Binding of Small Peptides to Immobilized Antibodies: Kinetic Analysis by Surface Plasmon Resonance

This unit describes a method for screening small viral peptides as specific antigens using a surface plasmon resonance (SPR) biosensor (Fägerstam et al., 1992; Malmqvist et al., 1997; Homola et al., 1999). Real-time biomolecular interaction analysis using affinity-based biosensors is more attractive than immunoenzymatic techniques such as ELISA because it is a fast means to get affinity and kinetic data for large numbers of biospecific interactions (Brigham-Burke et al., 1992; Dubs et al., 1992; VanCott et al., 1994; Saunal and Van Regenmortel, 1995; Wu et al., 1995; Cheskis and Freedman, 1996; Lessard et al., 1996; England et al., 1997; Oddie et al., 1997; Richalet-Sécordel et al., 1997; Ferrer et al., 1999; Houshmand et al., 1999) and does not require any labeling (see *UNIT 18.6* for further details on BIACORE technology). Since SPR response is directly related to mass changes on the sensor surface, in small antigen–antibody interaction studies it is usual to immobilize the (smaller) antigens and inject the (larger) antibodies as soluble analytes (Altschuh et al., 1992; Brigham-Burke et al., 1992; Lemmon et al., 1994; Wu et al., 1995; Chao et al., 1996; Lessard et al., 1996; Tamamura et al., 1996; England et al., 1997; Zeder-Lutz et al., 1997). Whenever this is not suitable, alternative SPR approaches are employed, such as multistep sandwich (Huyer et al., 1995; Cheskis and Freedman, 1996; Lookene et al., 1996; Shen et al., 1996) or indirect competitive analysis (Lasonder et al., 1994, 1996; Karlsson, 1994; Zeder-Lutz et al., 1995; Nieba et al., 1996). However, for antigen-antibody interaction studies one often aims to screen a high number of analogs against a small set of specific monoclonals. Thus, antibody immobilization is more appropriate, both from practical and analytical (comparability and reproducibility) points of view. Additionally, the size of antibodies makes them more prone to engage in steric hindrance and mass-transport effects that affect true binding kinetics.

The Basic Protocol in this unit is suited for direct single-step surface plasmon resonance (SPR) analysis of small ligand–large receptor interactions, where small peptides are used as analytes (injected in the continuous buffer flow) and monoclonal antibodies (MAbs) are immobilized on the SPR sensor chip surface. The Alternate Protocol is included for situations where kinetic analysis is not possible and uses a surface competition assay to indirectly measure the kinetics of small analyte binding.

DIRECT ASSAY FOR KINETICS OF SMALL PEPTIDE BINDING TO IMMOBILIZED MONOCLONAL ANTIBODIES USING AN SPR BIOSENSOR

BASIC PROTOCOL

The SPR assay described in this protocol is suited for the direct kinetic analysis of small viral peptides interacting with immobilized monoclonal antibodies (MAbs) and has been optimized and validated using foot-and-mouth disease virus (FMDV) peptides and anti-FMDV neutralizing MAb as binding partners (Gomes et al., 2000 a,b; Gomes et al., 2001a). Binding is detected due to changes in mass near the sensing surface where one of the binding partners has been previously immobilized (Fig. 18.9.1A). Real-time monitoring of the binding response (in resonance units, RU) provides a means to assess binding kinetics (Fig. 18.9.1B). This assay has been optimized and validated in a first-generation SPR biosensor, suited for the detection of analytes with molecular weights over 5 kDa. While the general approach in this type of instrumentation would be peptide immobilization followed by injection of MAb (or Fab) solutions, this is advisable neither from a practical nor an analytical point of view, because: (1) production, purification, and storage are more complex processes for MAb/Fab than for small peptide

Contributed by David Andreu and Paula Gomes
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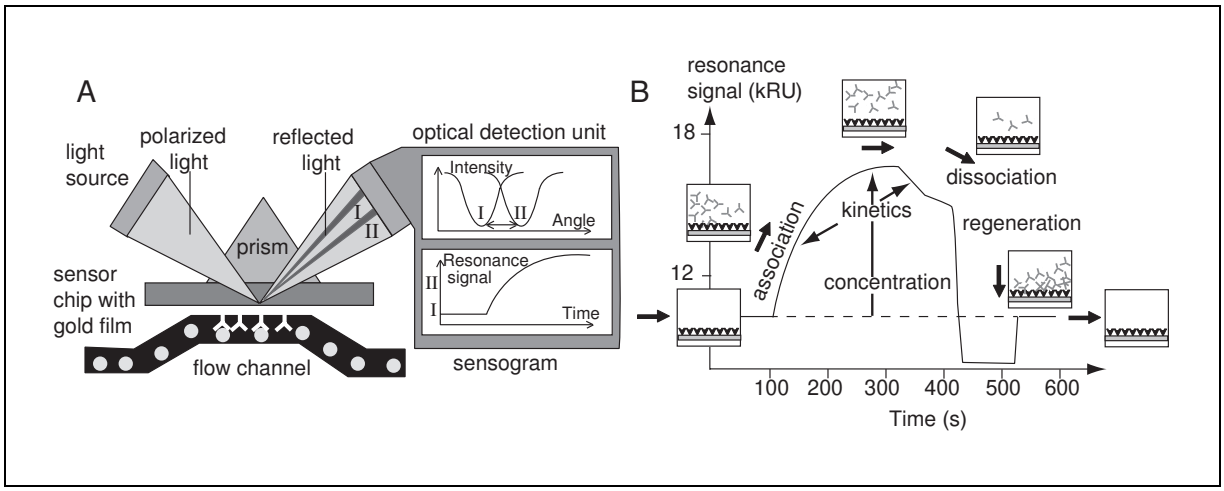

Figure 18.9.1 General schematic diagrams of SPR biosensor detection and monitoring of biospecific interactions (taken from *http://www.biacore.com/*): (**A**) Detection principle. (**B**) Sensorgram.

samples; (2) screening of high numbers of peptides against a small panel of MAbs is more common than vice versa; (3) antigenic ranking of peptide analogs is meaningful only if they are analyzed under identical conditions (e.g., using the same MAb surface); (4) reproducibility in peptide immobilization procedures is difficult to control; and (5) if used as a soluble analyte, the large size of MAb/Fab may give rise to mass transport limitations affecting true binding kinetics.

Assuming the size limitations associated with analyte detection by SPR, the authors' experimental setup relies on high peptide concentrations (up to 2.5 μM) and medium MAb surface densities (about 1.5 ng/mm^2) to ensure measurable binding levels. Also, fast buffer-flow rates (60 μl/min) are required to minimize diffusion-controlled kinetics and obtain reliable quantitative data (see Critical Parameters and Troubleshooting). Good reproducibility in kinetic parameters is observed under these analysis conditions (standard deviations below 10% of the mean values; see Tables 18.9.1 and 18.9.2) for systems with measurable binding kinetics (in the authors' case, measurable kinetic constants have ranged as follows: $3\times10^4 \le k_a \le 6\times10^5\ M^{-1}sec^{-1}$, $2\times10^{-4} \le k_d \le 5\times10^{-2}\ sec^{-1}$). Nonspecific peptide binding is practically negligible and can be suppressed upon subtraction of responses given by negative control peptides (e.g., random sequence peptide). Application of this protocol to determine the antigenic ranking of viral peptides shows an excellent agreement with ELISA assays on the same peptide/antibody systems (see Anticipated Results).

Materials

HBS-EP running buffer (BIACORE BR-1001-88; 6 × 200 ml; also see recipe)
Monoclonal antibody solutions (see recipe)
Immobilization buffers (see recipe)
Amine coupling kit (BIACORE BR-1000-50, for 50 immobilizations):
  750 mg *N*-ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDC)
  115 mg *N*-hydroxysuccinimide (NHS)
  10.5 ml ethanolamine hydrochloride
Regenerating solutions for MAb surface (see recipe), e.g., 50 mM HCl or 10 mM NaOH
BIAnormalizing solution (BIACORE BR-1003-22, 90 ml): for normalization of BIACORE probe signal
Peptide solutions (see recipe)
Scrambled peptide (i.e., same mol. wt. and amino acid composition as peptide of interest, but with randomized sequence) as negative control (prepare as described in recipe for peptide solutions)

Table 18.9.1 Kinetic and Affinity Data from the SPR Analysis of the Interaction Between Peptide A15[a] and MAb SD6[b]

Curve fitting[c]	[peptide] (nM)	k_a ($M^{-1}sec^{-1}$)	$k_{d\ (sec^{-1})}$	K_A (M^{-1})
Global (numerical integration)	--	6.2×10^4	2.6×10^{-3}	2.3×10^7
	152	6.0×10^4	2.4×10^{-3}	2.5×10^7
	305	5.8×10^4	2.6×10^{-3}	2.3×10^7
Local, simultaneous k_a/k_d (numerical integration)	610	5.9×10^4	2.6×10^{-3}	2.3×10^7
	1220	6.1×10^4	2.7×10^{-3}	2.3×10^7
	2440	6.2×10^4	2.9×10^{-3}	2.1×10^7
	152	6.7×10^4	2.4×10^{-3}	2.8×10^7
	305	6.2×10^4	2.6×10^{-3}	2.4×10^7
Local, separate k_a/k_d (analytical integration)	610	5.5×10^4	2.6×10^{-3}	2.1×10^7
	1220	5.8×10^4	2.8×10^{-3}	2.1×10^7
	2440	5.9×10^4	2.7×10^{-3}	2.2×10^7

[a]Peptide A15, reproducing the main antigenic site (loop GH of capsid protein VP1, residues 136-150) of FMDV isolate C-S8c1, has the amino acid sequence YTASARGDLAHLTTT-amide.
[b]MAb SD6 neutralizes FMDV C-S8c1 and binds peptide A15 with high affinity.
[c]Three different curve fitting methods were tested in order to evaluate consistency of the fitted parameters.

Personal computer running Microsoft Windows '95, '98, 2000 or NT
BIACORE 1000 SPR biosensor system including:
BIACORE control 3.1 software
CM5 sensor chips, certified grade (BIACORE BR-1000-12; three-chip pack): carboxymethylated dextran matrix, with ≥ 4000 RU binding capacity for a 40 -kDa protein standard; user-defined binding specificity.
BIAevaluation 3.0 software

Prepare the system

System preparation and routine maintenance are described in detail in instrumentation manuals. Routine procedures are simple and computer-controlled through an icon-based software for Windows. Instrument control command keys (BIAcore control 3.1 software) are underlined in the following protocol steps.

1. Dock the new sensor chip, replace the HBS running buffer bottle by a fresh one and prime the system.
2. Normalize the probe signal according to the manufacturer's instructions.

Choose immobilization buffer and identify appropriate MAb concentration

Steps 3 to 6 are "pre-concentration assays," i.e., preliminary assays for determining the optimal conditions.

3. Prepare different MAb and buffer solutions to test for the best immobilizing conditions using different MAb concentrations (e.g., 5, 10, and 50 µg/ml) and immobilization buffers (e.g., 10 mM sodium formate, pH 4.5; 10 mM sodium acetate, pH 5.0; 10 mM sodium acetate, pH 5.5; and 5 mM sodium maleate, pH 5.5 to 6.0.
4. Select one out of the four independent CM5 sensor chip flow cells and set the running buffer flow rate to 5 µl/min.

Table 18.9.2 Reproducibility in Kinetic Data Obtained in the Biosensor Analysis of the Interactions Between MAb 4C4[a] and 6 FMDV Peptides[b]

Peptide	Analysis no.[c,d]	k_a ($M^{-1}sec^{-1}$)[d]	k_d (sec^{-1})[d]
A15(138D)	1	n.i.	n.i.
	2	n.i.	n.i.
	3	n.i.	n.i.
	4	n.i.	n.i.
	5	n.i.	n.i.
	6	n.i.	n.i.
	mean value (±SD)	--	--
A15(138F)	1	5.54×10^5	5.06×10^{-3}
	2	6.00×10^5	6.23×10^{-3}
	3	4.70×10^5	5.44×10^{-3}
	4	5.68×10^5	5.55×10^{-3}
	5	5.70×10^5	6.04×10^{-3}
	6*	4.90×10^5*	1.16×10^{-3}*
	mean value ±SD	$(5.5 \pm 0.5) \times 10^5$	$(5.7 \pm 0.5) \times 10^{-3}$
A15(138K)	1	3.05×10^5	2.35×10^{-2}
	2	3.62×10^5	2.33×10^{-2}
	3	3.74×10^5	2.33×10^{-2}
	4	3.78×10^5	2.40×10^{-2}
	5	3.38×10^5	2.25×10^{-2}
	6*	4.65×10^5*	2.25×10^{-2}*
	mean value ±SD	$(3.5 \pm 0.3) \times 10^5$	$(2.32 \pm 0.06) \times 10^{-2}$
A15(138R)	1*	1.06×10^5*	2.16×10^{-2}*
	2	1.46×10^5	1.94×10^{-2}
	3	1.45×10^5	1.88×10^{-2}
	4	1.51×10^5	2.08×10^{-2}
	5	1.42×10^5	1.94×10^{-2}
	6	1.39×10^5	1.88×10^{-2}
	mean value ±SD	$(1.45 \pm 0.05) \times 10^5$	$(1.94 \pm 0.07) \times 10^{-2}$
A15(138V)	1*	2.56×10^5*	6.59×10^{-4}*
	2	2.90×10^5	1.34×10^{-3}
	3	2.49×10^5	1.15×10^{-3}
	4	2.67×10^5	1.34×10^{-3}
	5	2.72×10^5	1.60×10^{-3}
	6	2.83×10^5	1.62×10^{-3}
	mean value ±SD	$(2.7 \pm 0.2) \times 10^5$	$(1.4 \pm 0.2) \times 10^{-3}$

continued

Table 18.9.2 Reproducibility in Kinetic Data Obtained in the Biosensor Analysis of the Interactions Between MAb 4C4[a] and 6 FMDV Peptides[b],*continued*

Peptide	Analysis no.[c,d]	k_a ($M^{-1}sec^{-1}$)[d]	k_d (sec^{-1})[d]
A15(138Y)	1	4.46×10^5	1.41×10^{-3}
	2	3.99×10^5	1.32×10^{-3}
	3	4.05×10^5	1.12×10^{-3}
	4	3.82×10^5	1.41×10^{-3}
	5	3.80×10^5	1.48×10^{-3}
	6	3.82×10^5	1.34×10^{-3}
	mean value ±SD	$(4.0 \pm 0.3) \times 10^5$	$(1.3 \pm 0.1) \times 10^{-3}$

[a]MAb 4C4 neutralizes FMDV C-S8c1 and binds peptide A15 with high affinity.
[b]Peptides are single point mutants of A15 (Table 18.9.1, footnote *a*); the position and replacing residue are indicated in parentheses.
[c]Each peptide was characterized in 6 independent analyses.
[d]Data marked with asterisks (*) were not considered for calculating mean and standard deviation values; "n.i." signifies that no interaction could be reliably measured.

5. Inject, sequentially, 25 µl of each of the different MAb solutions prepared in step 3 (5-min injections), with short (1-min) pulses (i.e., injections) of 1 M ethanolamine hydrochloride, pH 8.5, between injections.

6. Examine carefully to see which combination of MAb concentration and immobilization buffer pH is most suitable for efficient ligand electrostatic pre-concentration on the sensor chip surface.

   This corresponds to the lowest ligand concentration and to the highest pH giving maximum response. Pre-concentration levels are evaluated by subtracting the initial baseline level from the final response levels (also see step 12) after every MAb solution injection (i.e., before each ethanolamine-regenerating pulse).

   Immobilization conditions leading to extremely high MAb attachment rates (steep ascent) should be avoided (see Critical Parameters and Troubleshooting and see Anticipated Results).

Immobilize MAb by covalent amine coupling

The amine coupling procedure involves chemical activation of the CM5 surface carboxyl groups and subsequent covalent binding to the MAb primary amino groups.

7. Prepare the activating mixture by mixing 35 µl of 0.05 M NHS with 35 µl of 0.2 EDC.

   NHS and EDC solutions (provided with BIACORE amine coupling kit) must be stored separately below 0°C, and should be mixed immediately before usage.

8. Select the flow cell and set the running buffer flow rate to 5 µl/min.

9. Inject 35 µl (7 min) of the activating mixture prepared in step 7

   A square-wave-like response curve will be observed due to the refractive index change caused by the EDC/NHS mixture.

10. Immobilize the ligand by injecting 35 µl (7 min) of the MAb solution chosen in the pre-concentration assays (step 6).

Carefully follow the slope of response ascent and the maximum level reached; injection can be interrupted if immobilization responses are unexpectedly high; conversely, repeated MAb injections can be performed at this stage, if immobilization responses are unexpectedly low. However, it is more advisable to re-evaluate immobilization conditions rather than resort to these improvised procedures.

11. Block the nonreacted surface active sites by injecting 35 µl (7 min) of 1 M ethanolamine hydrochloride adjusted to pH 8.5.

    This will also serve to break remaining ligand-surface electrostatic bonds; ethanolamine causes a resonance response similar to that referred in the annotation to step 9 for EDC/NHS.

12. Measure the amount of immobilized ligand by subtracting the initial ("empty" flow cell) from the final baseline level.

    One thousand resonance units (1000 RU) correspond to a 1 ng/mm^2 ligand surface density.

    When performing kinetic analyses, ligand density should be as low as possible, provided signal-to-noise ratios are adequate. Direct detection of small peptide antigens (~1.5 kDa) binding to MAbs (~150 kDa) on a BIACORE 1000 requires immobilization levels of ~1800 RU.

13. Test the regeneration conditions of the surface by repeated cycles of analyte injection (e.g, 25 µl of a 600 nM solution of the native peptide specific for the immobilized MAb) followed by a short pulse (1 to 3 min) of a regenerating solution.

    A suitable regenerating agent provides full recovery of baseline level at the end of each cycle while preserving ligand activity (checked by constancy of analyte binding level over repeated cycles).

Assay peptide binding to the immobilized MAb

14. Dock the sensor chip containing the immobilized MAb, replace the HBS bottle with a new one, prime the system, and normalize the probe signal according to the manufacturer's instructions.

15. Prepare solutions of the peptide of interest that are to be injected over the MAb surface by dilution of peptide stock solutions in HBS-EP running buffer (also see recipe for peptide solutions in Reagents and Solutions).

    Each peptide should be analyzed at least at six different concentrations (each corresponding to one injection cycle as described in step 20, below); each measurement takes ~30 min and should be run at least in triplicate; injections should preferably follow a random order. Flush the system whenever a new peptide is to be screened and prime the system once a day.

    Six or seven different peptide concentrations, e.g., a dilution series ranging from 2500 to 20 nM in HBS-EP running buffer, will suffice.

16. Prepare solutions of the scrambled peptide that are to be injected as negative controls (nonspecific binding).

    A dilution series must be prepared as in step 15 (also see recipe for peptide solutions in Reagents and Solutions) for a nonspecific (scrambled sequence) peptide.

17. Include one blank sample (HBS-EP running buffer only) and a negative control analyte (e.g., scrambled peptide) in the analyses.

18. Prepare the regeneration solution chosen in step 13.

19. Set the running buffer flow rate to 60 µl/min on the flow cell containing the immobilized MAb.

    Kinetic analyses require buffer flow rates higher than 30 µl/min to avoid diffusion-controlled kinetics.

20. Program the injection cycle:

    a. Use the the kinject command (minimizes sample dispersion and provides user-defined dissociation times in running buffer) and needle-cleaning operations (predip needle before analyte injection and extra clean-up after regeneration to avoid carry-over).

    Each cycle comprises two main steps:

    b. kinject 90 µl (1.5 min) of sample solution followed by 4 min (240 sec) dissociation in running buffer;
    c. Inject 60 µl (1 min) of the regenerating solution.

Process raw data and analyze results

Data processing is done by means of the BIAevaluation software. Experimental curves (i.e., sensorgrams) corresponding to the same peptide (at different concentrations) are simultaneously processed. The software includes several kinetic models and nonlinear least squares methods to optimize parameter values. Simple kinetic models perfectly described by integrated rate equations use analytical integration, while more complex ones (e.g., involving mass transport limitations, ligand or analyte heterogeneity, conformational changes, analyte multivalency, or ligand cooperativity) use numerical integration.

21. Open a new BIAevaluation file and, from there, access all the experimental curves corresponding to all concentrations injected for given peptide on a given MAb surface. From the same file, open the experimental curves corresponding to the blank run and to the negative-control peptide injections on the same MAb surface.
22. Adjust the time scale (X-transform) so that $t = 0$ (injection start) is the same for all curves and the baseline level (Y-transform) equals 0 RU in all sensorgrams.
23. Delete the useless parts of the sensorgrams (e.g., cut the regeneration pulses), then subtract the blank run (Y-transform) curve from all the others to eliminate buffer response and instrumental drifts or artifacts.
24. Subtract (Y-transform) from each peptide concentration curve the corresponding one from the scrambled peptide, to eliminate nonspecific binding.
25. Fit the set of binding curves by global curve fitting to those kinetic models compatible with your system. Judge which one gives the best fit and the most reliable parameters.

    A 1:1 Langmuirian behavior—pseudo–first order reaction—should be expected for the interaction between each antigen molecule and each of the two equivalent Fabs on the immobilized MAb (see the Appendix at the end of this unit).

    The fitting models are based on "blind" mathematical tools and the "best fit" depends on the ability of the fitting algorithm to converge for the true minimum and on the number of parameters that can be varied in the model, i.e., the complexity of the model (see Critical Parameters and Troubleshooting). Therefore, caution must be taken when judging the "best fit" from a purely mathematical point of view. Generally, the best choice is the simplest model of those giving reasonably good fits.

    Once the "best fit" is chosen, a further detailed evaluation should be performed in order to establish data consistency (Shuck and Minton, 1996). Different zones of the experimental curves should be used for fitting purposes; local fittings (each sensorgram separately) should be done and compared with globally fitted data; when applicable, analytical integration methods (separate fitting of association and dissociation phases) should be tested and compared with numerical integration methods. This means that, for a 1:1 interaction (pseudo-first-order kinetics), data should be fitted as follows: (1) global fitting

to the 1:1 interaction model (numerical integration); (2) local fitting (each concentration separately) to the 1:1 interaction model (numerical integration); (3) local fitting, separate k_a/k_d (analytical integration in each one of the separate association and dissociation phases). If kinetic parameters are consistent throughout all these fits, the kinetic model chosen is most probably correct and interaction data are meaningful.

ALTERNATE PROTOCOL

ALTERNATIVE KINETIC ANALYSIS OF SMALL PEPTIDE/LARGE ANTIGEN COMPETITION ON THE ANTIBODY SURFACE

As mentioned before, direct SPR detection of small analytes (mol. wt. <2 kDa) is often difficult, and such analytes often lack the multiple independent binding sites necessary for response enhancement with sandwich techniques. When the direct approach for kinetic analysis is not applicable, a possible alternative is the surface competition assay, where a high-molecular-weight competitor (HMWC) is employed to compete with the small target analyte for the same ligand binding site (Fig. 18.9.2A). Since response due to small analyte binding is unappreciable, only the response from the HMWC is monitored. Thus, the effect on the kinetics of macromolecule binding due to addition of the small competing analyte can be measured, and the kinetics of small analyte binding can be indirectly determined.

This alternative method follows the Basic Protocol through step 13, the only exception being that the amount of immobilized antibody should be adjusted (decreased) taking into account the larger size, and thus the higher responses, of the detected analyte (i.e., the HMWC). Subsequent steps also resemble those in the Basic Protocol, with the most significant modifications directed to sample concentration and composition.

Additional Materials *(also see Basic Protocol)*

High-molecular-weight competitor (HMWC) solutions (see recipe)
Peptide–HMWC mixtures (see recipe)

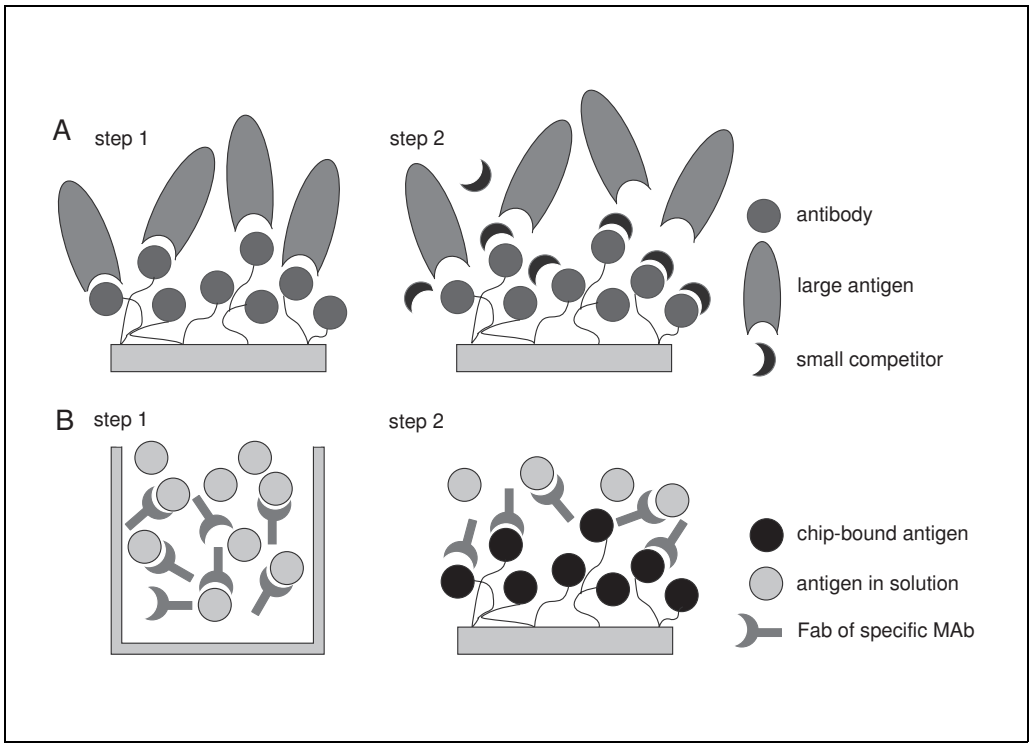

Figure 18.9.2 Indirect biospecific interaction analysis by SPR: (**A**) Surface competition assay. (**B**) Solution affinity assay.

1. Perform steps 1 to 13 of the Basic Protocol, except adjust MAb immobilization to a lower level as described below.

   When performing kinetic analyses, ligand density should be as low as possible, provided signal-to-noise ratios are adequate. Direct detection of HMWC (>5 kDa) binding to MAbs (~150 kDa) on a Biacore 1000 generally does not require more than 300 to 600 RU for immobilization levels.

   Immobilization levels are adjusted as in the pre-concentration steps (see Basic Protocol, steps 3 to 6). Amount of bound MAb can be decreased in several ways: by diluting MAb samples or decreasing MAb-surface contact times or by lowering pH or increasing the ionic strength of the MAb solution (see Critical Parameters and Troubleshooting).

2. Assay kinetics of HMWC binding to the immobilized MAb as follows:

   a. Perform steps 14 to 20 of the Basic Protocol, using HMWC solutions (10 to 300 nM) instead of the peptide solutions.

   b. Do not prepare a negative control peptide but inject HMWC samples (following steps 1 to 20 of the Basic Protocol) on a nonspecific mock surface under identical conditions to those used for injecting on the MAb-coated surface.

      Ideally, a mock surface should be coated with a MAb unrelated to the antibody-antigen systems under study, in order to reproduce as closely as possible the coating properties of the specific surface.

3. Process raw data and analyze results for HMWC–MAb interactions by peforming steps 21 to 25 of the Basic Protocol, taking into consideration differences in negative control procedures.

   In step 24 in the Basic Protocol, HMWC binding curves from the nonspecific surface (see step 2b of this protocol) must be subtracted (Y-transform) from the corresponding binding curves for the specific MAb surface.

4. Perform surface competition assays with HMWC–peptide mixtures by again carrying out steps 14 to 20 of the Basic Protocol, replacing the HMWC samples used previously (step 2a) with peptide–HMWC mixtures prepared as described in Reagents and Solutions. Again, use the mock surface (step 2b) for negative control injections.

   In the relevant peptide series, include one for the random-sequence peptide as a non-competitor control.

5. Process raw data and analyze results for HMWC–peptide competitions by performing steps 21 to 25 of the Basic Protocol, taking into account that data must now be fitted using the heterogeneous analyte kinetic model in the BIAevaluation software; see the Appendix at the end of this unit.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***APPENDIX 5****.*

HBS-EP running buffer

10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)
0.15 M NaCl
3.4 mM EDTA
0.005% (v/v) surfactant P20 (Tween 20)

HBS-EP running buffer may also be purchased from BIACORE (cat. no. BR-1001-88).

High-molecular-weight competitor (HWMC) solutions

Purified HMWC (e.g., native viral protein bearing the exposed antigenic site) in PBS (*APPENDIX 2A*) can be used as stock solutions for subsequent dilution in the immobilization buffer (see recipe); generally, HMWC stock solutions correspond to ~0.5 mg protein per ml of PBS and are diluted to ~300 nM in HBS-EP running buffer. Serial dilutions are subsequently made for SPR analysis.

Immobilization buffers

10 mM sodium acetate buffer, pH 5.5, is usually suitable for MAb immobilization by amine coupling onto a CM5 chip. Common immobilization buffers for CM5 are 10 mM sodium formate (pH 3.0 to 4.5), 10 mM sodium acetate (pH 4.0 to 5.5), and 5 mM sodium maleate (pH 5.5 to 6.0). Buffer solutions are evaluated in pre-concentration assays (see Basic Protocol and see Critical Parameters and Troubleshooting).

Monoclonal antibody solutions

Purified MAbs (*UNIT 2.5*) in PBS (*APPENDIX 2A*) can be used as stock solutions for subsequent dilution in the immobilization buffer (see recipe). MAb stock solutions usually contain ~20 mg antibody per ml PBS and are diluted to ~5 µg/ml in the immobilization buffer chosen.

Peptide-HMWC mixtures

Mix peptide solutions (see recipe) with HMWC solutions (see recipe) in different proportions, keeping the total HMWC concentration constant at ~75 nM, so that competitor peptide concentrations in the mixture range from 0 to 150 nM.

The same peptide solutions (see recipe) as in the Basic Protocol (2.5 mM stock solutions in water or 100 mM acetic acid) can be used for ~8000-fold and subsequent serial dilutions in HBS-EP running buffer. Thus, peptide dilutions in HBS now range from ~300 to 0 nM (instead of the 2500 to 20 nM dilutions specified in the recipe below, which are used in the Basic Protocol).

Peptide solutions

Peptide 2.5 mM stock solutions in water or 100 mM acetic acid can be prepared for 1000-fold and subsequent serial dilutions in HBS-EP running buffer (see recipe). Thus, peptide solutions injected on the biosensor typically range from 2500 to 20 nM in HBS-EP running buffer.

Regenerating solutions

Common regenerating agents in SPR are acids (10 to 100 mM HCl or H_3PO_4), bases (10 to 100 mM NaOH), salts (1 to 5 mM NaCl), detergents (0.5% w/v SDS), and denaturants (8 M urea, 6 M guanidine hydrochloride). However, one must bear in mind that MAb surfaces are delicate.

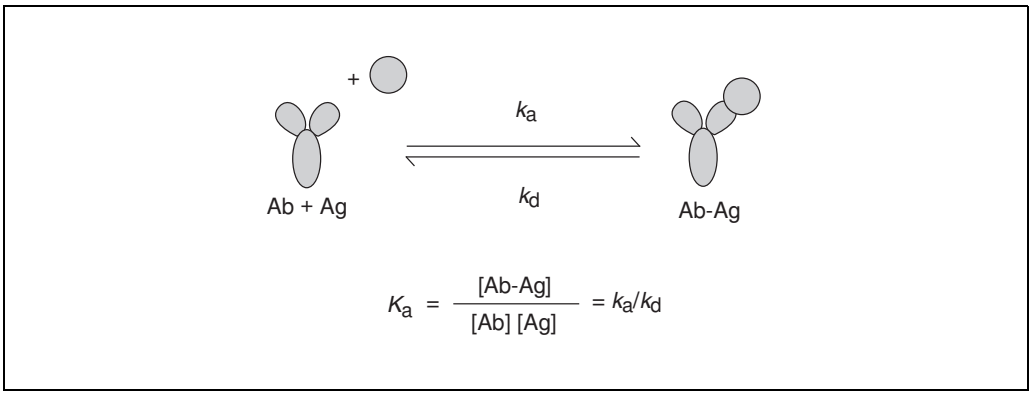

Figure 18.9.3 Schematic definition of the affinity constant in an antibody-antigen interaction.

COMMENTARY

Background Information

Kinetic constants measurable by direct SPR

Antibody affinity is defined as depicted in Figure 18.9.3. Normally, IgGs have one effective binding site if an affinity interaction is taking place. Thus, for a monovalent antigen, 1:1 affinity binding is to be expected. When both Fab fragments of the same IgG molecule interact with a multivalent antigen, then an avidity interaction is taking place and higher stabilization of the Ab-Ag antibody is observed. Avidity phenomena are extremely important and must be considered for multivalent antigens (natural antigens binding to several Ig molecules and forming the so-called immune complexes) and for multivalent Ig molecules, such as IgM (Abbas et al., 1997). In the case of antibody–natural antigen interactions, equilibrium constants usually range from 10^7 to 10^{10} M^{-1}, and immunoglobulins with $K_A \leq 10^4$ M^{-1} for a particular antigen are ineffective.

The range of rate constants amenable to study by SPR varies with the molecular weight of the analyte and with the sensitivity of the particular biosensor employed. BIACORE 1000 is suitable to determine k_a between 10^3 and 10^6 $M^{-1}sec^{-1}$ and k_d between 10^{-5} and 10^{-1} sec^{-1} for large analytes (Hall et al., 1996). These limits are narrowed when molecular weight decreases, and the authors of this unit have observed that quantitative kinetic studies of small peptides (~10^3 Da) are only possible for interactions with k_a between 10^4 to 5×10^5 $M^{-1}sec^{-1}$ and k_d between 10^{-4} and 5×10^{-2} sec^{-1} (Hall et al., 1996). This creates some difficulties in kinetic analysis of peptide-antibody interactions. Direct quantitative evaluation of interaction kinetics outside these limits is often impossible, even when raw data are processed by numerical integration and global fit. In such cases, alternative methodologies might be required, often entailing loss of kinetic information (e.g., only solution affinity experiments can be carried out). Indeed, most of these alternative methodologies have limitations concerning reliability or even applicability of SPR as a tool for kinetic studies. The best alternative for direct kinetic analysis of small analyte binding is the surface competition assay described here as the Alternate Protocol (Karlsson, 1994). However, even this method has an important limitation: macromolecules sharing the same binding specificity with the small target analyte (e.g., viral proteins) are not easily available. Other alternative SPR methodologies are briefly commented upon below.

Direct multi-step approach

This consists of ligand immobilization followed by a first injection of the specific analyte, and, subsequently, by the injection of a second binding partner (which binds the first analyte); each binding stage is monitored in real time. This approach is often employed for binding site analysis (Dubs et al., 1992) and analyte response enhancement (Van Regenmortel et al., 1994), but is not applicable for small analytes lacking multiple binding sites. One possible way to circumvent this limitation is analyte biotinylation, where, in a first step, the biotinylated small analyte is allowed to bind the immobilized receptor, and in a second step streptavidin is injected so that a response can be detected (due to analyte–biotin–streptavidin binding). This tactic poses new questions regarding, for instance, conservation of analyte biospecificity and interaction kinetics after biotinylation, or the reliability of kinetic parameters which are now influenced by streptavidin-biotin interactions.

Solution affinity experiments

This approach is widely employed for small analyte detection, but it does not provide kinetic information. Solution affinity experiments resemble competition ELISA in the sense that a suitable analyte (e.g., native peptide antigen) is immobilized on the sensor surface and preincubated mixtures of analyte-receptor (e.g., other peptide antigens plus specific antibody) are injected. Incubating variable analyte concentrations with a constant receptor concentration allows building of inhibition curves (i.e., free receptor concentration versus analyte concentration), from which binding constants can be obtained (Nieba et al., 1996; Gomes et al., 2001b, 2002).

Alternative ligand immobilization procedures

Amine coupling chemistry has so far been adequate for MAb covalent immobilization in MAb-peptide interaction studies by SPR. However, this coupling chemistry shares with some other immobilization procedures (thiol and aldehyde coupling chemistries; O'Shannessy et al., 1992) the problem of being a random process (Kortt et al., 1997; Catimel et al., 1997) in which ligand can bind the activated surface through several reactive groups (primary amino groups in the case of amine coupling), with different possible orientations. This implies an heterogeneous ligand surface, with such heterogeneity possibly affecting kinetic parameters measured on this surface. On the other hand, covalent coupling chemistries may cause loss of ligand bioactivity if groups reactive for covalent coupling are placed in a biologically relevant region. Problems inherent in random covalent immobilization strategies can be avoided through affinity capture procedures. In the case of MAb immobilization, affinity capture consists of two main steps: standard amine coupling immobilization of a goat or rabbit anti–mouse-Fc antibody followed by injection of the relevant MAb, which is then biospecifically captured (Karlsson and Fält, 1997). Although the first immobilization step (anti-Fc antibody) is also a randomly oriented process, all molecules of the relevant MAb will be immobilized through their Fc region, so that Fab regions are free for antigen binding. Even so, this affinity capture approach still has two main limitations: (1) as in random coupling chemistries, if too much antibody is immobilized, then not all MAb molecules within the dextran layer will be equally accessible for antigen binding, which is a source of heterogeneity; and (2) when the relevant ligand is not covalently linked to the dextran matrix, surface decay occurs and this requires complex mathematical models for correct interpretation of kinetic rate constants (Joss et al., 1998).

In summary, and taking the authors' own experience into account, amine coupling chemistry remains one of the best MAb immobilization strategies, as long as surface density of MAb (i.e., immobilization level) is kept at the minimum possible, so that heterogeneity effects are minimized.

Critical Parameters and Troubleshooting

Immobilization levels

Immobilization levels depend on five main factors, i.e., (1) ligand concentration, (2) pH, (3) activation time (EDC/NHS mixture), (4) injection time (ligand), and (5) ionic strength. Lower ligand binding levels can be reached by decreasing the first four factors or by increasing the fifth. Conversely, higher concentrations and higher activation or contact times, as well as lower ionic strength, contribute to increased ligand immobilization levels.

Baseline levels

For the present protocols only covalent immobilization of the biospecific ligand (MAb) was considered (amine coupling chemistry). Thus, baseline level decrease over repeated cycles, which is often a problem in affinity capture immobilization strategies (e.g., anti-Fc antibodies for MAb capture), is not to be expected. However, baseline increase over repeated cycles can be observed. Inefficient regeneration steps are often to blame, with bound analyte not being fully washed off after each binding cycle; alternative regeneration agents must be tested and a cocktail approach (Andersson et al., 1999 a,b) may be required.

Binding levels

Absence or gradual loss of an expected biospecific response can be observed. This problem is due to ligand inactivation under the analysis conditions employed (e.g., inadequate buffers or regeneration agents) or to blockage of ligand binding sites (extremely strong analyte-ligand interactions, ineffective regeneration steps). Different immobilization methodologies (e.g., thiol coupling or affinity capture) or regeneration conditions may solve the problem.

Buffer refractive index response

When sample and running buffers are different, nonspecific bulk refractive index (RI) jumps take place (square-wave shaped signals superim-

pose to the binding curves). Such bulk RI response may be eliminated by subtraction of a blank run, but useful information from stages immediately after the injection pulse may be lost. Thus, sample buffer should resemble the running buffer as closely as possible. Beware of "exotic" buffers, especially those including organic solvents; these can cause dramatic compression effects on the dextran hydrogel matrix, preventing binding phenomena from occurring.

Nonspecific binding

Nonspecific binding may become a problem when using inadequately purified samples, such as cell lysates or hybridomas. In antigen-antibody systems where purified peptide and MAb samples are employed, significant nonspecific responses are not to be expected. Nevertheless, nonspecific binding should be checked by one of the following methods.

(1) Perform the sample injection on both the specific cell and a reference cell. This reference cell must be prepared in a manner as similar as possible to that used for the specific one (e.g., using same coupling chemistry to immobilize a similar amount of inactivated ligand).

(2) Inject a nonspecific analyte (e.g., a peptide with randomized sequence). This approach may per-

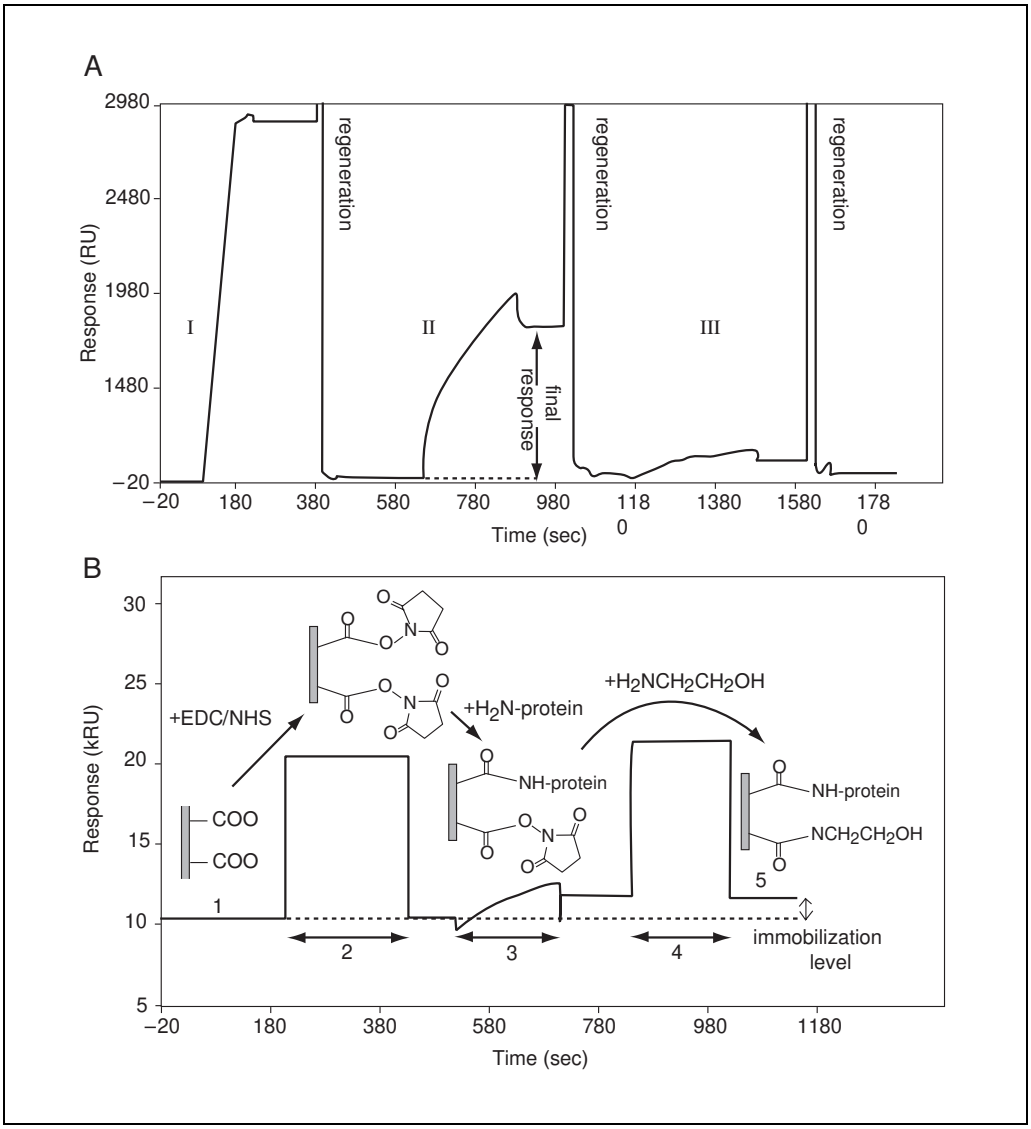

Figure 18.9.4 Stages involved in the covalent immobilization of MAb on the chip: (**A**) Three sequential pre-concentration assays: **I**, 50 μg/ml MAb in 10 mM acetate buffer, pH 5.5; **II**, 5 μg/ml MAb in 10 mM acetate buffer, pH 5.5; **III**, 5 μg/ml MAb in 10 mM formate buffer, pH 4.5. (**B**) Steps in covalent MAb immobilization via amine coupling: **1**, free carboxymethyl dextran layer; **2**, activation of carboxyl groups with an EDC/NHS mixture; **3**, injection of MAb solution; **4**, unreacted groups blocked by ethanolamine injection; **5**, final MAb surface.

haps be more appropriate than method (1) and is described in the Basic Protocol.

Fitting data

Antigen-antibody (Fab) interactions are expected to display a Langmuirian behavior on the biosensor. Deviations from pseudo-first-order kinetics, one of the most difficult problems to solve in biosensor analysis (Morton et al., 1995; O'Shannessy and Windzor, 1996; Hall et al., 1996), can result from several factors. Whatever they may be (see below), one must keep in mind that, for kinetic studies, mass transport effects must be minimized. This can be achieved by decreasing ligand immobilization levels (to the minimum giving a satisfactory signal-to-noise ratio), increasing buffer flow rate (higher than 30 µl/min), and increasing analyte concentration (as long as surface capacity is not saturated). Mass transport limitations can be tested through analysis of effects of different buffer flow rates on initial binding rates (slopes at the beginning of association steps). Another precaution aimed at eliminating mass transport effects in complex dissociation (i.e., rebinding) consists of replacing buffer with ligand solution during the dissociation phase.

Other common sources of deviation are ligand or analyte heterogeneity. The first is mainly due to random immobilization procedures and can be minimized by lowering binding levels or using oriented methodologies such as streptavidin–biotin or anti-Fc–Fc indirect immobilization. Analyte heterogeneity can be reduced through additional sample purification steps.

The sources of deviation most difficult to deal with are those intrinsic to the binding partners or phenomena, such as analyte multivalency, avidity, or complex binding mechanisms (e.g., involving conformational changes). When these effects are present, the only way to take them into account is to use the more complex fitting models, although it may be difficult to judge whether a better fitting model corresponds to the real interaction mechanism (Schuck, 1997).

Anticipated Results

Pre-concentration assays and antibody immobilization

Figure 18.9.4 illustrates the results of three sequential pre-concentration assays (Fig. 18.9.4A) followed by covalent immobilization of antibody under the selected conditions (Fig. 18.9.4B). Using ~1700 RU as a reasonable immobilization level for the direct kinetic assay of small peptide binding to an antibody surface, situation II in Fig. 18.9.4A (5 µg/ml MAb in 10 mM acetate buffer, pH 5.5) is clearly the most satisfactory. In I (50 µg/ml MAb in 10 mM acetate buffer, pH 5.5) a fast MAb uptake by the surface results in a too high MAb final density, while in situation III (5 µg/ml MAb in 10 mM formate buffer, pH 4.5) MAb response increases rather slowly and the final MAb level is insufficient. Choosing conditions as in II, a standard ligand covalent immobilization can be performed and monitored by SPR (Fig. 18.9.4B). The free carboxylate groups (1) in the dextran hydrogel matrix of chip CM5 are activated by injection of an EDC/NHS mixture, and an increase in the SPR signal is observed (2) due to refractive index changes in the sensing area. The MAb solution is then injected and the binding event (3) can be followed in real time. Once the adequate binding level is reached, the remaining active carboxyl-NHS esters are blocked with ethanolamine hydrochloride, causing a significant change in the bulk refractive index (4). The biospecific MAb surface is then ready to be used (5).

Binding assays

Binding assays consist of sequential analyte injection plus regeneration cycles. Figure 18.9.5A shows the three main stages observed when monitoring the biospecific interaction in real time, i.e., analyte-ligand association (1), analyte dissociation from ligand in running buffer (2), and ligand surface regeneration (3). Each cycle corresponds to a new sample, so that all blanks, controls, different analyte concentrations, and assay repeats are covered. When a full set (i.e., all concentrations of a given analyte) of injection cycles is finished, the corresponding sensorgrams can be transformed in order to eliminate irrelevant regions (e.g., regeneration pulses) and to normalize the time and response axes. This results in the superposition of several sensorgrams (Fig. 18.9.5B), ready to be processed by the curve-fitting software.

Competition assays are experimentally similar to binding assays, except that target analyte plus competitor HMWC mixtures are used instead of pure analyte samples. Figs. 18.9.5C and D show sensorgrams from recent experiments at our laboratory.

Reliability and biological relevance of SPR data

As shown in Fig. 18.9.6, the authors' direct single-step approach for the SPR analysis of small peptide–immobilized MAb binding kinetics shows excellent agreement with classical

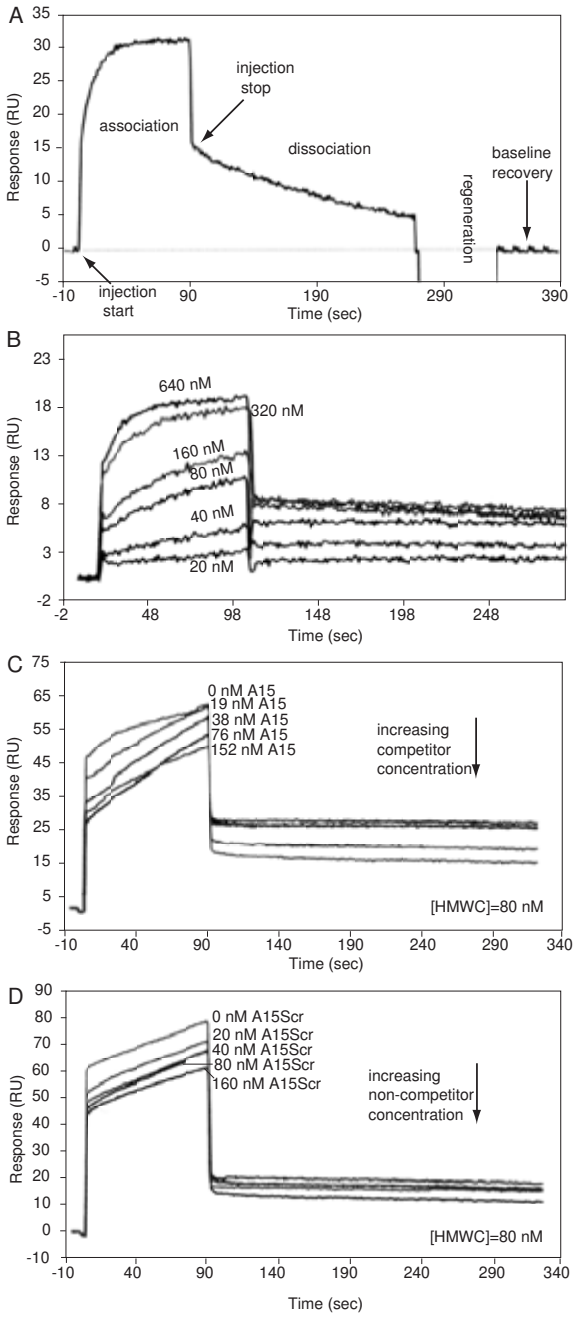

Figure 18.9.5 Experimental sensorgrams in different situations: (**A**) interaction of FMDV peptide A15 (600 nM in HBS) with immobilized MAb SD6 (see Table 18.9.1, footnotes a and b). (**B**) Full set of binding curves for the interaction between peptide A15(140P) (A15 with a Ser→Pro mutation at position 140; see Table 18.9.2, footnotes *a* and *b*) and immobilized MAb 4C4. (**C**) Surface competition assay between varying concentrations of native peptide A15 and a constant concentration of protein JX249A (an engineered β-galactosidase containing the immunodominant site of FMDV strain C-S8c1) on a 4C4 surface. The competition is shown by the fact that JX249A-antibody association and dissociation rates respectively decrease (decreasing positive slopes during first 90 sec) and increase (increasing negative slopes after 90 sec) with increasing concentrations of the target analyte (peptide A15). (D) Surface competition assay between varying concentrations of negative control peptide A15Scr (contains the same amino acid residues of A15, but randomized) and a constant concentration of antigenic protein JX249A on a 4C4 surface. In contrast to panel C, no decrease in slope during the association phase (or corresponding increase during the dissociation phase) is observed in this case.

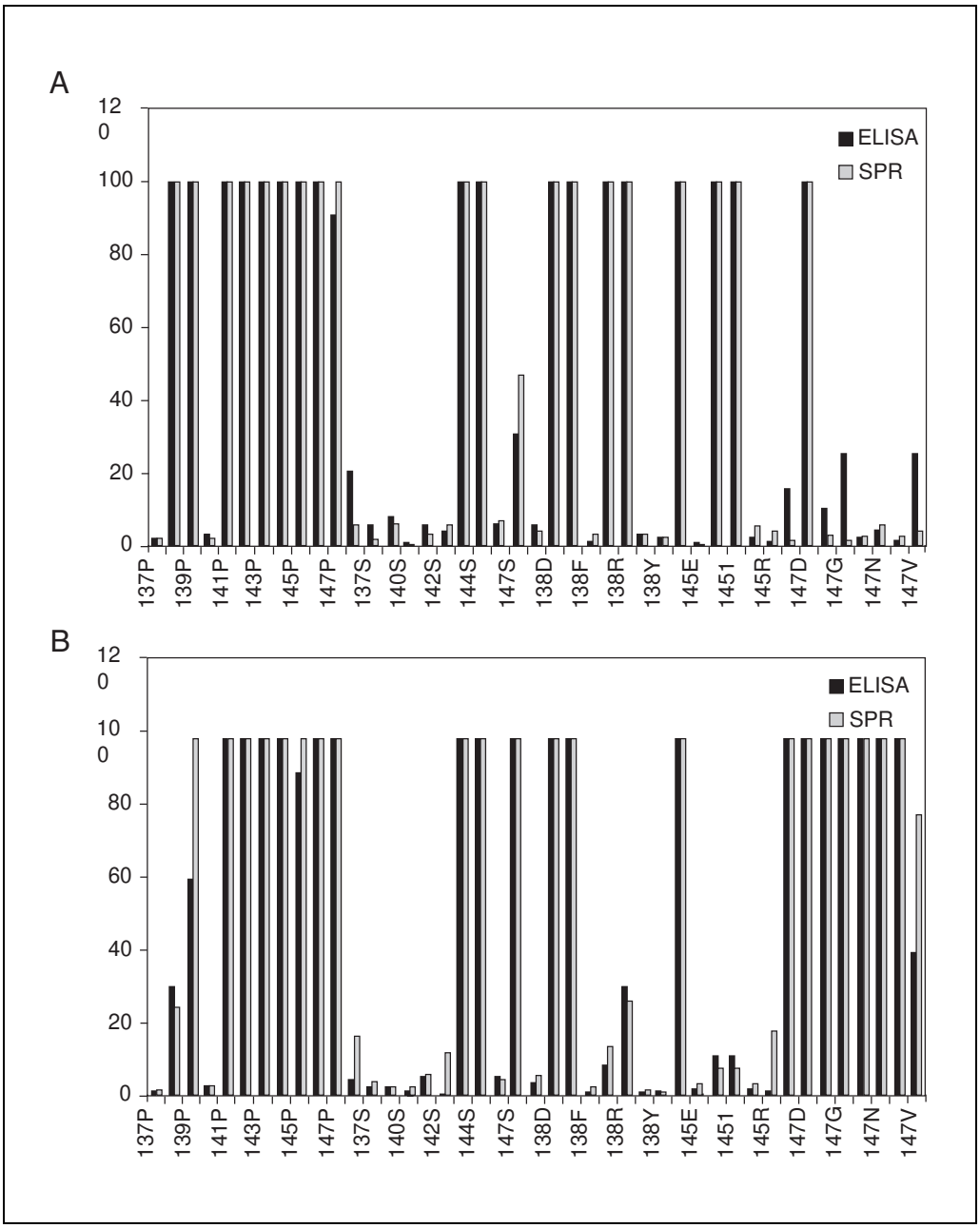

Figure 18.9.6 Comparison of antigenic ranking of FMDV peptides (series of 43 A15 single-point mutants) by SPR and ELISA: (**A**) Interaction of FMDV peptides with MAb SD6. (**B**) Interaction of FMDV peptides with MAb 4C4. On both panels, the vertical axis depicts relative IC_{50} and K_D values for ELISA and SPR data, respectively. Relative IC_{50} is defined as the quotient IC_{50}(mutant peptide)/IC_{50}(A15 peptide). Similarly, relative K_D = K_D(mutant peptide)/K_D(A15 peptide).

techniques such as competition ELISA. Peptide binding affinities to MAbs SD6 (Fig. 18.11.6A) and 4C4 (Fig. 18.11.6B) are represented as relative IC_{50} values, defined as the ratio between the IC_{50} of the peptide analyte and that of the native sequence A15. SPR data are expressed as relative K_D values, defined similarly as the ratio between the K_D of a given analyte and that of the reference A15. These SPR data fully validated previous studies on the antigenic structure of the C-S8c1 isolate of FMDV (Hernández et al., 1996; Mateu et al., 1996; Verdaguer et al., 1995, 1998).

Time Considerations

Full kinetic analysis of one peptide-antibody system on a previously prepared MAb surface requires about 2 to 3 hr, including data processing and analysis. If ligand immobi-

lization and maintenance procedures are also considered, then 4 to 5 hr will be required.

Detailed time considerations (*i.e.*, step by step) are as follows:

Pre-concentration assays: 60 min;

Covalent immobilization: 30 min;

Evaluation of regeneration conditions: 30 min;

Two blank runs: 20 min;

One analyte run (sample plus regeneration agent): 20 min;

Data analysis: 60 min (variable, depending on the quality of the fit);

System priming: 10 min;

Weekly "desorb" operation: 30 min;

Monthly "sanitize" operation: 40 min;

Signal calibration ("normalizing"): 40 min.

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O'Shannessy, D.J., Brigham-Burke, M., Soneson, K.K., Hensley, P., and Brooks, I. 1993. Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: Use of non-linear least squares methods. *Anal. Biochem.* 212:457-468.

All of the above are original papers on the fundamentals, applications, limitations and strategies for real-time biospecific interaction analysis using SPR biosensors.

Garland, P.B. 1996. Optical evanescent wave methods for the study of biomolecular interactions. *Quart. Rev. Biophys.* 29:91:117.

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Homola et al., 1999. See above.

The above are review papers on SPR biosensor technology for real-time biospecific interacion analysis.

BIAcore Instrument Handbook. 1994. Pharmacia Biosensor AB, Uppsala, Sweden.

BIAevaluation Software Handbook: version 3.0, 1997. BIAcore AB, Uppsala, Sweden.

BIApplications Handbook, 1994. Pharmacia Biosensor AB, Uppsala, Sweden.

The three publications above are key sources on biosensor technology, from the manufacturer BIOSENSOR AB (Uppsala, Sweden), including instrumentation, software, and application manuals.

Internet Resources

http://www.biacore.com

Web site of BIAcore.

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APPENDIX: RATE EQUATIONS USED FOR CURVE FITTING WITH BIAevaluation 3.0

In all of the following equations, A represents injected analyte, B represents immobilized receptor, AB represents the complex between A and B, C represents total analyte concentration, RI represents bulk refractive index, t_{on} represents the time (in sec) at the beginning of the injection pulse, R_{max} represents the maximum resonance response level (in RU), and mw represents the molecular weight.

(a) Simultaneous k_a/k_d Fit: 1:1 Langmuirian Binding

Differential equations

$d[B]/dt = -(k_a[A][B] - k_d[AB])$

$d[AB]/dt = k_a[A][B] - k_d[AB]$

Total response

[AB] + RI

Reaction scheme

$A + B \Leftrightarrow AB$

$[A] = C$, $[B]_0 = R_{max}$, $[AB]_0 = 0$

(b) Simultaneous k_a/k_d Fit: 1:1 Binding with Drifting Baseline

Differential equations

The same as in (a), above.

Total response

$[AB] + drift(t - t_{on}) + RI$

Reaction scheme

The same as in (a), above.

(c) Simultaneous k_a/k_d Fit: 1:1 Binding with Mass Transfer

Differential equations

The same as in (a), above, plus:

$d[A]/dt = k_t(C - [A]) - (k_a[A][B] - k_d[AB])$

Total response

[AB] + RI

Reaction scheme

$A_{bulk} \Leftrightarrow A + B \Leftrightarrow AB$

$[A]_{bulk} = C$, $[B]_0 = R_{max}$, $[AB]_0 = 0$

(d) Simultaneous k_a/k_d Fit: Heterogeneous Ligand (Two Different Binding Sites)

Differential equations

$d[B_1]/dt = -(k_{a1}[A][B_1] - k_{d1}[AB_1])$

$d[AB_1]/dt = k_{a1}[A][B_1] - k_{d1}[AB_1]$

$d[B_2]/dt = -(k_{a2}[A][B_2] - k_{d2}[AB_2])$

$d[AB_2]/dt = k_{a2}[A][B_2] - k_{d2}[AB_2]$

Total response

$[AB_1] + [AB_2] + RI$

Reaction scheme

$A + B_1 \Leftrightarrow AB_1$

$A + B_2 \Leftrightarrow AB_2$

$[A] = C$

$[B_1]_0 = R_{max1,}\ [B_2]_0 = R_{max2},\ [AB_1]_0 = [AB_2]_0 = 0$

(e) Simultaneous k_a/k_d Fit: Heterogeneous Analyte (Competition Between Two Different Analytes)

Differential equations

$d[B]/dt = -(k_{a1}[A_1]mw_1[B] - k_{d1}[A_1B])/mw_1n_1 - (k_{a2}[A_2]mw_2[B] - k_{d2}[A_2B])/mw_2n_2$

$d[A_1B]/dt = k_{a1}[A_1]mw_1[B] - k_{d1}[A_1B]$

$d[A_2B]/dt = k_{a2}[A_2]mw_2[B] - k_{d2}[A_2B]$

Total response

$[A_1B] + [A_2B] + RI$

Reaction scheme

$A_1 + B \Leftrightarrow A_1B$

$A_2 + B \Leftrightarrow A_2B$

$[A_1] = C_1,\ [A_2] = C_2,\ [B]_0 = R_{max}/mw_1,\ [A_1B]_0 = [A_2B]_0 = 0$

(f) Simultaneous k_a/k_d Fit: Bivalent Analyte

Differential equations

$d[B]/dt = -(k_{a1}[A][B] - k_{d1}[AB]) - (k_{a2}[AB][B] - k_{d2}[AB_2])$

$d[AB]/dt = (k_{a1}[A][B] - k_{d1}[AB]) - (k_{a2}[AB][B] - k_{d2}[AB_2])]$

$d[AB_2]/dt = k_{a2}[AB][B] - k_{d2}[AB_2]$

Total response

$[AB] + [AB_2] + RI$

Reaction scheme
$A + B \Leftrightarrow AB$

$AB + B \Leftrightarrow AB_2$

$[A] = C$, $[B]_0 = R_{max}$, $[AB]_0 = [AB_2]_0 = 0$

(g) Simultaneous k_a/k_d Fit: Conformational Change (Two-State Reaction)

Differential equations
$d[B]/dt = -(k_{a1}[A][B] - k_{d1}[AB])$

$d[AB]/dt = (k_{a1}[A][B] - k_{d1}[AB]) - (k_{a2}[AB] - k_{d2}[AB^*])$

$d[AB^*]/dt = k_{a2}[AB] - k_{d2}[AB^*]$

Total response
[AB] + [AB*] + RI

Reaction scheme
$A + B \Leftrightarrow AB \Leftrightarrow AB^*$

$[A] = C$, $[B]_0 = R_{max}$, $[AB]_0 = [AB^*]_0 = 0$

(h) Separate k_a/k_d Fit: 1:1 Langmuirian Binding

Integrated rate equations

$$R = \frac{Ck_a R_{max}[1 - e^{-[(Ck_a + k_d)t]}}{Ck_a + k_d}$$

$$R = R_0 e^{-k_d t} + \text{offset}$$

Total response
[AB] + RI

[AB] + offset

Reaction scheme
The same as in (a), above.