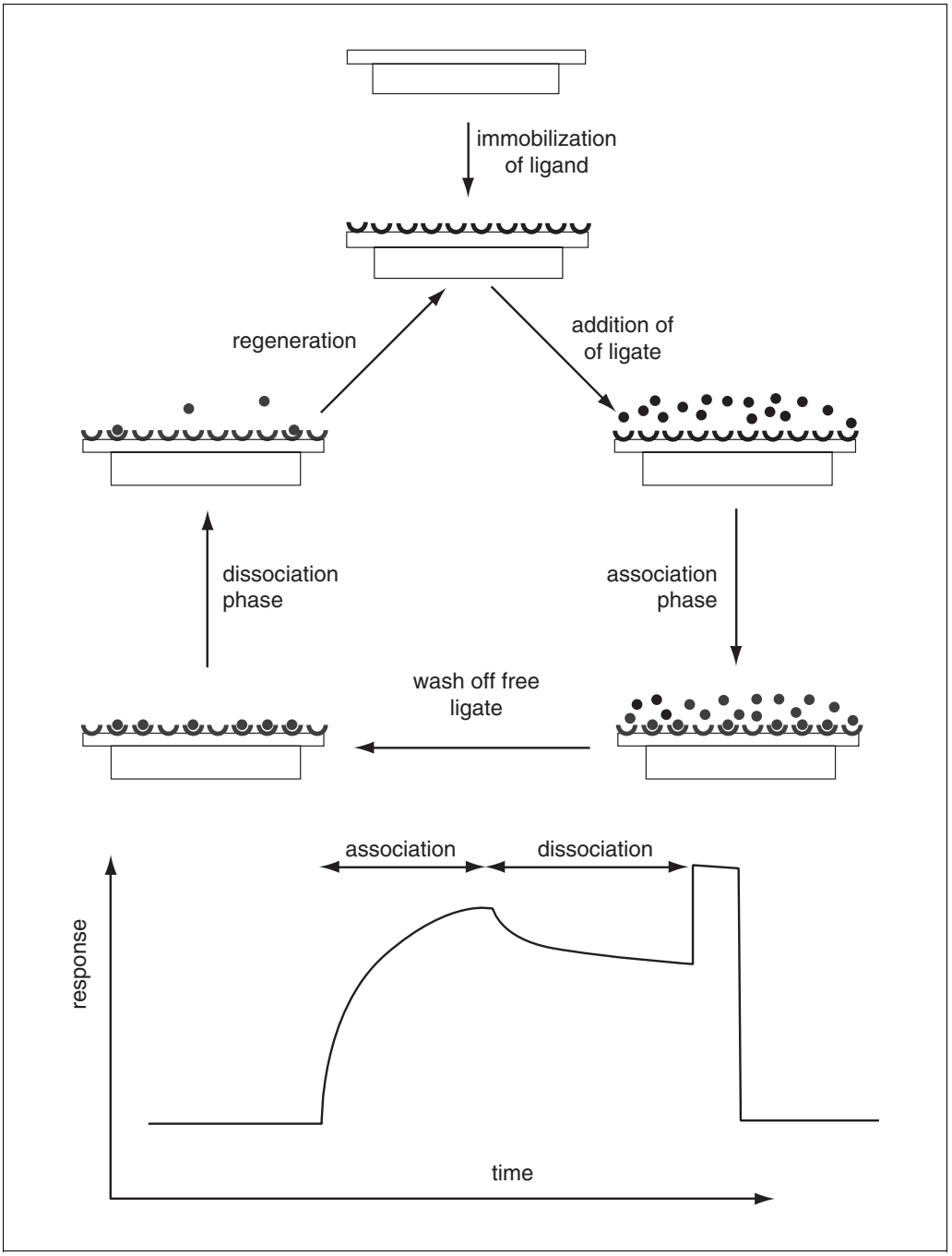

Figure 18.5.2 Plan of typical experiment using biosensor. This figure illustrates the typical stages in an experiment using a biosensor. Initially the ligand is immobilized on the sensing surface. The ligate is then added and the binding is followed for a period of time (association phase). The sensing surface is then washed to remove free ligate, leaving ligate bound to the surface. The dissociation of the ligate from the ligand is then followed over time. Finally, any ligate remaining bound to the ligand is removed during the regeneration phase. The surface can then be used for further association-dissociation-regeneration cycles. At the bottom of the figure is shown a typical trace, with time plotted on the *x* axis and the amount of material bound to the surface plotted on the *y* axis. The trace shows typical association and dissociation phases. The regeneration phase is the almost square wave seen immediately following the dissociation, which returns the response to the baseline. The sudden change in the response during the regeneration phase is due to changes in buffer leading to a bulk shift. The response (or amount of binding) is measured by a change in the resonant angle (see Background Information), the units for which are arc seconds.

following its association to the ligand, including the kinetics of binding (see Support Protocol 2); (3) washing of free ligate from chamber and following the dissociation of the ligand-ligate complex; (4) regeneration of the surface (see Support Protocol 3); repetition of phases 2 and 3; and (5) data analysis (see Support Protocol 4). Throughout the unit, the convention will be used whereby the molecule that is bound to the surface is termed the ligand and that in solution the ligate. However, the reader should be aware that this terminology is not universally used (e.g., some authors use the term "analyte" instead of "ligate"). All parameters mentioned in this unit will be for the manual IAsys system, rather than the automated IAsys+. A protocol (Support Protocol 1) is also included for optimization of ligand pH for immobilization to the carboxymethylated dextran surfaces described in this unit.

The response (or amount of binding) is measured by a change in the resonant angle (see Background Information), the units for which are arc seconds.

STRATEGIC PLANNING

A strategy must be developed for immobilization of the ligand to the sensing surface. There are several sensing surfaces available for the IAsys. The most commonly used is a dextran hydrogel, consisting of a carboxymethylated dextran layer that extends ~200 nm from the sensing surface. The carboxymethyl groups offer suitable sites for covalent attachment of molecules. Alternative surfaces consist of an aminosilane surface in which the aminosilane is attached directly to the sensing surface, or biotinylated aminosilane surfaces, in which biotin moieties have been attached to the amino groups.

The carboxymethylated (CM) dextran hydrogel surfaces are similar to those used in the BIACORE machines (see *UNIT 18.6*). They provide a large number of potential attachment sites for the molecule. The most common form of linkage uses standard 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-*N*-hydroxysuccinimide (EDC-NHS) chemistry to cross-link to amino groups present on the protein. However, alternatives include the use of thiol chemistry or linking to carbohydrate groups. The aminosilane surfaces do not have the dextran hydrogel, so the amount of material that can be bound to the surface is considerably less. However, if large objects (such as cells) are being used, a hydrogel will prevent them from getting close to the sensing surface and will result in a weaker signal. As a result, the "flat" surfaces may be advantageous in such circumstances. In addition, it has been shown that the hydrogel can, under some circumstances, introduce artifacts in the kinetic analysis, due to trapping of molecules within the dextran matrix (Edwards et al., 1995). The flat surfaces might also be useful in the attachment of lipid vesicles to the sensing surface (tethered liposomes).

The manufacturer of the IAsys apparatus produces a variety of protocols for immobilization to surfaces. These include the use of EDC-NHS chemistry to immobilize onto "flat" surfaces (i.e., lacking the dextran hydrogel) that have been prepared with free carboxylate groups, immobilization of biotinylated ligates onto biotin-coated "flat" surfaces via streptavidin, and deposition of a lipid monolayer onto a hydrophobic surface. However, the authors of this unit do not have experience with these approaches, and the reader is referred to the technical literature supplied by the manufacturer. The two protocols given here (see Basic Protocol and Alternate Protocol) should suffice for most routine purposes.

BASIC PROTOCOL

IMMOBILIZATION OF LIGANDS TO CARBOXYMETHYLATED DEXTRAN HYDROGEL SURFACES USING EDC/NHS CHEMISTRY

This protocol will describe immobilization to carboxymethylated dextran using the NHS coupling kit from IAsys. In the immobilization, a mixture of EDC and NHS is first added to activate the carboxymethyl groups (Fig. 18.5.3). The ligand is then added, which will be immobilized via its amine groups. Finally, any remaining unreacted activated groups on the dextran gel are blocked by ethanolamine. This protocol is suitable for proteins and peptides containing lysine residues (so long as these are not important in the ligate-binding properties of the molecule). In the case of short peptides, the authors have found that coupling the peptides to a carrier protein (such as bovine serum albumin) can simplify conjugation.

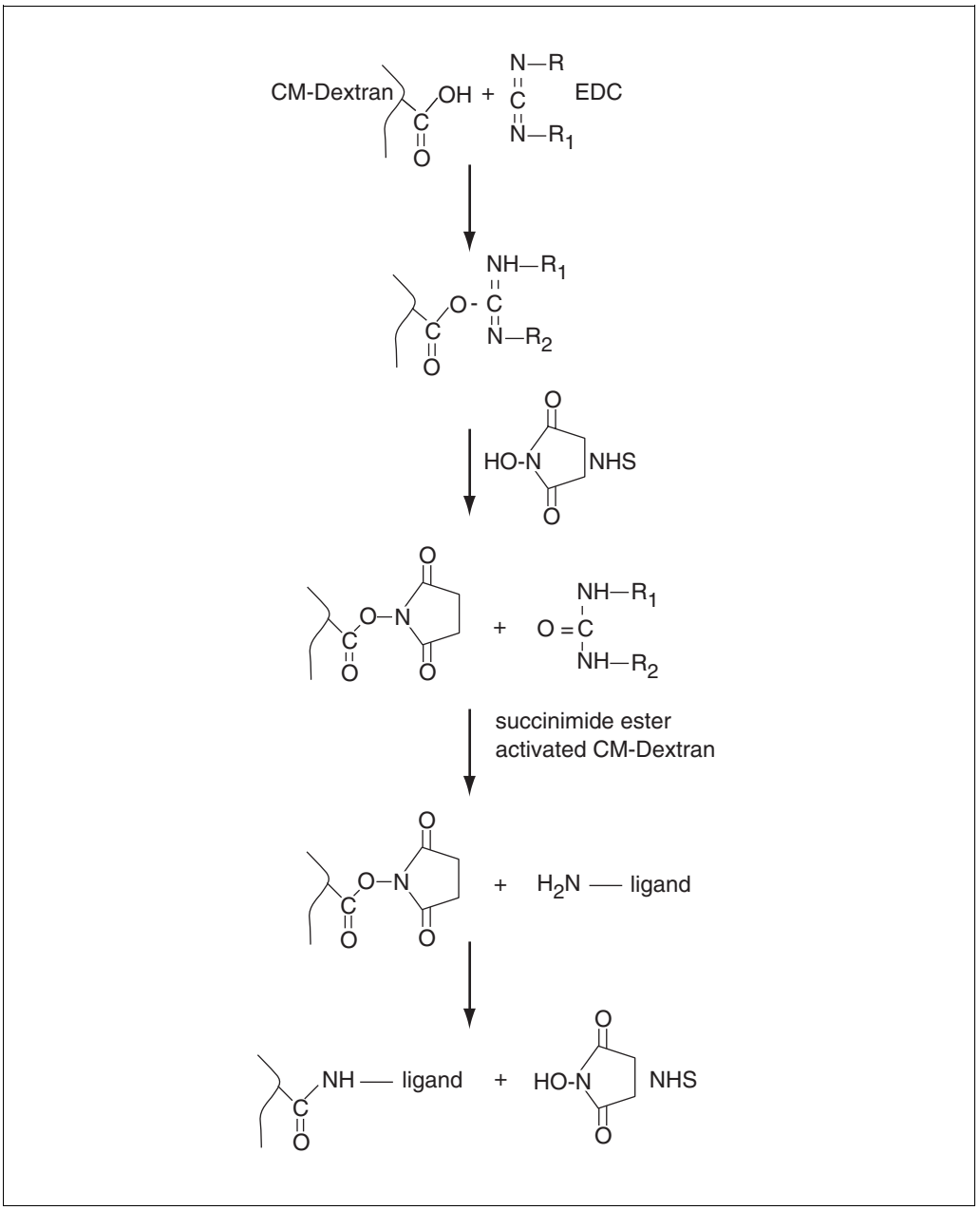

Figure 18.5.3 EDC-NHS reaction scheme.This figure illustrates immobilization of the ligand using EDC-NHS chemistry. The carboxymethylated dextran surface is first activated with EDC and NHS. This then couples, via lysyl amino groups, to the ligand. Figure reprinted with permission from Affinity Sensors.

Coupling of the ligand onto these surfaces relies on electrostatic concentration between the negatively charged carboxylate groups on the hydrogel and the positively charged ligand. It is necessary to optimize the pH of the coupling for any particular ligand in order to optimize this process (see Support Protocol 1). The coupling occurs between free amine groups on the ligand and succinimide ester–activated carboxymethyl groups on the dextran hydrogel.

Materials

PBS-Tween: phosphate-buffered saline (PBS; *APPENDIX 2*) containing 0.05% (v/v) Tween 20

NHS coupling kit (IAsys NHS-2005; Affinity Sensors), with aliquots prepared according to the manufacturer's instructions (also see recipe)

Ligand of interest at as high a concentration as possible

1 M ethanolamine, pH 8.5

100 mM acetate buffer of appropriate pH (see Support Protocol 1)

IAsys resonant mirror biosensor system (Affinity Sensors; see Background Information)

IAsys CM-Dextran cuvettes (Affinity Sensors; FCD-0101 for manual machine; FCD5101 for Auto+; see Background Information)

1. Place a CM-dextran cuvette into the IAsys instrument with the stirrer set at 4000 rpm (for machines using vibrating disc, see manual) and the temperature at 25°C. Add 200 µl of PBS-Tween and allow to equilibrate 5 to 10 min.

   The machine takes readings of the resonant angle every 0.2 to 0.3 sec. These data are collected by the computer at a rate that is determined by the experimenter, using the control software supplied with the instrument. For kinetic analysis the data should be collected at the maximum rate. For coating, the data collection can be limited to one data point every second, thereby reducing the size of the computer files generated.

   If a cuvette has been used for optimization of the pH (see Support Protocol 1), then the same cuvette may be used for immobilization. If this is done, wash the chamber three times with PBS-Tween and allow the cuvette to equilibrate for ~5 min.

2. Thaw one aliquot of NHS and one aliquot of EDC.

   See manufacturer's instructions and see Reagents and Solutions for preparation of the aliquots.

3. Add 100 µl of the thawed aliquot of NHS to 100 µl of the thawed aliquot of EDC and immediately replace the contents of the cuvette with this mixture (by pressing the button on the machine which then aspirates the contents of the cuvette to the waste bottle). Leave for 7 min.

4. Wash cuvette with 200 µl PBS-Tween, leaving the solution in the cuvette for 30 sec.

   Washing is performed by removing the contents of the cuvette (see step 3) and immediately replacing with PBS-Tween.

5. Remove the PBS-Tween and add 200 µl of 10 mM acetate at the appropriate pH containing 50 µg/ml of the ligand of interest.

   The ligand should be provided at as high a concentration as possible. Make it at 50 µg/ml in 10 mM acetate buffer of the appropriate pH (prepare the buffer using 100 mM sodium acetate buffer and distilled water). Note that if the dilution that must be performed to obtain the ligand at 50 µg/ml is not very high, the pH may not be the same as the original pH of the buffer. To some extent, this does not matter so long as the pH optimization has been performed under the same conditions. It is also important to keep the salt concentration low, so as not to interfere in the electrostatic concentration.

The solution left over from the pH optimization experiment (see Support Protocol 1) can be used.

6. Leave for 5 to 15 min depending on the degree of immobilization required.

   How much material should be immobilized onto the surface depends on the nature of the experiments being performed. If kinetic analysis is to be carried out, then the lowest level of ligand consistent with obtaining a satisfactory signal upon addition of the ligate should be immobilized. This will depend on such features as the molecular weight of the ligate and the degree to which the binding epitopes on the ligand are destroyed by immobilization. However, as a rule of thumb, it is probably desirable to immobilize <1000 arc sec (note that as the response is approximately proportional to the mass of material bound to the surface this figure may need to be adjusted for small or large molecules). If the biosensor is being used for concentration analyses, then the amount of immobilized ligand should be as high as possible. It is possible to vary the amount of immobilization by varying the time of immobilization, the concentration of the ligand, and the pH.

7. Wash cuvette three times with 200 µl PBS-Tween, each time leaving the solution in the cuvette for 30 sec after the final wash.

8. Replace contents of cuvette with 200 µl 1 M ethanolamine, pH 8.5. Leave for 3 min.

9. Wash cuvette three times with PBS-Tween (see step 7).

   A typical immobilization profile is shown in Figure 18.5.4.

   The manufacturer recommends adding regeneration buffer (see Support Protocol 3) at this stage. However, especially when using a new ligand, the author prefers to check that the ligand is available to bind to its ligate and has not been inactivated by the immobilization, and therefore prefers to add the ligate and carry out the binding sequence before adding regeneration buffer. The same binding sequence is then carried out an additional two times to check the surface. Frequently there is some loss of binding between the first addition of

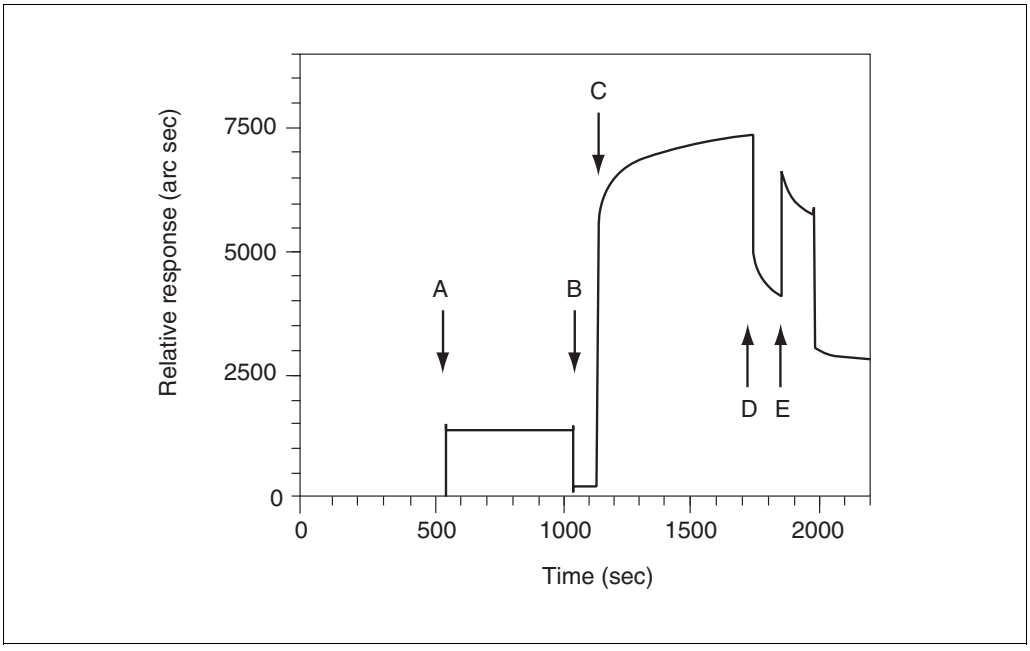

Figure 18.5.4 Immobilization of ligand. The results of a typical immobilization protocol are shown. The resonant angle (in arc sec) is shown on the *y* axis, and the time of the experiment (in sec) on the *x* axis. In this case the ligand is a recombinant ED-B fibronectin fragment. At point A the activating EDC-NHS mixture is added. It is washed off at point B, resulting in a "square wave" due to the bulk shifts consequent on buffer changes. At point C, the ligand in low-salt, optimal-pH buffer is added. This is washed off at D, and the blocking ethanolamine solution is added at E. The degree of immobilization seen here would be used typically for concentration analysis or epitope mapping, rather than kinetic analysis, where lower levels should be immobilized.

the ligate and the second, because the regeneration buffer removes weakly bound ligand. The first sequence cannot, therefore, be used for data analysis. However, it does allow one to confirm whether the immobilization has been successful, and also whether the regeneration buffer destroys the ligand.

If the ligand of interest is particularly precious, it is possible to reduce the volume in which it is added to the cuvette to 50 µl. In such cases it is also possible to recover material by pipetting it out of the cuvette.

OPTIMIZATION OF pH FOR IMMOBILIZATION OF LIGANDS TO CARBOXYMETHYLATED DEXTRAN HYDROGEL SURFACES

SUPPORT PROTOCOL 1

In order to ascertain that the protein ligand will bind optimally to the surface for IAsys assay, its binding properties should be tested at different pH conditions. When the pH is too low, the protein is insufficiently charged to show strong electrostatic concentration. When it is too high, the surface loses its charge. It is reasonably simple to gauge the optimum pH for carrying out the immobilization by picking the pH at which the greatest uptake of the ligand was seen.

Once the pH for a particular ligand has been optimized, it should be unnecessary to repeat this optimization procedure for future immobilizations, assuming that the conditions (such as concentration of ligand and type of buffer) do not change substantially.

Materials

PBS-Tween: phosphate-buffered saline (PBS; *APPENDIX 2*) containing 0.05% (v/v) Tween 20
100 mM sodium acetate buffers at pH 3, 3.5, 4, 4.5, 5, 5.5, and 6
Ligand at as high a concentration as available

IAsys resonant mirror biosensor system (Affinity Sensors; see Background Information)
IAsys CM-Dextran cuvettes (Affinity Sensors; FCD-0101 for manual machine, FCD5101 for Auto+; see Background Information)

1. Place a new CM-dextran cuvette into the IAsys with the stirrer set at 4000 rpm and the temperature at 25°C. Add 200 µl of PBS-Tween and allow to equilibrate for 5 to 10 min.
2. For each of the seven different-pH acetate buffers, add 50 µl of 100 mM acetate buffer to each of two microcentrifuge tubes. Label each tube with the pH of the buffer to be added and label one with a "+" and the other with a "–".
3. To the tubes labeled "+" (seven in total) add 25 µg of the ligand.
4. Make all the tubes (+ and –) up to 500 µl with distilled water.

   The tubes will now all contain 10 mM acetate buffer, and the "+" tubes will have 50 µg/ml of the ligand.

   If the ligand is particularly precious, it is possible to scale the volumes from 500 µl down to 50 µl. It is also possible to check only 2 or 3 pH values, as it may not be necessary for a fully optimal pH to be used.

5. Remove the PBS-Tween from the chamber of the cuvette and immediately add 200 µl of the pH 3 "–" buffer. Leave for 20 sec.
6. Replace the contents of the chamber with 200 µl of the pH 3 "+" buffer. Leave for 2 min.
7. Replace the contents with 200 µl of PBS-Tween.

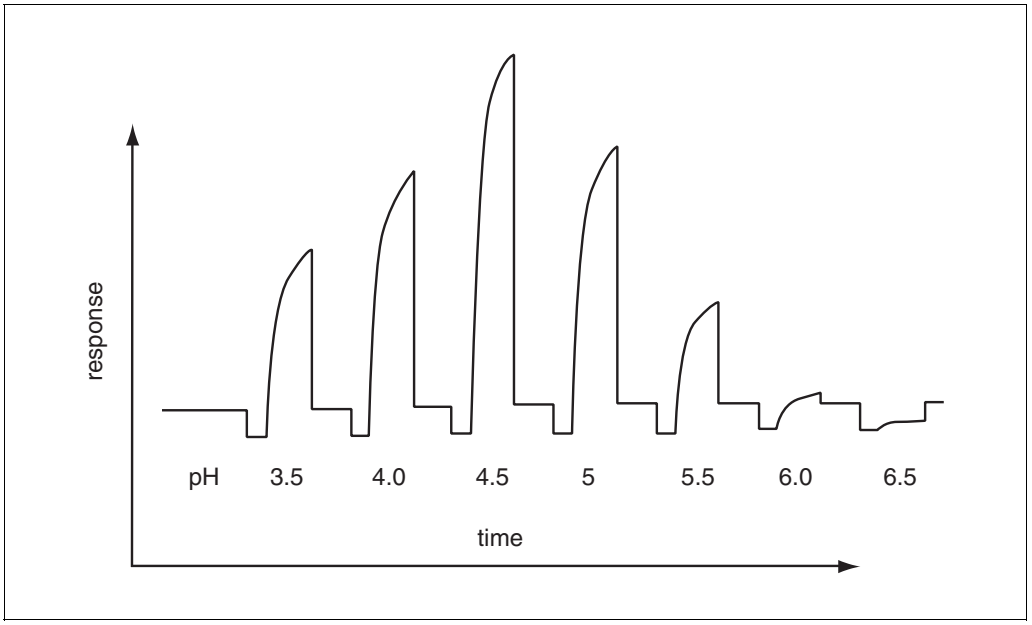

Figure 18.5.5 pH optimization. This figure illustrates what a pH optimization experiment might look like, according to Support Protocol 1. Whenever the 10 mM acetate buffer is added, there is a bulk shift down, due to the change in the buffer. When the protein is added in the same buffer it binds to the surface of the cuvette, due to electrostatic interactions. The amount of binding seen is dependent on the pH. In this case maximal binding is seen at pH 4.5. When the surface is washed with PBS-Tween, this causes the bound material to rapidly dissociate from the surface, and the baseline to return to the original one. The exact shapes of the binding curves can vary somewhat.

8. Repeat steps 5 to 7 with each of the other buffers.

A profile similar to that shown in Figure 18.5.5 should be obtained. The height of the peaks (relative to the baseline obtained with the appropriate acetate buffer alone) is used to determine which pH results in the highest levels of electrostatic concentration. The height of the peaks can be readily obtained using the software supplied, while the experiment is in progress. Thus, if one is in a hurry, it is possible to stop the optimization process early, once it is clear that the optimal pH has been discovered. For example, in the experiment shown in Figure 18.5.5, it would not be necessary to proceed beyond pH 5.5, since by then one would be confident that the optimal pH (4.5) had been found.

ALTERNATE PROTOCOL

IMMOBILIZATION OF LIGANDS ONTO AMINOSILANE SURFACE VIA GLUTARALDEHYDE

This protocol describes a simple method for immobilizing ligand onto aminosilane surfaces, as an alternative to the hydrogel used in the Basic Protocol. As described above (see Strategic Planning), such surfaces have advantages in particular settings.

The chemistry of the immobilization is shown in Figure 18.5.6. This process relies on the formation of glutaraldehyde polymer. This polymerization can be determined by a wavelength scan between 200 and 350 nm, with a peak at 280 nm representing the monomer and another at 234 nm the polymer. However, the author himself has never carried out such a scan.

Materials

5% (v/v) glutaraldehyde (prepare using 25% aqueous solution purchased from Sigma)
0.1 M NaOH
0.1 M HCl

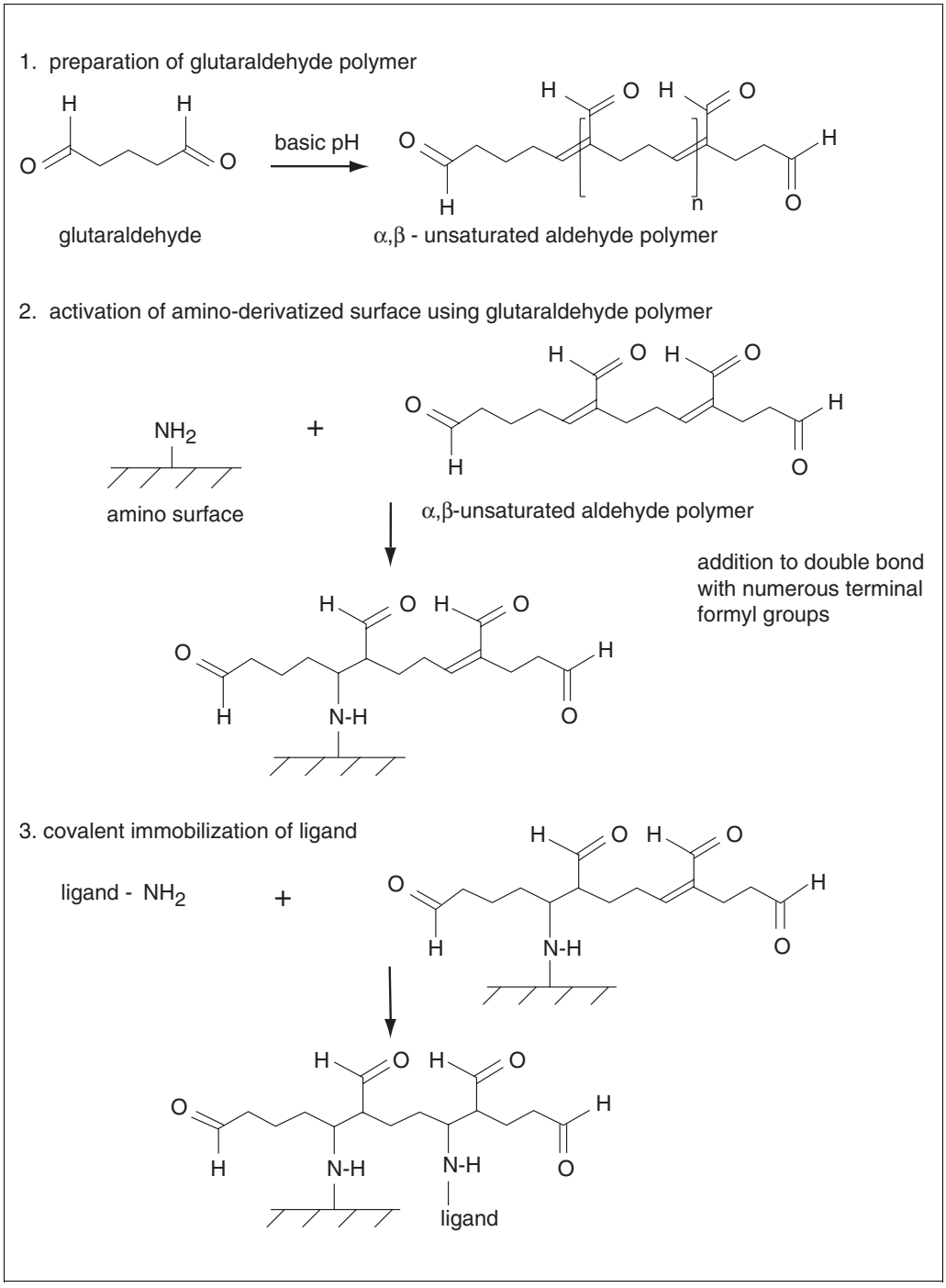

Figure 18.5.6 Glutaraldehyde immobilization reaction scheme. This figure illustrates immobilization of a ligand to an amino surface using polymerized glutaraldehyde. One of several possible reaction routes is shown. Figure reprinted with permission from Affinity Sensors.

10 mM sodium phosphate, pH 7.7
100 µg/ml ligand in 10 mM sodium phosphate, pH 7.7
10 mg/ml bovine serum albumin (BSA) in 10 mM sodium phosphate, pH 7.7

Aminosilane cuvettes (Affinity Sensors; FCA-0201 for manual machine; FCA-5201 for Auto+; see Background Information)

IAsys resonant mirror biosensor system (Affinity Sensors; see Background Information)

Prepare polymerized glutaraldehyde

1. To 5 ml of 5% (v/v) glutaraldehyde in water, add 500 µl of 0.1 M NaOH. Leave for 30 min at room temperature.
2. Add 500 µl of 0.1 M HCl to neutralize, then divide into 250-µl aliquots and freeze at −20°C.

The aliquots of polymerized glutaraldehyde may be stored up to 6 months at −20°C.

Immobilize ligand

3. Place aminosilane cuvette in biosensor and wash twice with 10 mM sodium phosphate, pH 7.7. After final wash, leave for 5 to 10 min to equilibrate in 10 mM sodium phosphate buffer, pH 7.7.

Washing is performed by removing the contents of the cuvette (by pressing the button on the machine which then aspirates the contents of the cuvette to the waste bottle) and immediately replacing with the wash buffer.

4. Thaw an aliquot of polymerized glutaraldehyde. Replace contents of cuvette with 200 µl of the polymerized glutaraldehyde and leave for 30 min.
5. Wash cuvette with 200 µl 10 mM sodium phosphate, pH 7.7, then leave the buffer in the cuvette for 1 min.
6. Replace the contents of the cuvette with 200 µl of ligand at 100 µg/ml in 10 mM sodium phosphate, pH 7.7. Leave for 30 min.
7. Wash twice, each time with 200 µl of 10 mM sodium phosphate, buffer pH 7.7. Leave 2 min after final wash.
8. Add 200 µl of 10 mg/ml BSA in 10 mM sodium phosphate, pH 7.7. Leave for 10 min to block remaining sites.
9. Wash twice with 10 mM sodium phosphate, pH 7.7. Leave 5 min after final wash.

As with the immobilization using EDC-NHS chemistry (see Basic Protocol), the manufacturer recommends a regeneration step before adding the ligate. However, the author of this unit suggests testing the binding of the ligand to the ligate first. It is, however, necessary to perform a regeneration step before data that can be analyzed are obtained.

SUPPORT PROTOCOL 2

DETERMINATION OF THE KINETICS OF BINDING OF BIOMOLECULES TO LIGANDS USING IAsys

In order to determine the binding kinetics of biomolecules, the ligate (in this case an antibody against the ligand) is added to the cuvette containing the immobilized ligand at a range of concentrations. The association of the two molecules is then followed, and the cuvette is washed extensively. The dissociation of bound ligate can then be followed. The steps below provide a good starting point for the experiment; however, this will need to be modified depending on the nature of the interaction.

Materials

PBS-Tween: phosphate-buffered saline (PBS; *APPENDIX 2*) containing 0.05% (v/v) Tween 20

Ligate

Cuvette with immobilized ligand (see Basic Protocol or Alternate Protocol)

Additional reagents and equipment for regeneration of the binding surface of the cuvette (see Support Protocol 3) and analysis of data (see Support Protocol 4)

Prepare cuvette and ligate dilution series

1. If the cuvette has been stored overnight or longer, regenerate the surface (see Support Protocol 3).

2. Place a cuvette with the immobilized ligand into the IAsys instrument with the stirrer set at 4000 rpm and the temperature at 25°C. Wash the cuvette six times with 200 µl of PBS-Tween, allowing the cuvette to equilibrate with the solution for 10 min after the final wash.

   Washing is performed by removing the contents of the cuvette (by pressing the button on the machine which then aspirates the contents of the cuvette to the waste bottle) and immediately replacing with the wash buffer.

Prepare the ligate

3. During the 10-min incubation in step 2, prepare a series of solutions of the ligate (antibody against the ligand) at different concentrations, at 50 µl volume and 10× the required final concentrations, in PBS-Tween.

   These can be most easily prepared by making doubling dilutions from a starting stock. The correct starting concentration depends upon the affinity of the interaction; as a rule of thumb start at 10× the estimated K_d of the interaction (i.e., this should be the final concentration, therefore make the solution at 100× the K_d). For example, for an unknown antibody/antigen interaction the author typically starts with 20 µg/ml final concentration of the antibody (assuming that the antigen is the ligand). Make ~10 two-fold dilutions. Store the ligate dilution series in the temperature-controlled compartment of the IAsys.

Collect and analyze data

4. Replace the 200 µl PBS-Tween in the cuvette with 180 µl PBS-Tween. Leave for 90 sec and check that the data collection rate is set to the maximum possible, using the control software supplied with the instrument.

5. Add 20 µl of the highest concentration of the antibody. Leave for 5 min in order to follow the association.

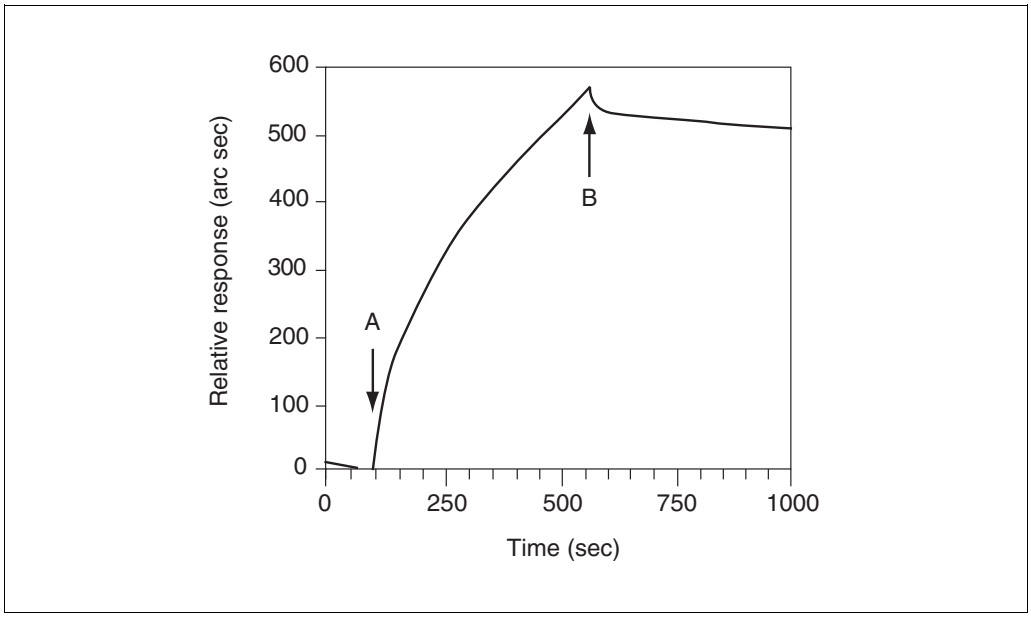

Figure 18.5.7 A typical binding/dissociation cycle. In this case the immobilized ligand is ED-B fibronectin. At the time indicated by A, the soluble ligate was added (in this case a monoclonal antibody against the fibronectin). At time B, the free antibody was washed off the surface and the dissociation followed. The regeneration of the surface is not shown in this figure.

6. Wash rapidly three times, each time with 200 µl PBS-Tween. Leave for 5 min in order to follow the dissociation.
7. Regenerate the surface (see Support Protocol 3).
8. Allow cuvette to equilibrate in PBS-Tween for 5 min.
9. Repeat steps 4 to 8 using the next concentration of the ligate. Continue the process until a significant response is no longer seen.

   The results of a typical experiment are shown in Figure 18.5.7. The response obtained will be dependent on the molecular weight of the ligate, but relative responses of 10 to 200 arc sec can be easily analyzed.

10. Analyze data (see Support Protocol 4).

SUPPORT PROTOCOL 3

REGENERATION OF IAsys LIGAND BINDING SURFACES

One of the critical stages in designing an experiment is the regeneration. This stage aims to strip the bound ligate off the ligand, thus allowing the surface to be reused for the next binding experiment. However, while the surface can withstand harsh conditions, the ligand is often sensitive to the regeneration buffer. This leads to a loss of binding and can also increase the nonspecific binding.

Regeneration involves: (1) replacing the contents of the cuvette with 200 µl of regeneration buffer, (2) waiting for as short a time as necessary (typically 2 min), and (3) washing well (six times), each time with 200 µl PBS-Tween (see Basic Protocol).

The following are possible regeneration (elution) buffers for use with the IAsys resonant mirror biosensor. Experimentation will be required to determine which buffer is optimal for a particular ligand/ligate pair. The buffers marked "see above" are recommended by the manufacturer, but the author has had no experience with them.

Acid:
20 mM HCl
20 mM or 50 mM glycine·HCl, pH 2.2
10 to 50 mM H_3PO_4 (see above)

Alkaline:
50 mM Na_2CO_3/$NaHCO_3$, pH 9.6
50 mM Na_2CO_3 (see above)

Hydrophobic:
20% (v/v) ethanol (see above)
30% (v/v) ethylene glycol (see above)

Ionic:
1 M NaCl (see above)

Other:
Gentle Elution Buffer (Pierce)

The choice of buffer is dictated by the ligand, and is frequently empirical. If it is known that the ligand is unstable at a certain pH, that will limit the buffers that can be used. If the ligand has been used successfully in an affinity column, the elution buffer used with the column is a good starting point. The author has found that Gentle Elution Buffer, sold by Pierce for affinity chromatography, is a good general-purpose regeneration buffer.

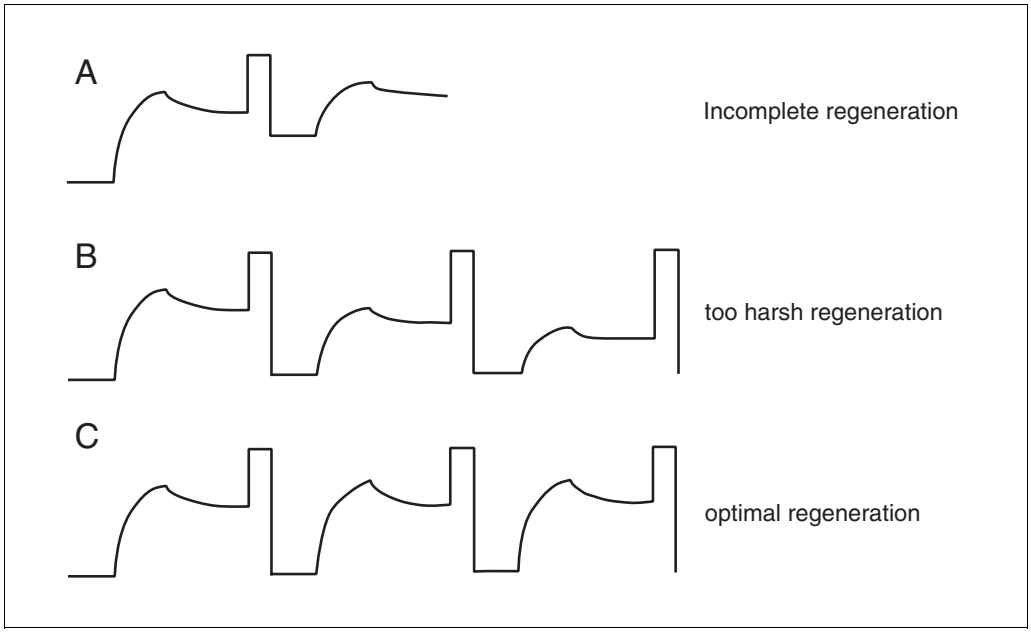

Figure 18.5.8 Optimization of regeneration. This figure illustrates three traces that might be obtained when regenerating the surface. In each case, successive binding curves are shown using the same concentration of ligate for each cycle. Each cycle contains the standard association and dissociation phases, followed by a regeneration step. In (**A**) incomplete regeneration is shown; the trace does not return to its original position. It is necessary to either use harsher conditions or to increase the length of the regeneration step. In (**B**) the effect of using too harsh regeneration conditions is shown; there is a decrease in the binding after each regeneration stage, suggesting that the ligand had been damaged in some way. In such circumstances it is necessary to choose less harsh conditions. Finally, in (**C**) the optimal regeneration conditions are shown. Each binding curve is identical, and the trace returns to the original baseline after each regeneration.

When using a new ligand–regeneration buffer combination, it is a good idea to repeat the typical binding experiments three times with a single concentration of ligate. If the profile shown in Figure 18.5.8A is obtained, the regeneration conditions are not harsh enough. If the profile shown in Figure 18.5.8B is obtained, the regeneration conditions are damaging the ligand. The profile shown in Figure 18.5.8C is ideal.

ANALYSIS OF IAsys DATA USING FASTfit SOFTWARE

SUPPORT PROTOCOL 4

It is not the intention of this protocol to describe in any detail how to use the FASTfit data analysis software package provided by Affinity Sensors with the IAsys machine, as such information will change with different versions of the software, and is available in the manual. Nor is it the intention to derive or justify the assumptions implicit in the equations used in the analysis, which can be found in George et al. (1995a).

It should be remembered that there are several levels of sophistication of IAsys user. Some users simply want to rank the binding kinetics of their antibodies and get "order of magnitude" results to allow them to choose molecules for further development. Others want highly precise data on a particular interaction for biophysical studies, while other users require an intermediate degree of precision. If one is in the former category, it is reasonably simple and fast to obtain the data needed; however, to obtain very precise data, one needs to put in the time and effort, and use different experimental formats. It should also be remembered that the accuracy of the results is dependent on factors such as the concentration of the ligate. It is pointless to worry about a 10% difference in the results obtained by different methods of analyses if one does not know the concentration of the ligate to that degree of precision.

The basis of the FASTfit package is to define particular regions on the data corresponding to the baseline, association, and dissociation regions. The software then uses an iterative process to fit the data to selected equations. For most purposes the data should be fitted to obtain "k_{on} single phase," "k_{diss} single phase," and "k_{diss} to baseline, single phase." It is important when selecting the areas for analysis to check (using the options provided in the software) that there is no systematic error and that the regions analyzed correspond to <80% the maximal levels of binding or dissociation. In other words, when analyzing the association part of the curve, one should reduce the area of the region analyzed so as not to include the "plateau" of the curve.

The term k_{on} can be confused with the association rate constant (termed k_{ass}). The author of this unit therefore prefers to call it the observed rate constant of the reaction (k_{obs}). The k_{obs} or k_{on} is related to k_{ass} by the following equation:

$$k_{obs} = k_{ass}[L] + k_{diss}$$

where [L] is the concentration of ligate. In order to obtain the k_{ass} it is necessary to plot k_{obs}/k_{on} against the concentration of the ligate. This should result in a straight line whose slope is k_{ass} (Fig. 18.5.9). At high concentrations of the ligate, the straight line can appear to flatten out, and such data can be discarded.

The k_{diss} terms do not need further analysis, although choosing which equation to use (k_{diss} single phase or k_{diss} to baseline, single phase) can be difficult. The k_{diss} single phase fits the curve to the following equation, which makes no assumption about where the dissociation curve will end:

$$R_t = R_\infty + (R_0 - R_\infty)e^{-k_{diss}t}$$

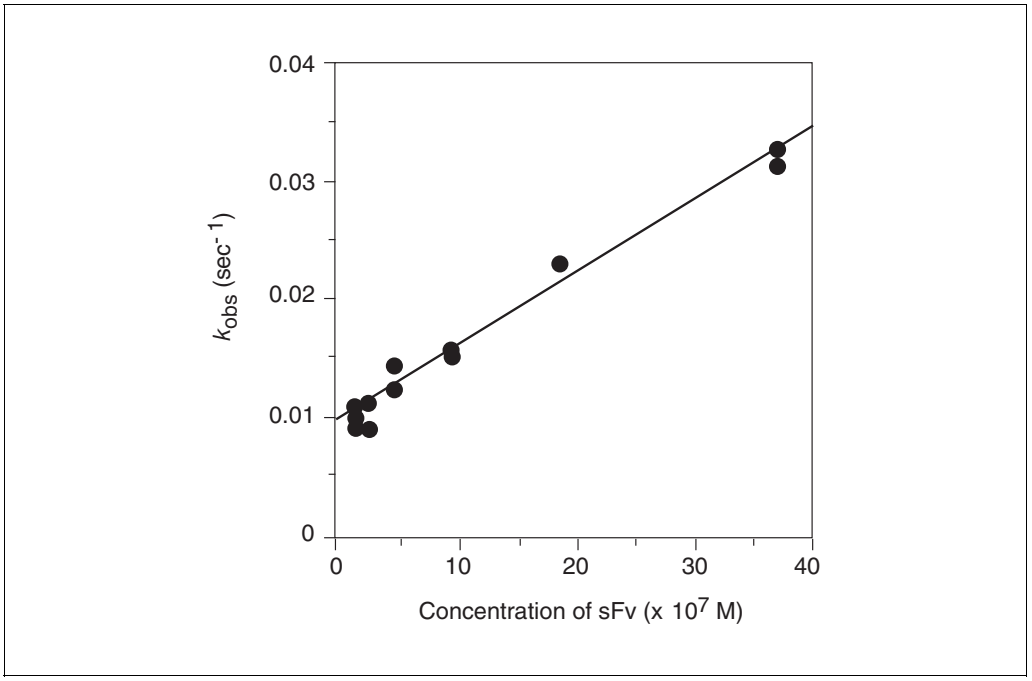

Figure 18.5.9 Plot of k_{obs} against concentration. The figure illustrates the expected result when k_{obs} is plotted against the concentration of ligate. In this case the immobilized ligand is ouabain coupled to bovine serum albumin, and the soluble ligate is the 26-10 single-chain antibody, which recognizes digoxin and related cardiac glycosides such as ouabain (George et al., 1995b).

where R_t is the response at time t.

In the author's hands this equation has sometimes produced bizarre results, with the fitted curve predicting that only a very small proportion of the ligate will dissociate. The k_{diss} to baseline, single phase, fits to the following equation:

$$R_t = R_0 \cdot e^{-k_{diss}t}$$

This assumes that the ligate will, in time, completely dissociate from the ligand, which is clearly more attractive in theory. However, there are dangers to beware of. First, the slightest drift or inconsistency in the baseline over the course of the experiment can wreak havoc, as one is forcing the k_{diss} to tend towards this false baseline. It is therefore important to use the FASTfit options to check the baseline. In addition, when using the carboxymethyl dextran, it is possible that not all of the ligate will be free to dissociate from the ligand (Edwards et al., 1995). Thus, ligate buried deep in the hydrogel may have the opportunity to rebind spare ligand and remain trapped on the sensing surface. One can reduce this by keeping the concentration of the ligand as low as possible, by carrying out the dissociation in the presence of free ligand, or by using a "flat surface" (see Strategic Planning).

The basic forms of analysis have been described. There are others that can be useful, such as inhibition studies (Karlsson, 1994). However, at this stage in the development of the technology, these are probably not yet in routine use by the casual user.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Coupling reagents, preparation of aliquots

Dissolve 1.15 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.2 g *N*-hydroxysuccinimide (NHS) in 15 ml of water. Divide into 150-µl aliquots and immediately freeze at −20°C (stable up to 6 months at this temperature).

As these reagents are relatively unstable they should only be thawed once they are about to be used. Any unused reagents should be thrown away and not refrozen.

COMMENTARY

Background Information

Biosensors, including the BIACORE and the IAsys, are becoming increasingly important tools in understanding biomolecular interactions (George et al., 1997). They are now widely accessible both to sophisticated users, who will be designing their own experiments, and the relative novice who wants relatively straightforward data. However, while it is relatively easy to generate data using these machines, it is important to design the experiments so that the data are useful and meaningful. It is therefore important to bear in mind considerations such as the effect of multivalency on the interactions being measured. Choice of which partner will be the ligate and which will be the ligand is dictated by the availability and nature of the two molecules, but should also be influenced by the type of data that one wants to obtain. Thus if one wants to look at the binding of an IgG antibody to a multivalent antigen, it is probably most appropriate to immobilize the antigen onto the surface and use the antibody as the ligate. Clearly, such binding will still be influenced by the bivalent nature of the antibody causing an avidity advantage, but, after all, in most settings where that antibody might be used, it will be free to bind in a bivalent way—and so it may be more useful than looking at the interaction of Fab fragments. If used correctly and with care, the biosensors offer immunologists a wealth of information that was previously difficult to obtain.

How the resonant mirror biosensor works

The principle of the resonant mirror device is shown in Figure 18.5.10 and discussed in more detail in the literature (Buckle et al., 1993; Davies and Pollard-Knight, 1993). The resonant mirror is made up of a glass prism covered by low-refractive-index silica. The top layer of the mirror consists of high-refractive-index silicon nitride. On top of the resonant mirror is the sensor surface, which in some cases consists of a carboxymethylated dextran hydrogel. Polarized laser light is directed at the prism, at a variety of different incident angles. Some of the light tunnels through the silica layer and, at one particular angle (the resonant angle), propagates along the high refractive index layer (which acts as a monomode waveguide) before tunneling out of the silica layer and leaving the prism to be detected. This resonant angle can be detected owing to a phase shift that occurs in some of the reflected light, which can be

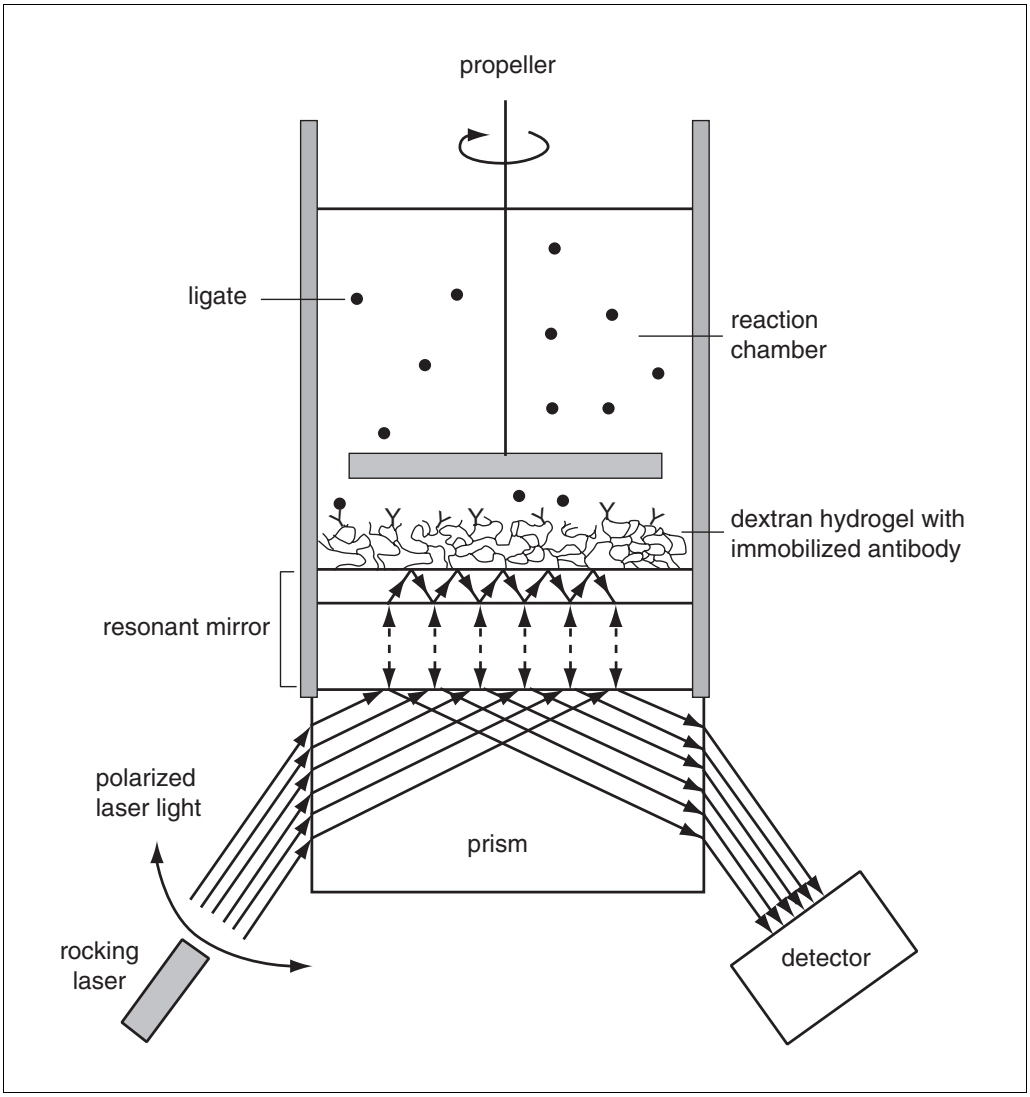

Figure 18.5.10 How the IAsys biosensor works: a schematic representation of the IAsys resonant mirror biosensor. The sample cuvette contains a reaction chamber into which the sample is added. Here, the carboxymethylated dextran sensing surface, which is probably the most commonly used surface, is illustrated. The ligand (in this case represented by Y-shaped antibody molecules) is immobilized to the surface, which is placed on top of the resonant mirror. The contents of the reaction chamber are efficiently mixed either with a propeller (shown here) or by a vibrating disc. Laser light is directed at a prism underlying the resonant mirror at a range of angles. At one angle (the resonant angle), the light tunnels through the low refractive index layer of the mirror and propagates along the high refractive index layer before tunneling out of the mirror. Alterations in the amount of material bound at or near the sensing surface affect the position of the resonant angle, and so the binding of the ligate (black circles) to the ligand can be followed by determining the angle. Reproduced with permission from George et al. (1997).

translated into an intensity peak using phase optics.

When the light propagates along the waveguide, it sets up an evanescent wave. This penetrates 670 nm above the sensing surface, though as it exponentially decays the effective distance is probably 200 nm. The evanescent wave is an electromagnetic field that propagates along the sensing surface, whose strength decays exponentially with distance from the surface. Alterations in the medium through which the evanescent wave propagates, such as changes in the refractive index, alter the properties of the wave, and so influence the resonant angle. Thus binding events that occur at or near the sensing surface can be detected by measuring the change in the resonant angle (in arc sec).

IAsys machines

There are two forms of IAsys machines now available: the IAsys manual machine and the IAsys Auto+, which is an automated version. Both machines, in contrast to the BIACORE series, use cuvette rather than flow systems. The cuvettes contain the resonant mirror transducer, the sensing surface, and a small reaction chamber to which appropriate solutions can be added. The manual machine has a single chamber, which can hold up to 250 µl of solution (though more typically 200 µl reaction volumes are used). The automated machine has two chambers, each of which holds up to 80 µl of solution.

The cuvettes are placed into the IAsys machine. The contents of the cuvette are efficiently stirred using either a propeller or, in more recent versions of the machine, a vibrating disc. In the manual machine the cuvette contents are accessed by lifting a small flap and adding the solutions manually, using conventional micropipets. The contents of the chamber are either vacuum aspirated or evacuated by means of a peristaltic pump. The automated machine uses a needle attached to a robotic arm and suitable pumps to add solutions to the chambers. The temperature of the cuvette is controlled using a circulating water bath or a Peltier device.

The cuvette system is different in concept from the flow system used by the BIACORE series of instruments. Kinetic analysis in both machines assumes that the concentration of reactants in solution is not altered by binding or dissociation events. Flow systems try and achieve this by continually replenishing the solution, so that the concentration of material is not reduced by binding, and also so that any material that dissociates from the surface is rapidly removed and cannot rebind. The same problem is addressed in the cuvette systems by keeping the volume of the cuvette large in comparison to the amount of material attached to the sensing surface. Thus, any material binding to the surface should not significantly alter the concentration in solution, and material dissociating from the surface is rapidly diluted. These problems can be minimized in both systems by reducing the amount of material covalently coupled to the surface.

Cuvette systems may have advantages when used to measure interactions with samples that have the potential to clog up the microfluidic systems of flow devices. Thus the cuvette system has been used with whole cells.

Critical Parameters

The results that will be obtained, of course, depend upon the nature of the molecular interactions being studied. However, there are several critical parameters that must be born in mind during the design of the experiments. One of the most important of these is valency. When a multivalent interaction occurs (for example, between immobilized antigen and a soluble antibody molecule) the functional affinity of the interaction is increased as a result of the binding. This effect is most pronounced in the k_{diss} of the interaction. It is therefore important to take this into account when designing the experiment. If one wishes to study monovalent interactions, it is necessary to make sure that the soluble species is monovalent (e.g., in the example of an antibody/antigen interaction, either the antibody must be immobilized and a monovalent antigen used as a ligate, or Fab fragments of the antibody must be created). In particular be aware that any aggregation of the ligate can produce multivalent binding. As the functional affinity of these species will be much higher than that of any monovalent material, they may dominate the binding analysis. Of course, in many cases, one may be happy to measure the "functional affinity" of an antibody binding to an antigen—i.e., it may reflect the biological situation under investigation.

A second critical parameter that can influence the design of the experiment is the effect of immobilization on the ligand. In some cases, the coupling reaction can destroy the relevant binding site on the molecule or render the molecule heterogeneous with respect to its binding characteristics. If this occurs, it may be possible to use alternative coupling strategies (for example site-specific coupling to carbohy-

drate or thiol groups), or to turn the experiment "upside down" by using the molecule as a soluble ligate.

It is necessary that the stock solution of the ligand be sufficiently concentrated that addition of it to a concentration of 50 µg/ml in the 10 mM acetate buffer will not result in a high salt concentration that will block the immobilization, or alter the pH significantly. It does not matter if the final pH of the solution is not exactly the original pH of the 100 mM acetate buffer, since the pH conditions will be optimized and the optimal pH will be used for immobilization. However, if the buffer that the ligand is in has a particularly high pH or salt content, this might interfere with the immobilization. In such cases it is necessary to either use the protein at a lower concentration (final concentration 10 µg/ml) or dialyse it against an appropriate buffer.

While it is important to optimize the pH, clearly other factors may be important. If the ligand is unstable at low pH, then it may be necessary to either use an alternative coupling strategy, or compromise by using a suboptimal pH for immobilization.

Kinetics of binding

It is critical to have a flat baseline, especially when determining the k_{diss} of the reaction (see below). Sometimes, when starting an experiment the baseline takes some time to stabilize. There are several ways to help overcome this.

1. Leave the machine turned on (with the laser also on) all the time, unless it is not to be used for a considerable period.

2. Allow the cuvette to equilibrate to the machine temperature (2 to 3 min).

3. If the baseline is drifting, wash every 3 to 4 min with PBS-Tween. Try regenerating the surface a second time if that does not work.

It is always important to carry out appropriate controls, especially where the interaction being studied is weak and nonspecific interactions are more dominant, and in cases where there is some chance that the ligand (or indeed the ligate) has been denatured and is "sticky." This can be seen sometimes towards the end of an experiment, when the surface has gone through a number of regeneration cycles. The correct control will depend on the particular experiment, but might include an isotype-matched antibody or a cuvette coated with an irrelevant ligand.

If the k_{diss} is very low there will be trouble in obtaining a consistent result, due to minor fluctuations in the baseline. An alternative to get around this is to perform an association/dissociation binding experiment similar to that in Support Protocol 1, but using a high concentration of the ligate, follow the association for just 2 to 3 min, and then follow the dissociation for 5 min in the normal manner. In the author's laboratory, this is repeated three to four times and the data are then used for analyzing the k_{diss}.

Changes in the buffer cause changes in optical properties, resulting in "bulk shifts" that are seen as immediate changes in the response. In order to avoid these, the ligate should be in the same buffer as that used to collect the baseline, and the cuvette should be washed. The bulk shifts need not affect the analysis of data, but it is best to avoid sudden changes in buffer during kinetic analysis wherever possible.

One of the advantages of the biosensor technology is that the molecules being studied need not be pure. This particularly applies to the ligate, though in the author's experience, ligands comprising ~20% of the protein in the preparation have been successfully immobilized. However, beware of side interactions involving other molecules. It is important to carry out appropriate negative controls to check the specificity of the interaction (for example, using an irrelevant antibody prepared in the same way as the test antibody). It can be particularly important to carry out these controls both at the start and the end of an experimental session. Sometimes the repeated rounds of binding and regeneration can denature some of the immobilized ligand, causing it to become "sticky" and exhibit nonspecific binding. This can be a particular problem when the ligate is not pure (e.g., a serum sample).

Time Considerations

It takes ~3 hr to optimize and immobilize a ligate onto the surface of the cuvette for the first time. Subsequent immobilizations take ~1 hr. Each cycle of binding, dissociation, and regeneration should take ~20 min (allowing time to achieve a flat baseline). The data analysis can be performed (once the operative is confident in the techniques) while the experiments are running.

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Internet Resources

http://www.affinity-sensors.com/

Affinity sensors Web site.

Contributed by Andrew J.T. George
Imperial College School of Medicine
Hammersmith Hospital
London, United Kingdom