CHAPTER 3

Biosensor-Surface Plasmon Resonance Methods for Quantitative Analysis of Biomolecular Interactions

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Abstract

The surface plasmon resonance (SPR) biosensor method has emerged as a very flexible and powerful approach for detecting a wide diversity of biomolecular interactions. SPR monitors molecular interactions in real time and provides significant advantages over optical or calorimetric methods for systems with strong binding and low spectroscopic signals or reaction heats. The SPR method simultaneously provides kinetic and equilibrium characterization of the interactions of biomolecules. Such information is essential for development of a full understanding

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of molecular recognition as well as for areas such as the design of receptor-targeted therapeutics. This article presents basic, practical procedures for conducting SPR experiments. Initial preparation of the SPR instrument, sensor chips, and samples are described. This is followed by suggestions for experimental design, data analysis, and presentation. Steady-state and kinetic studies of some small molecule–DNA complexes are used to illustrate the capability of this technique. Examples of the agreement between biosensor-SPR and solution studies are presented.

I. Introduction

Transcriptional activators and repressors bind to DNA; drugs form complexes with membrane components and in cells they target sites on nucleic acids and proteins; antibodies bind to proteins of disease organisms; and a host of other biomolecular interactions are essential for organisms and their cells to function. To understand the functional processes that drive biological systems, it is essential to have detailed information on the array of biomolecular interactions that drive and control cellular function. The binding affinity (the equilibrium constant, K, and Gibbs energy of binding, ΔG), stoichiometry (n, the number of compounds bound to the biopolymer), cooperative effects in binding, and binding kinetics (the rate constants, k, that define the dynamics of the interaction) are the basic quantitative characteristics of all biomolecular interactions. The more of these key parameters that can be determined experimentally, the better will be our understanding of the underlying interaction and how it affects cellular functions.

Because of the varied properties of biological molecules and the changes in properties that occur on complex formation, it is frequently difficult to find a method that can characterize the full array of interactions under an appropriate variety of conditions. For complexes that involve very tight binding, it is necessary to conduct experiments at very low concentrations, down to the nanomolar or lower levels, that fall below the detection limit for many systems. In such cases, radiolabels or fluorescent probes have been used for added sensitivity in detection. Biosensors with surface plasmon resonance (SPR) detection provide an alternative method, which responds to the refractive index or mass changes at the biospecific sensor surface on complex formation (Jonsson *et al.*, 1991; Karlsson *et al.*, 1994; Malmqvist and Granzow, 1994; Malmqvist and Karlsson, 1997; Myszka, 2000; Nagata and Handa, 2000). Since the SPR signal responds directly to the amount of bound compound in real time, as versus indirect signals at equilibrium for many physical measurements, it provides a very powerful method to study biomolecular interaction thermodynamics and kinetics (Karlsson and Larsson, 2004; Katsamba *et al.*, 2002a,b, 2006; Morton and Myszka, 1998; Myszka, 2000; Rich *et al.*, 2002; Svitel *et al.*, 2003; Van Regenmortel, 2003). Use of the SPR response to monitor biomolecular reactions also removes many difficulties with labeling or characterizing the diverse properties of biomolecules.

II. Rationale: Biomolecular Interactions with SPR Detection

The biosensor-SPR methods described in this chapter refer to Biacore instruments (Biacore International AB), which have been most widely used in the SPR analysis of biomolecular interactions (Rich and Myszka, 2005b). The description will be divided into three primary areas that define the SPR experiment: (i) instrument and sensor chip preparation; (ii) immobilization of one reaction component on the sensor chip surface; and (iii) data collection and analysis. For reaction of biomolecules, B1 and B2, to give a complex, the reaction is:

$$\mathrm{B1} + \mathrm{B2} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{C} \quad K_A = \frac{k_a}{k_d} \tag{1}$$

Figure 1 shows the basic components for biosensor-SPR analysis of this bimolecular interaction with B1 in the flow solution and B2 immobilized. In this example, B2 is linked to dextran in a hydrogel matrix, a typical method for Biacore sensor chips. Reactant B1 is at a fixed concentration in the solution that flows over the biospecific surface containing B2. A number of solutions with different concentrations can be injected to cover a full binding profile. Detection of binding is through a change in refractive index that is monitored in real time by a change in the SPR resonance angle that occurs when the molecules form a complex on the surface (Fig. 1). In a typical Biacore experiment, a four channel sensor chip is used with one flow cell left blank as a control, while the remaining three cells have reaction components immobilized (such as B2 and other target biomolecules). Whether the experiment will have sufficient signal to noise for accurate data analysis depends on two primary factors that are described in the following sections: the moles of B2 immobilized and the mass of B1 bound (moles of B1 bound $\times$ MW of B1) at any time point (since the SPR signal is related to the refractive index change on binding). The molecular weight of binding molecule is thus a key consideration in SPR-biosensor experiments.

Typical Biacore sensor chips, such as the one in Fig. 1, are derivatized with a carboxymethyl-dextran (CM-dextran) hydrogel that provides many possibilities for biomolecular immobilization (BIACORE, 1994a). The Biacore web site has descriptions of a range of sensor chip surfaces and immobilization chemistries (https://www.biacore.com/lifesciences/index.html), and it is generally possible to find an appropriate surface and immobilization chemistry for any biological interaction application. It is obviously essential that the immobilization method, of whatever type, not significantly perturb the binding interactions relative to what occurs in free solution. Covalent coupling of molecules to the surface should use groups that are well away from the binding site and that do not interfere with the interaction.

As shown in Fig. 1, when a solution of a reaction component is injected into the Biacore flow system and passes over the sensor chip biospecific surface, complex

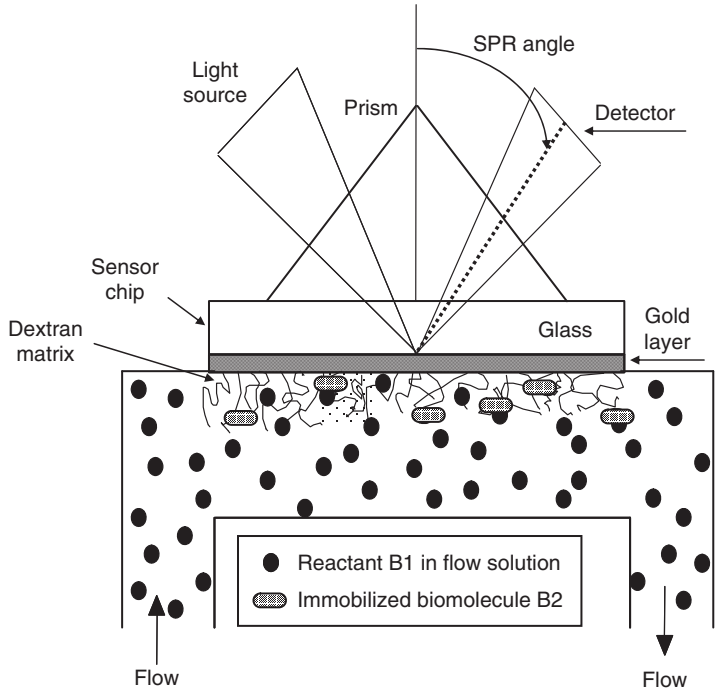

Fig. 1 The SPR signal and biosensor surfaces. The components of a biosensor-SPR experiment are illustrated: the optical unit that generates and measures the SPR angle, the sensor chip with a gold layer, the chip surface with immobilized matrix (dextran on this chip) and reaction component (B2 in this experiment), and the flow control system and solution that provide the other reaction component(s), such as B1. As more of the B1+B2 complex forms, the SPR angle changes as a function of time. Analysis of the signal change with time can provide the kinetic constants for the reaction.

formation occurs and is monitored in real time by a change in SPR angle. After a selected time, reactant flow is replaced by buffer flow and dissociation of the complex is monitored over time. The time course of the experiment shown in Fig. 1 creates a sensorgram such as the one illustrated in Fig. 2. Buffer flow establishes an initial baseline and injection of component B1 leads to the association phase. As the association reaction continues, a steady-state plateau is eventually reached such that the rate of association equals the rate of dissociation of the complex and no change of signal with time is observed. The time required for the steady state to be reached depends on the reactant concentrations and reaction kinetics. If the added molecule does not bind, the SPR angle change in the sample and reference flow cells will be the same and a zero net response, which is indicative of no binding, will be observed after subtraction. When binding does occur, the added molecule is bound at the sensor surface and the SPR angle changes more in the sample than in the reference cell to give the time course of the sensorgram (Fig. 2). Since the amount of unbound compound in the flow solution is the same

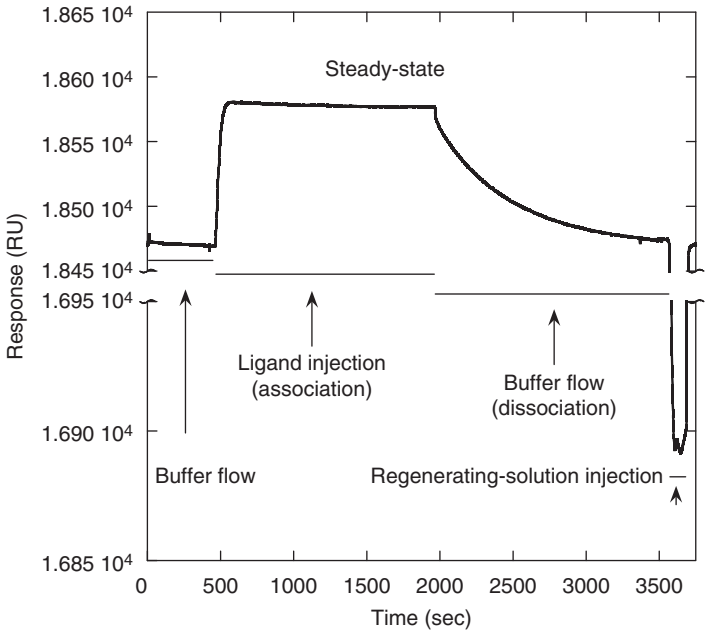

Fig. 2 An experimental sensorgram illustrating the steps in an SPR experiment. These steps include buffer flow for baseline, followed by an association phase, then another buffer flow for dissociation which is followed by injection of the regeneration solution to bring the surface back to the starting conditions. The injections are repeated at a range of concentrations to generate a set of sensorgrams.

in the sample and reference flow cells, it can be subtracted and only the bound reactant generates an SPR signal. The concentration of unbound molecule is constant and is fixed by the concentration in the flow solution.

Several sensorgrams can be obtained at different concentrations of the injected compound and they can be simultaneously fit (global fitting) to obtain the most accurate kinetic (k) and equilibrium (K) constants (Myszka, 1999a, 2000). As will be described below, equilibrium constants can be determined independently from ratios of rate constants or by fitting the steady-state response versus the concentration of the binding molecule in the flow solution over a range of concentrations. The SPR signal change is an excellent method to determine binding stoichiometry, since the refractive index change in SPR experiments generates essentially the same response for each bound molecule and depends on the molecular weight of the binding molecules. The maximum signal increase in an SPR experiment thus provides a direct determination of the stoichiometry, provided the amount of immobilized biomolecule is known. For complexes that have quite slow dissociation rates, the biosensor surface can be regenerated before complete dissociation by using a solution that causes rapid dissociation of the complex, but does not significantly degrade the surface (Fig. 2). The angle change in Biacore instruments

is converted to resonance units (RU) and a 1000 RU response is equivalent to a change in surface concentration of about 1 ng/mm^2 of bound protein or DNA (the relationship between RU and ng of material bound varies with the refractive index of the bound molecule) (Davis and Wilson, 2000, 2001).

The equilibrium and kinetic constants that describe the reaction in Eq. (1) are obtained by fitting the sensorgrams or steady-state RU versus concentration plots to a 1:1 binding model. More elaborate binding models are necessary for more complex interactions, and these models are the same for all types of binding experiments and are not unique to SPR methods. It should be emphasized that to obtain accurate kinetic information about a binding reaction, it is essential that the kinetics for transfer of the binding molecule (diffusion through the hydrogel—see below for better description) to the immobilized biomolecule (mass transfer) be much faster than the binding reaction (Karlsson, 1999). When this is not true, various alternatives to deal with the mass transfer problem are available and will be described below. Annual surveys on methods, applications, and appropriate experimental approaches using SPR by Myszka and colleagues provide many additional helpful suggestions on experimental protocols to obtain high quality biosensor data (Myszka, 1999b; Rich and Myszka, 2000, 2001, 2002, 2003, 2005a,b).

III. Materials and Methods

A. Instrument Preparation

It is recommended by Biacore to run *Desorb* weekly and *Sanitize* once a month for maintaining the instrument. *Desorb* is a general method that uses a series of solutions injected through the instrument internal flow system to remove any absorbed compounds from previous experiments. *Sanitize* is a method to insure that no microbial growth is present in the liquid injection and flow system. Before beginning an experiment, it is very important to ensure that the instrument is running properly. The goal is to determine if a stable baseline can be maintained throughout a series of replicate buffer injections across a nonderivatized sensor surface. A simple method for cleaning is described below. If the baseline is not stable, for example, if the baseline drifts more than ± 1.0 RU/min, additional cleanings may be needed.

1. Required Materials, Chemicals, and Solutions

- Maintenance chip with a glass flow cell surface
- CM5 chip
- Running buffer: HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20)
- 0.5% SDS (BIAdesorb solution 1)

- 50 mM glycine pH 9.5 (BIAdesorb solution 2)
- 1% (v/v) acetic acid solution
- 0.2 M sodium bicarbonate solution
- 6 M guanidine HCl solution
- 10 mM HCl solution

2. Methods for Preliminary Cleaning and Checking Baseline

a. Set instrumment temperature to 25 °C

b. *Dock* a maintenance chip and *Prime* once with distilled water (*Prime* is a method for priming the liquid system by flushing pumps, integrated μ-fluidic cartridge (IFC) and autosampler with water or buffer. This procedure is used at start up, when the buffer is changed, and also to remove small air bubbles from the system).

c. Run *Desorb*.

d. After *Desorb, Prime* several times with warm water (50–60 °C).

e. *Undock* the maintenance chip and *Dock* a fresh research grade CM5 sensor chip and *Prime* once with water.

f. Switch to running buffer and *Prime* several times.

g. Prepare aliquots of 200 μl running buffer into individual vials and run Method 1 (below).

This method will collect a set of sensorgrams of replicated buffer injections across an unmodified CM5 sensor chip. These sensorgrams should overlay after double-referenced subtraction and this indicates a very stable system that is ready for experiments. Methods are written as text files, and may be created or modified with any text editor. The BIA software for instrument control converts the text file to instrument commands (BIACORE, 1994b).

Method 1:

```
MAIN
  RACK      1 thermo_c
  RACK      2 thermo_a
  FLOWCELL      1,2,3,4
  LOOP Buffer STEP
  APROG drug %sample2 %position2 %volume2 %conc2
  ENDLOOP
  APPEND Continue
END
DEFINE APROG buffer
  PARAM %sample2 %position2 %volume2 %conc2
  KEYWORD Concentration %Conc2
```

```
  CAPTION %conc2 %sample2 over (gradient surface)
  FLOW 25
  FLOWPATH 1,2,3,4
  WAIT 5:00
  KINJECT %position2 %volume2 180
  EXTRACLEAN
  EXTRACLEAN
  WAIT 5:00
END
DEFINE LOOP Buffer
  LPARAM %sample2 %position2 %volume2 %conc2
  TIMES 1
        Buffer   r2a1    100    0.000u
        Buffer   r2a2    100    0.000u
        Buffer   r2a3    100    0.000u
        Buffer   r2a4    100    0.000u
        Buffer   r2a5    100    0.000u
        Buffer   r2a6    100    0.000u
        Buffer   r2a7    100    0.000u
        Buffer   r2a8    100    0.000u
        Buffer   r2a9    100    0.000u
        Buffer   r2a10   100    0.000u
  END
```

3. Additional Cleaning Methods

After running the above method, if the baseline is not stable within ±1.0 RU/min (note: this specification may change as different instruments become available), the following methods, designed and provided by Biacore, may be used:

a. Super Clean (As Needed)

1. Insert a maintenance chip into the instrument and *Dock*.
2. Run *Desorb* using SDS and glycine.
3. Run the following method with warm (50–60 °C) filtered water as flowing solution.

```
main
  prime
  prime
```

```
  unclog
  rinse
  flush
  prime
  prime
  append standby
end
```

4. Run *Desorb* using 1% (v/v) acetic acid in place of SDS and glycine.
5. *Prime* the instrument to wash out the acetic acid residuals.
6. Run *Desorb* using 0.2 M sodium bicarbonate in place of SDS and glycine.
7. *Prime* the instrument to wash out the sodium bicarbonate residuals.
8. Run *Desorb* using 6 M guanidine HCl for the SDS (solution 1) and 10 mM HCl for glycine (solution 2).
9. *Prime* the instrument a few times to thoroughly clean all residuals.

b. Super Desorb (Monthly)

1. *Dock* a maintenance chip and run *Prime* using 0.5% SDS.
2. Run *Prime* using 10 mM glycine, pH 9.5.
3. Run *Prime* at least three times using filtered water.

B. Sensor-Chip Surface Preparation

In general, there are two ways to capture biomolecules on the sensor chips: covalent and noncovalent captures. Covalent capture will be illustrated with streptavidin and this surface can then be used to immobilize biotin-labeled biomolecules. Although other immobilization techniques are available, the biotin–streptavidin coupling is popular in Biacore SPR experiments and it is particularly useful for nucleic acids immobilization (Bates *et al*., 1995; Bischoff *et al*., 1998; Hendrix *et al*., 1997; Mazur *et al*., 2000; Nair *et al*., 2000; Nieba *et al*., 1997; Rutigliano *et al*., 1998; Wang *et al*., 2000). The large affinity constant for the biotin–streptavidin complex results in a stable surface for binding studies under physiological conditions. In the example below, immobilization of streptavidin and biotin-labeled DNA will be described.

An important step in sensor chip immobilization is to decide how much biomolecule to immobilize. For kinetic experiments, it is usually best to immobilize the smallest amount of materials, while maintaining the necessary signal-to-noise ratio, in order to minimize mass transport effects. Mass transport effects of ligand to the surface will influence kinetic data when the rate of mass transport is slower than or on the same time scale as the kinetics of the interaction (BIACORE, 1994c; Karlsson, 1999; Myszka *et al*., 1998). Since a high concentration of surface binding

sites rapidly consumes the ligand at the surface, the more material immobilized the greater the contribution from mass transport. However, when the ligand is a small molecule, it becomes necessary to increase the immobilized compound surface density since the instrument response from small-molecule binding is low.

1. Immobilization of Streptavidin

For immobilizing biotin nucleic acids on a sensor chip, the sensor chip must be modified to a streptavidin surface. Biacore offers pre-made streptavidin sensor chips (SA sensor chip) that are ready for immediate use. However, it is possible and in some cases worthwhile to prepare streptavidin sensor chips (BIACORE, 1994c; Hendrix *et al.*, 1997) using standard (CM5) dextran surfaces or CM4 chips with features such as a low density carboxyl surface. The low density carboxyl surface uses dextran but has less negative charge and so may be advantageous when investigating the interactions between highly charged biomolecules. The procedure outlined below is used for immobilizing streptavidin on CM5 or CM4 sensor chips. The Biacore website has many references that describe other methods to immobilize biomolecules to different sensor chip surfaces.

a. Required Materials, Chemicals, and Solutions

- A CM5 or CM4 sensor chip that has been at room temperature for at least 30 min
- HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20) (running buffer)
- 100 mM *N*-hydroxsuccinimide (NHS) freshly prepared in water
- 400 mM *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC) freshly prepared in water
- 10 mM acetate buffer pH ~4.5 (immobilization buffer)
- 200–400 μg/ml streptavidin in immobilization buffer
- 1 M ethanolamine hydrochloride in water pH 8.5 (deactivation solution)

b. Procedures for Streptavidin Immobilization

1. *Dock* the CM4 or CM5 chip, *Prime* with running buffer. Start a sensorgram in all flow cells with a flow rate of 5 μl/min.

2. With NHS (100 mM) in one vial and EDC (400 mM) in other, use the *Dilute* command to make a 1:1 mixture of NHS/EDC.

3. *Inject* NHS/EDC for 10 min (50 μl) to activate the carboxymethyl surface to reactive esters.

4. Using *Manual Inject* with a flow rate of 5 μl/min, load the loop with ~100 μl of streptavidin in the appropriate buffer and inject streptavidin over all flow cells. Track the number of RUs immobilized which is available in real time readout and

stop the injection after the desired level is reached (typically 2500–3000 RU for CM5 chip and 1000–1500 RU for CM4 chip).

5. *Inject* ethanolamine hydrochloride for 10 min (50 μl) to deactivate any remaining reactive esters.

6. *Prime* several times to ensure surface stability.

2. Immobilization of Nucleic Acids

Derivatized nucleic acids with biotin at either the 5′ or 3′ end are ready to be immobilized on a streptavidin-coated sensor chip (SA Chip). This immobilization method provides rapid kinetics and high affinity binding of the nucleic acid to the surface. Relatively short oligonucleotide hairpins (<50 bases) do not require high salt for immobilization. A solution of ~25 nM oligonucleotide (5′-biotin nucleic acid) in HBS-EP buffer is used when immobilizing nucleic acids less than 50 bases. It may be necessary to increase the concentration when using larger nucleic acids. A concentration that is too high, however, will make control over the amount of nucleic acid immobilized very difficult. Typically, an immobilization amount of 300–450 RUs of hairpin nucleic acid (~20–30 bases in length) is immobilized for running steady-state experiments and 100–150 RUs for kinetic experiments to minimize mass transfer effects.

a. Required Chemicals, Materials, and Solutions

- Streptavidin-coated sensor chip (SA chip or prepared as outlined above)
- HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20) (running buffer)
- Activation buffer (1 M NaCl, 50 mM NaOH)
- Biotin-labeled nucleic acid solutions (~25 nM of strand or hairpin dissolved in HBS-EP buffer)

b. Immobilization of Nucleic Acids on a Streptavidin Surface (or on SA Chips)

If two or more different nucleic acid hairpins are to be immobilized on different flow cells, there are two options for immobilization level: equal RU amount or equal moles. For an equal RU amount, different nucleic acid hairpins can be immobilized with the same total response units (RU) on each flow cells. For equal moles, the amount of nucleic acid to be immobilized is proportional to its molecular weight. A higher level is required for a higher molecular weight hairpin because the observed response per bound ligand (RU_{obs}) is proportional to mass bound (moles bound $\times$ MW). This option is useful to visualize and illustrate a difference in stoichiometry.

1. *Dock* a streptavidin-coated chip, *Prime* a few times with HBS-EP buffer, and start a sensorgram with a 20 μl/min flow rate.

2. *Inject* activation buffer (1 M NaCl, 50 mM NaOH) for 1 min (20 μl) five to seven times to remove any unbound streptavidin from the sensor chip.

3. Allow buffer to flow at least 5 min before immobilizing the nucleic acids.

4. Start a new sensorgram with a flow rate of 2 μl/min and select one desired flow cell on which to immobilize the nucleic acid. Take care not to immobilize nucleic acid on the flow cell chosen as the control flow cell. Generally, flow cell 1 (''fc1'') is used as a control and often left blank. It is often desirable to immobilize different nucleic acids on the remaining three flow cells. A nonbinding nucleic acid may be immobilized on fc1 to provide a more similar control surface for subtraction.

5. Wait a few minutes for the baseline to stabilize. Use *Manual Inject*, load the injection loop with $\sim$100 μl of a 25 nM nucleic acid solution and inject over the flow cell. Track the number of RUs immobilized and stop the injection after a desired level is reached.

6. At the end of the injection and after the baseline has stabilized, use the software crosshair to determine the RUs of nucleic acid immobilized and record this amount. The amount of nucleic acid immobilized is required to determine the theoretical moles of small molecule binding sites for the flow cell.

7. Repeat steps 4 to 6 for another flow cell (e.g., fc3 or fc4).

C. Sample Preparations

The solution of small molecule must be prepared in the same buffer used to establish the baseline—the running buffer. If the small molecule is not very soluble in buffer, it can be dissolved in water as a concentrated stock solution and diluted in the running buffer. If the small molecule requires the presence of a small amount of an organic solvent (e.g., $<$5% DMSO) to maintain solubility, the same amount of this organic solvent should be in the running buffer to minimize the refractive index difference.

Preliminary studies may be needed to obtain some information about the compounds being studied such as solubility, stability, or an estimated binding constant. Such information is useful in setting parameters for data collection. Sample concentrations should vary over a wide range (at least 100-fold). In general, the sample concentration range should vary from well below to well above $1/K_a$. If the K_a is unknown, a broad concentration range should be used in a preliminary experiment to obtain an estimate of the K_a. Ideally, the order of sample injection should be randomized. Injecting samples from low to high concentration is useful for eliminating artifacts in the data from adsorption or carry over. It is also useful to inject the same concentration twice to check for reproducibility. For binding constants of 10^6–10^9 M^{-1}, as observed with many small molecules DNA complexes, small molecule concentrations from 0.01 nM to 10 μM in the flow solution allow accurate determination of binding constants.

D. Data Collection and Processing

1. Data Collection

A sample method used to collect steady-state small molecule data on nucleic acid surfaces is shown below. This method is set for a flow rate of 10 μl/min (**FLOW 10**) over all flow cells (**FLOWPATH 1,2,3,4**). The samples are injected as written (**STEP**) from low to high concentration. Note that before any analyte is injected, buffer injections are done to enable double referencing. In addition, the volume of analyte injected is set as a variable so that the least amount of volume required to reach a steady state is used for each concentration. Much less time is required for the association reactions at high concentrations of the injected compound. In this sample method, the small molecule solution of Hoechst 33258 (or analyte) is injected over immobilized DNA (or macromolecule). Note that with the steady-state method, equilibrium, but not kinetics, constants can be obtained even when mass transfer effects dominate the observed kinetics. Much higher injection flow rates are used when collecting kinetics data.

At the end of the compound solution injection, a regeneration step may be necessary to remove any complex remaining on the surface. To subsequently remove any regeneration buffer remaining after this step, two 1-min injections of running buffer are used prior to the end of the cycle followed by a 5-min wait with running buffer flowing. After the next cycle has begun, a 5-min waiting period is set to ensure the baseline has stabilized before the next sample injection. When working with small molecules (or small responses), it is essential that the baseline does not drift significantly during the injection. To reduce carry overs (of sample and regenerating solution), a *Mix* command is added to rinse the injection tube and the injection is conducted from low to high concentrations. If needed, multiple injections of buffer at the end of the cycles are useful to check for carry over.

```
MAIN
   RACK     1 thermo_c
   RACK     2 thermo_a
   FLOWCELL 1,2,3,4
   DETECTION 2–1, 3–1, 4–1
   LOOP Hoechst33258 STEP
   APROG Flow10 %sample2 %position2 %volume2 %conc2
   ENDLOOP
   APPEND Continue
END
DEFINE APROG Flow10
   PARAM %sample2 %position2 %volume2 %conc2
   KEYWORD Conc %Conc2
   CAPTION %conc2 %sample2 over AATT_TTAA_TATA (gradient surface)
```

```
   FLOW          10
   FLOWPATH 1,2,3,4
   WAIT          5:00
   KINJECT       %position2 %volume2 300
−0:20 RPOINT     −b BASELINE
2:30 RPOINT      %sample2
   QUICKINJECT r2f3 10        ! 10 mM Glycine pH 2.5
   EXTRACLEAN
   MIX r2f7 300               ! buffer
   QUICKINJECT r2f4 10        ! buffer
   EXTRACLEAN
   QUICKINJECT r2f5 10        ! buffer
   EXTRACLEAN
   WAIT          5:00
END
DEFINE LOOP Hoechst33258
LPARAM %sample2  %position2 %volume2 %conc2
   TIMES 1
   Buffer          r2a1     200     0.0000u
   Buffer          r2a2     100     0.0000u
   Buffer          r2a3     50      0.0000u
   Buffer          r2a4     200     0.0000u
   Buffer          r2a5     100     0.0000u
   Buffer          r2a6     50      0.0000u
   Buffer          r2a7     200     0.0000u
   Buffer          r2a8     100     0.0000u
   Buffer          r2a9     50      0.0000u
   Buffer          ra10     200     0.0000u
   Buffer          r2b1     100     0.0000u
   Buffer          r2b2     50      0.0000u
   Buffer          r2b3     200     0.0000u
   Buffer          r2b4     100     0.0000u
   Buffer          r2b5     50      0.0000u

   Hoechst33258    r2c1     200     0.0001u
   Hoechst33258    r2c2     200     0.0002u
   Hoechst33258    r2c3     200     0.0004u
   Hoechst33258    r2c4     200     0.0006u
```

Hoechst33258	r2c5	200	0.0008u
Hoechst33258	r2c6	200	0.0010u
Hoechst33258	r2c7	200	0.0020u
Hoechst33258	r2c8	200	0.0040u
Hoechst33258	r2c9	200	0.0060u
Hoechst33258	r2c10	100	0.0080u
Hoechst33258	r2d1	100	0.0100u
Hoechst33258	r2d2	100	0.0200u
Hoechst33258	r2d3	100	0.0400u
Hoechst33258	r2d4	100	0.0600u
Hoechst33258	r2d5	100	0.0800u
Hoechst33258	r2d6	100	0.1000u
Hoechst33258	r2d7	50	0.2000u
Hoechst33258	r2d8	50	0.4000u
Hoechst33258	r2d9	50	0.6000u
Hoechst33258	r2d10	50	0.8000u
Buffer	r2f5	200	0.0000u
Buffer	r2f5	100	0.0000u
Buffer	r2f5	50	0.0000u

END

2. Data Processing

After the data has been collected, there are several processing steps that must be performed before any quantitative information can be extracted. A number of software programs are available for processing Biacore data, including BIAevaluation (Biacore, Inc.), Scrubber2, and CLAMP (Myszka and Morton, 1998). The results can also be exported and presented in graphing software such as KaleidaGraph for either PC or Macintosh computers (Mazur *et al.*, 2000; Wang *et al.*, 2000). Zeroing on the *y*-axis (RU) and then *x*-axis (time) are the first steps in data processing. Because the flow cell surfaces are not identical to each other, the refractive index of each surface is different causing the flow cells to register at different positions on the *y*-axis. Zeroing the data on the *y*-axis is necessary to allow the responses of each flow cell to be compared. Generally the average of a stable time region of the sensorgram, prior to sample injection, should be selected and set to zero. Because the flow cells are aligned in series, sample is not injected across the flow cells simultaneously. Zeroing on the *x*-axis aligns the beginnings of the injections with respect to each other.

The two data-processing steps outlined below help to minimize offset artifacts and also to correct for the bulk shift that results from slight differences in injection buffer and running buffer. In the first step, the control flow cell (fc1) sensorgram is subtracted from the reaction flow cell sensorgrams (i.e., fc2-fc1, fc3-fc1, and fc4-fc1). This removes the bulk shift contribution to the change in RUs. The next step in data processing is required to remove systematic deviations that are frequently seen in the sensorgrams. In this step, the effect of buffer injection on a reaction flow cell is subtracted from the compound injections (different concentrations) on the same flow cell. These processes are referred to as ''double referencing'' (Myszka, 1999a), and remove the systematic drifts and shifts in baseline that are frequently observed even in control cell-subtracted sensorgrams. In the data collection method shown above, buffer injections are performed for each volume amount used for sample injection. Typically, multiple buffer injections are performed and averaged before subtraction. In double referencing, plots are made for each flow cell separately overlaying the control flow cell-corrected sensorgrams from buffer and all sample injections. The buffer sensorgram is then subtracted from the sample sensorgrams. At this point, the data should be of optimum quality and is ready for fitting to determine the thermodynamic and/or kinetic values that characterize the reaction.

IV. Results and Data Analysis

Even when it is not possible to get kinetic constants, equilibrium constants can be extracted from SPR data in a correctly performed experiment. The equilibrium constant can be obtained from fitting steady-state data, or from kinetics. The association equilibrium constant (K_A) is the ratio of the observed association (k_a) and dissociation rate (k_d) constants in Eq. (1). Comparing the K_A value obtained by different methods can help to evaluate the models used to fit the data. Kinetic constants, true k_a and k_d values, can be obtained when the reaction is not dominated by mass transfer.

Knowledge of the stoichiometry of the system is essential for obtaining correct kinetic and binding constants as well as for obtaining a complete description of the system being studied. Because the refractive index increments (R_{II}s) of small molecules can be very different from those of proteins and nucleic acids, it is essential that such a difference be accounted for during data interpretation to correctly determine stoichiometry, and subsequently kinetic and equilibrium constants. The maximum Biacore instrument response for a 1:1 binding interaction can be predicted with Eq. (2).

$$\mathrm{RU}_{\max} = \mathrm{RU}_{\text{biopolymer}} \times \left(\frac{\mathrm{MW}_{\text{compound}}}{\mathrm{MW}_{\text{biopolymer}}}\right) \times R_{II} \tag{2}$$

where RU_{max} is the response for binding of one molecule to the biopolymer; $RU_{biopolymer}$ is the amount of immobilized biopolymer, in response units; MW is molecular weight of compound and biomolecule, respectively; and R_{II} is the refractive index increment ratio of compound to the immobilizing biopolymer where:

$$R_{II} = \frac{(\partial n/\partial C)_{compound}}{(\partial n/\partial C)_{biopolymer}}.$$

The R_{II} value is close to one for proteins and DNA but can deviate considerably from 1.0 for small molecules (Davis and Wilson, 2000). Reference for R_{II} values and methods for determination are given in Davis and Wilson (2000). One way to determine the R_{II} is by comparison of the predicted value from Eq. (2) to the experimental observed value RU_{max}. Small molecules may have more than a single binding site in biomolecular complexes. Nonspecific, secondary binding can occur with cationic molecules and nucleic acids for example, and the R_{II} ratio is critical for accurate determination of stoichiometry.

A. Equilibrium Analysis

After double subtraction, the average of the data in the steady-state region of each sensorgram (RU_{avg}) can be converted to r ($r = RU_{avg}/RU_{max}$) and is plotted as a function of analyte concentration. Equilibrium constants can be obtained by fitting the results with either a single site model (Eq. (3) with $K_2 = 0$) or with the two-site model in Eq. (3):

$$r = \left(\frac{K_1 \times C_{free} + 2 \times K_1 \times K_2 \times C_{free}^2}{1 + K_1 \times C_{free} + K_1 \times K_2 \times C_{free}^2}\right) \quad (3)$$

where K_1 and K_2, the macroscopic thermodynamic binding constants, are the variable parameters to fit; r is the moles of compound bound/mole DNA-hairpin $= RU_{avg}/RU_{max}$; and C_{free} is the concentration of the compound in the flow solution. Although more complex models could be used in data fitting, it is unlikely that a unique fit to the results would be obtained. In such complex cases, other experimental methods should be used to fix some of the variables before fitting the SPR results.

The monocationic Hoechst 33258 DNA minor groove binder has strong preference for A/T rich sequences (Weisblum and Haenssler, 1974). Its DNA binding affinity has been studied with different biophysical methods (Bontemps *et al.*, 1975). A crystallographic structure of the Hoechst 33258 bound to an –AATT– site is available (Pjura *et al.*, 1987; Quintana *et al.*, 1991; Teng *et al.*, 1988). Three different biotin-labeled DNA hairpin duplexes containing AATT, TTAA, TATA sites (Fig. 3) were immobilized on a streptavidin chip (as described above) and

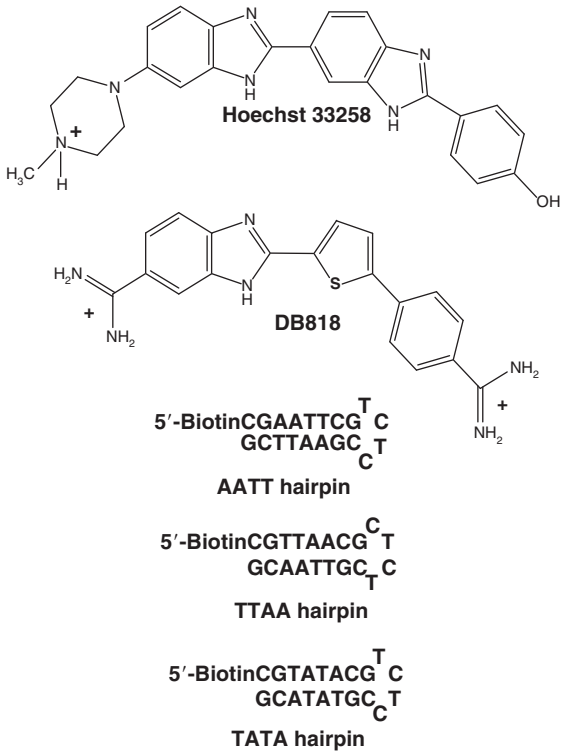

Fig. 3 Structure of Hoechst 33258, DB818, and 5′-biotin-labeled DNA hairpins.

different concentrations of Hoechst 33258 (Fig. 3) were injected onto the surface. Sensorgrams of binding of Hoechst 33258 to AATT and TTAA along with binding plots are shown in Figs. 4 and 5. The binding stoichiometry and affinity for this type of interaction are readily extracted. The binding stoichiometry can be obtained from comparing the maximum response with the predicted response per compound (Eq. (2)).

Because equal moles of DNA hairpins were immobilized, the difference in maximum responses among the sets of sensorgrams is readily seen and directly reflects the difference in binding stoichiometry (Figs. 4 and 5). Under these experimental conditions, the Hoechst ligand binds with a 1:1 ratio to the AATT site (Fig. 4) but with a 2:1 ratio to TTAA (Fig. 5) or TATA (not shown). Plotting the data in Scatchard form can visually reveal considerable information about the binding constants, stoichiometry of specific and nonspecific binding, and cooperativity (Fig. 6). In this figure, the differences in binding constants, stoichiometry and cooperativity for binding of a low molecular weight aromatic cation, Hoechst 33258, to two different DNA hairpins, AATT and TTAA are illustrated. The cooperative binding of two molecules of Hoechst 33258 to TTAA is clear.

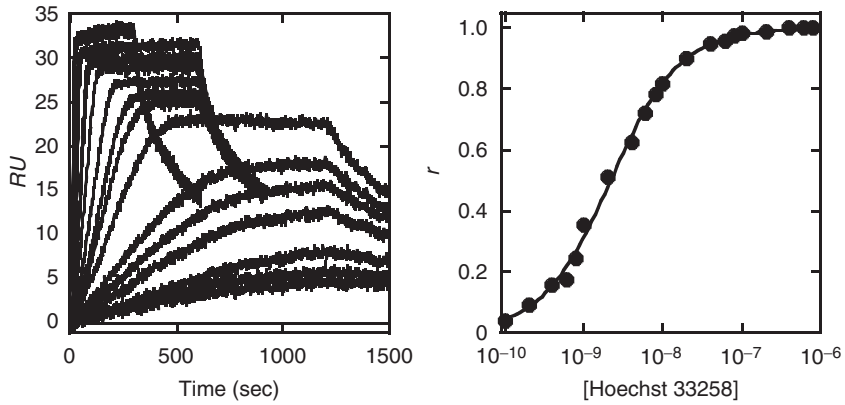

Fig. 4 Sensorgrams for the interaction of Hoechst 33258 with the 5′-biotin-labeled AATT DNA (Fig. 3). The sensorgrams (left) were collected in 0.1 M NaCl, 0.01 M MES (2-(*N*-morpholino)ethanesulfonic acid), 0.001 M EDTA, pH 6.25. The individual sensorgrams represent responses at different Hoechst concentrations; the concentrations were from 0.1 nM (lowest sensorgram) to 0.8 μM (highest sensorgram). Hoechst 33258 solutions were injected at a flow rate of 10 μl/min. The volume of Hoechst 33258 injected is set as a variable (see the method) so that the least amount of volume required to reach a steady state is used for each concentration. Much less time is required for the association reactions at high concentrations. Conversion of these sensorgrams to the binding isotherm (right) was done by dividing the averaged plateau or steady-state responses by the predicted maximum response per ligand ($RU_{pred\text{-}max} = 35$ in this case) as described in the text. The data were fitted (solid line) with a one-site model, Eq. (3), to obtain an equilibrium binding constant of $K = 4.6 \times 10^8$ M^{-1}. This value is in excellent agreement with K values from solution studies (see text).

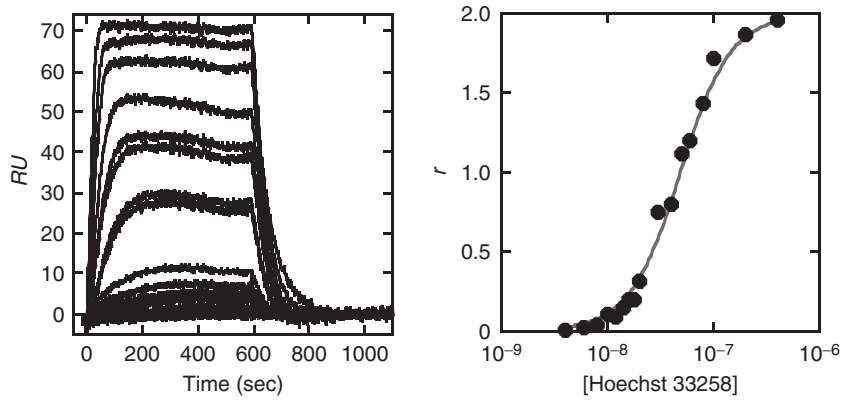

Fig. 5 Sensorgrams for the interaction of Hoechst 33258 with 5′-biotin-labeled TTAA DNA (Fig. 3). The sensorgrams (left) were collected in the same buffer as shown in Fig. 4. The concentrations were from 1.0 nM (lowest sensorgram) to 0.4 μM (highest sensorgram). Hoechst 33258 solutions were injected at a flow rate of 25 μl/min. Conversion of these sensorgrams to the binding isotherm (right) was done by dividing the averaged plateau or steady-state responses by the predicted maximum response per ligand ($RU_{pred\text{-}max} = 35$ as in this case) as described in the text. The data were fitted with a two-site model, Eq. (3), to obtain macroscopic equilibrium binding constants of $K_1 = 1.5 \times 10^6$ M^{-1} and $K_2 = 3.7 \times 10^8$ M^{-1}.

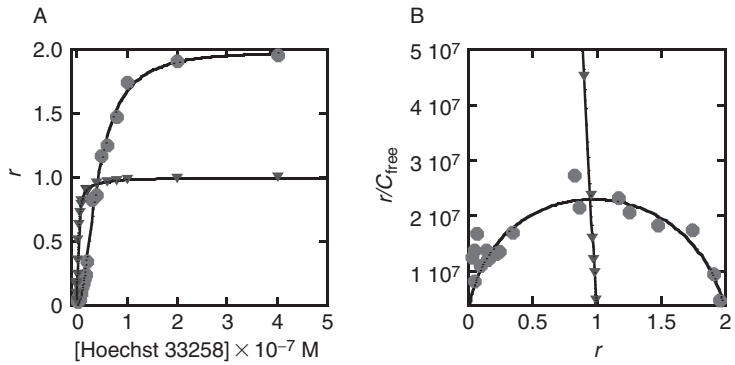

Fig. 6 Binding isotherms (A) and Scatchard plot (B) for the Hoechst 33258 complexes with the AATT and TTAA hairpins. The results are from the sensorgrams in Figs. 4 and 5. The result with the AATT hairpin is typical for AT specific minor groove agents and indicates one strong binding site. The result with the TTAA hairpin is very unusual. Two molecules bind to this oligomer with positive cooperativity. The lines in the figures were obtained by nonlinear least-squares fits of the data to one- and two-site binding equations.

The positive cooperativity in binding of Hoechst to TTAA can be easily seen from a convex shape of the Scatchard plot (Fig. 6). (See the Chapter by Garbett and Chaires for a more complete discussion of analysis of binding data.) A similar trend was observed with the TATA hairpin. The binding constants of Hoechst 33258 to the TATA hairpin are $K_1 = 6.6 \times 10^6\ \mathrm{M}^{-1}$ and $K_2 = 2.7 \times 10^7\ \mathrm{M}^{-1}$. This type of information is very difficult to obtain by other methods. Many systems involve specific binding at one or two sites followed by additional nonspecific binding at higher concentration. The SPR result of Hoechst binding to the AATT hairpin is in agreement with recent results from other methods (Breusegem *et al.*, 2002; Han *et al.*, 2005; Kiser *et al.*, 2005; Loontiens *et al.*, 1990).

B. Kinetic Analysis

Kinetic analysis was performed by global fitting of SPR data with non-mass-transport and mass transport kinetic binding models. In the non-mass-transport 1:1 binding model, Eqs. (4) and (5) are used for global fitting, while in a mass transport limitation model, Eqs. (4–7) are used for global fitting:

$$\mathrm{A} + \mathrm{B} \leftrightarrow \mathrm{AB}$$

$$[\mathrm{A}]_{t=0} = 0, [\mathrm{B}]_{t=0} = \mathrm{RU}_{\max}, [\mathrm{AB}]_{t=0} = 0$$

$$K_\mathrm{a} = \frac{[\mathrm{AB}]}{[\mathrm{A}][\mathrm{B}]} \tag{4}$$

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB] \tag{5}$$

$$\frac{d[A]}{dt} = k_t([A_{bulk}] - [A]) - (k_a[A][B] - k_d[AB]) \tag{6}$$

$$\frac{d[B]}{dt} = -k_a[A][B] + k_d[AB] \tag{7}$$

where [A] and $[A_{bulk}]$ are the concentration of the compound at the sensor surface and the in the bulk solution flow, respectively; [B] is the concentration of the immobilized DNA; [AB] is the concentration of the complex; k_a is the association rate constant; k_d is the dissociation rate constant, and k_t is the mass transport coefficient, defined by Eq. (6).

The fitting can be performed with BIAevaluation software or with CLAMP (Myszka and Morton, 1998) and should be preferentially done with a global analysis method that includes fitting of association and dissociation phases of all sensorgrams (Morton and Myszka, 1998). In cases where a steady-state plateau is reached, the ratio of the rate constants (k_a/k_d) should be compared to the steady-state K_A value. An agreement between the two methods suggests that the binding constant, K_A, is correct but does not necessarily mean that the k_a and k_d values are correct due to possible mass transfer effects and possible correlation of the constants. Some considerations for kinetic fitting have been previously outlined (Nguyen *et al*., 2007). To illustrate a kinetic fit, the interaction between a DNA minor groove binder and a DNA hairpin was studied. DB818, a DNA minor groove binding agent (Fig. 3), forms a 1:1 complex in the duplex minor groove at AATT site (Mallena *et al*., 2004). An SPR experiment for the interaction of DB818 with a DNA hairpin containing the –AATT– site (Fig. 3) was conducted at high ionic strength (1 M NaCl) with flow rate of 50 μl/min.

From this experiment, the kinetic and steady-state analyses are obtained from the same set of sensorgrams to illustrate the agreement of the binding constants obtained from the two analysis methods. The high ionic strength in this experiment with DB818 reduced K_a and k_a to minimize the mass transfer effects. Sensorgrams for the interaction are shown in Fig. 7 and the results are analyzed by both steady-state and kinetic methods (Table I). The sensorgrams increase in response as the DB818 concentration is increased. Note that it takes longer to reach a steady-state plateau at low concentration as expected for a bimolecular reaction. The smooth lines in the figure are the best fit lines using global fitting with a single site kinetic model with a mass transport term. The steady-state RU values for DB818/DNA sensorgrams from the same experiment are converted to r and graphed directly onto a direct plot in Fig. 7 with different concentrations for fitting with Eq. (3) with $K_2 = 0$. The binding constants obtained from steady-state and kinetic analyses are in excellent agreement, and the results are summarized in Table I. The kinetic

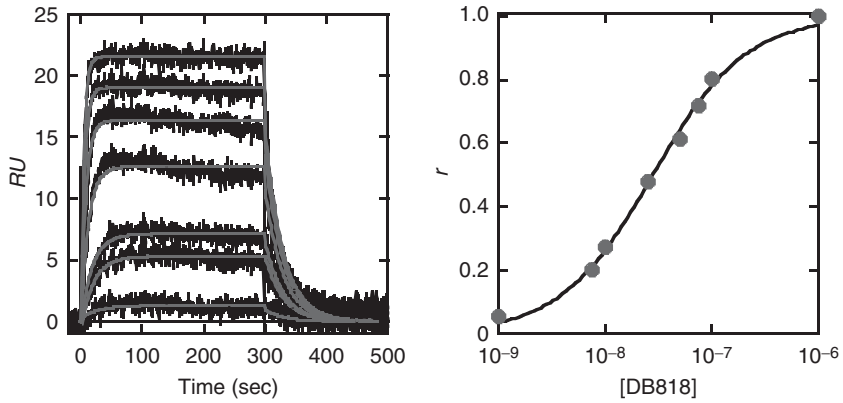

Fig. 7 Sensorgrams to evaluate the kinetics of the DB818–DNA interaction. The sensorgrams were collected with a BIACORE 2000 with flow rate of 50 μl/min at 25 °C and immobilized AATT DNA (Fig. 3) in 1.0 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.4. The concentrations in this experiment from the bottom to the top sensorgrams are 0 (reference line), 1, 7.5, 10, 25, 50, 75, and 100 nM. The kinetic analysis was performed by global fitting of the binding data with mass transport kinetic 1:1 binding models. Conversion of these sensorgrams to the binding isotherm (right) also was done by dividing the averaged plateau or steady-state responses by the predicted maximum response per ligand. The data were fitted with a one-site model, Eq. (3), to obtain an equilibrium binding constant of 3.6×10^7 M^{-1}.

Table I
Summary of Biacore Kinetics and Steady-State (S.S.) Results of DB818 Binding to DNA AATT Hairpin

Exp.	Flow rate (μl/min)	RU_{max} (RU)	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_A (1/M) k_a/k_d	k_t [RU/(Ms)]	Chi^2 $(RU)^2$	$k_a \times RU_{max}/k_t$
Kinetics	50	20.6	2.9×10^6	0.065	4.5×10^7	4.3×10^7	0.287	1.4
S.S.					3.6×10^7		0.234	

The results are obtained from steady-state and kinetic analysis of sensorgrams in Fig. 7. The steady-state and kinetic analyses yield similar binding constants.

fitting results meet the criteria previously outlined ($k_a \times RU_{max}/k_t \leq 5$) (Karlsson, 1999). In addition, the half-life $t_{1/2}$ from the dissociation phase of sensorgram is close to the calculated half-life using the fitted value ($t_{1/2} = \ln 2/k_d$) suggesting the mass transport effect is minimized (Nguyen *et al.*, 2007).

V. Summary

The SPR-biosensor method is excellent for studying small molecule–macromolecule interactions and in the short time that commercial instrument have been available, it has assumed a major role in quantitative analysis of

biomolecular complexes. For strong binding complexes, which are generally observed in biomolecular systems of interest, working at low concentration is required. However, many small molecules have optical properties that make low concentration measurements a clear disadvantage. The SPR method is very useful in such cases since it detects the mass change upon complex formation and can operate at very low compound concentrations. In many cases, the binding kinetics can be observed in real time and extracted from the sensorgrams. A number of studies have now shown that SPR results are comparable to those from other biophysical methods. Although this chapter has focused on small molecule–biopolymer interactions, the methods described above, with minor modifications, can be used to characterize biopolymer–biopolymer complexes.

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References

Bates, P. J., Dosanjh, H. S., Kumar, S., Jenkins, T. C., Laughton, C. A., and Neidle, S. (1995). Detection and kinetic studies of triplex formation by oligodeoxynucleotides using real-time biomolecular interaction analysis (BIA). *Nucleic Acids Res.* **23,** 3627–3632.

BIACORE. (1994a). "BIAapplications Handbook." Pharmacia Biosensor AB, Uppsala, Sweden.

BIACORE. (1994b). "BIACORE 2000: Instrument Handbook." Biacore AB, Uppsala, Sweden.

BIACORE. (1994c). "BIAtechnology Handbook." Pharmacia Biosensor AB, Uppsala, Sweden.

Bischoff, G., Bischoff, R., Birch-Hirschfeld, E., Gromann, U., Lindau, S., Meister, W. V., de, A. B. S., Bohley, C., and Hoffmann, S. (1998). DNA-drug interaction measurements using surface plasmon resonance. *J. Biomol. Struct. Dyn.* **16,** 187–203.

Bontemps, J., Houssier, C., and Fredericq, E. (1975). Physico-chemical study of the complexes of "33258 Hoechst" with DNA and nucleohistone. *Nucleic Acids Res.* **2,** 971–984.

Breusegem, S. Y., Clegg, R. M., and Loontiens, F. G. (2002). Base-sequence specificity of Hoechst 33258 and DAPI binding to five (A/T)4 DNA sites with kinetic evidence for more than one high-affinity Hoechst 33258-AATT complex. *J. Mol. Biol.* **315,** 1049–1061.

Davis, T. M., and Wilson, W. D. (2000). Determination of the refractive index increments of small molecules for correction of surface plasmon resonance data. *Anal. Biochem.* **284,** 348–353.

Davis, T. M., and Wilson, W. D. (2001). Surface plasmon resonance biosensor analysis of RNA-small molecule interactions. *Methods Enzymol.* **340,** 22–51.

Han, F., Taulier, N., and Chalikian, T. V. (2005). Association of the minor groove binding drug Hoechst 33258 with d(CGCGAATTCGCG)2: Volumetric, calorimetric, and spectroscopic characterizations. *Biochemistry* **44,** 9785–9794.

Hendrix, M., Priestley, E. S., Joyce, G. F., and Wong, C. H. (1997). Direct observation of aminoglycoside-RNA interactions by surface plasmon resonance. *J. Am. Chem. Soc.* **119,** 3641–3648.

Jonsson, U., Fagerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Lofas, S., Persson, B., Roos, H., Ronnberg, I., Sjolander, S., Stenberg, E., *et al.* (1991). Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Biotechniques* **11,** 620–627.

Karlsson, R. (1999). Affinity analysis of non-steady-state data obtained under mass transport limited conditions using BIAcore technology. *J. Mol. Recognit.* **12,** 285–292.

Karlsson, R., and Larsson, A. (2004). Affinity measurement using surface plasmon resonance. *Methods Mol. Biol.* **248,** 389–415.

Karlsson, R., Roos, H., Fagerstam, L., and Persson, B. (1994). Kinetic and concentration analysis using BIA technology. *Methods* **6,** 99–110.

Katsamba, P. S., Bayramyan, M., Haworth, I. S., Myszka, D. G., and Laird-Offringa, I. A. (2002a). Complex role of the beta 2-beta 3 loop in the interaction of U1A with U1 hairpin II RNA. *J. Biol. Chem.* **277,** 33267–33274.

Katsamba, P. S., Park, S., and Laird-Offringa, I. A. (2002b). Kinetic studies of RNA-protein interactions using surface plasmon resonance. *Methods* **26,** 95–104.

Katsamba, P. S., Navratilova, I., Calderon-Cacia, M., Fan, L., Thornton, K., Zhu, M., Bos, T. V., Forte, C., Friend, D., Laird-Offringa, I., Tavares, G., Whatley, J., *et al.* (2006). Kinetic analysis of a high-affinity antibody/antigen interaction performed by multiple Biacore users. *Anal. Biochem.* **352,** 208–221.

Kiser, J. R., Monk, R. W., Smalls, R. L., and Petty, J. T. (2005). Hydration changes in the association of Hoechst 33258 with DNA. *Biochemistry* **44,** 16988–16997.

Loontiens, F. G., Regenfuss, P., Zechel, A., Dumortier, L., and Clegg, R. M. (1990). Binding characteristics of Hoechst 33258 with calf thymus DNA, poly[d(A-T)], and d(CCGGAATTCCGG): Multiple stoichiometries and determination of tight binding with a wide spectrum of site affinities. *Biochemistry* **29,** 9029–9039.

Mallena, S., Lee, M. P., Bailly, C., Neidle, S., Kumar, A., Boykin, D. W., and Wilson, W. D. (2004). Thiophene-based diamidine forms a "super" at binding minor groove agent. *J. Am. Chem. Soc.* **126,** 13659–13669.

Malmqvist, M., and Granzow, R. (1994). Biomolecular interaction analysis. *Methods* **6,** 95–98.

Malmqvist, M., and Karlsson, R. (1997). Biomolecular interaction analysis: Affinity biosensor technologies for functional analysis of proteins. *Curr. Opin. Chem. Biol.* **1,** 378–383.

Mazur, S., Tanious, F. A., Ding, D., Kumar, A., Boykin, D. W., Simpson, I. J., Neidle, S., and Wilson, W. D. (2000). A thermodynamic and structural analysis of DNA minor-groove complex formation. *J. Mol. Biol.* **300,** 321–337.

Morton, T. A., and Myszka, D. G. (1998). Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. *Methods Enzymol.* **295,** 268–294.

Myszka, D. G. (1999a). Improving biosensor analysis. *J. Mol. Recognit.* **12,** 279–284.

Myszka, D. G. (1999b). Survey of the 1998 optical biosensor literature. *J. Mol. Recognit.* **12,** 390–408.

Myszka, D. G. (2000). Kinetic, equilibrium, and thermodynamic analysis of macromolecular interactions with BIACORE. *Methods Enzymol.* **323,** 325–340.

Myszka, D. G., and Morton, T. A. (1998). CLAMP: A biosensor kinetic data analysis program. *Trends Biochem. Sci.* **23,** 149–150.

Myszka, D. G., He, X., Dembo, M., Morton, T. A., and Goldstein, B. (1998). Extending the range of rate constants available from BIACORE: Interpreting mass transport-influenced binding data. *Biophys. J.* **75,** 583–594.

Nagata, K., and Handa, H. (eds.) (2000). "Real-Time Analysis of Biomolecular Interactions: Applications of BIACORE." Springer, New York.

Nair, T. M., Myszka, D. G., and Davis, D. R. (2000). Surface plasmon resonance kinetic studies of the HIV TAR RNA kissing hairpin complex and its stabilization by 2-thiouridine modification. *Nucleic Acids Res.* **28,** 1935–1940.

Nguyen, B., Tanious, F. A., and Wilson, W. D. (2007). Biosensor-surface plasmon resonance: Quantitative analysis of small molecule-nucleic acid interactions. *Methods* **42,** 150–161.

Nieba, L., Nieba-Axmann, S. E., Persson, A., Hamalainen, M., Edebratt, F., Hansson, A., Lidholm, J., Magnusson, K., Karlsson, A. F., and Pluckthun, A. (1997). BIACORE analysis of histidine-tagged proteins using a chelating NTA sensor chip. *Anal. Biochem.* **252,** 217–228.

Pjura, P. E., Grzeskowiak, K., and Dickerson, R. E. (1987). Binding of Hoechst 33258 to the minor groove of B-DNA. *J. Mol. Biol.* **197,** 257–271.

Quintana, J. R., Lipanov, A. A., and Dickerson, R. E. (1991). Low-temperature crystallographic analyses of the binding of Hoechst 33258 to the double-helical DNA dodecamer C-G-C-G-A-A-T-T-C-G-C-G. *Biochemistry* **30,** 10294–10306.

Rich, R. L., Hoth, L. R., Geoghegan, K. F., Brown, T. A., LeMotte, P. K., Simons, S. P., Hensley, P., and Myszka, D. G. (2002). Kinetic analysis of estrogen receptor/ligand interactions. *Proc. Natl. Acad. Sci. USA* **99,** 8562–8567.

Rich, R. L., and Myszka, D. G. (2000). Survey of the 1999 surface plasmon resonance biosensor literature. *J. Mol. Recognit.* **13,** 388–407.

Rich, R. L., and Myszka, D. G. (2001). Survey of the year 2000 commercial optical biosensor literature. *J. Mol. Recognit.* **14,** 273–294.

Rich, R. L., and Myszka, D. G. (2002). Survey of the year 2001 commercial optical biosensor literature. *J. Mol. Recognit.* **15,** 352–376.

Rich, R. L., and Myszka, D. G. (2003). A survey of the year 2002 commercial optical biosensor literature. *J. Mol. Recognit.* **16,** 351–382.

Rich, R. L., and Myszka, D. G. (2005a). Survey of the year 2003 commercial optical biosensor literature. *J. Mol. Recognit.* **18,** 1–39.

Rich, R. L., and Myszka, D. G. (2005b). Survey of the year 2004 commercial optical biosensor literature. *J. Mol. Recognit.* **18,** 431–478.

Rutigliano, C., Bianchi, N., Tomassetti, M., Pippo, L., Mischiati, C., Feriotto, G., and Gambari, R. (1998). Surface plasmon resonance for real-time monitoring of molecular interactions between a triple helix forming oligonucleotide and the Sp1 binding sites of human Ha-ras promoter: Effects of the DNA-binding drug chromomycin. *Int. J. Oncol.* **12,** 337–343.

Svitel, J., Balbo, A., Mariuzza, R. A., Gonzales, N. R., and Schuck, P. (2003). Combined affinity and rate constant distributions of ligand populations from experimental surface binding kinetics and equilibria. *Biophys. J.* **84,** 4062–4077.

Teng, M. K., Usman, N., Frederick, C. A., and Wang, A. H. (1988). The molecular structure of the complex of Hoechst 33258 and the DNA dodecamer d(CGCGAATTCGCG). *Nucleic Acids Res.* **16,** 2671–2690.

Van Regenmortel, M. H. (2003). Improving the quality of BIACORE-based affinity measurements. *Dev. Biol. (Basel)* **112,** 141–151.

Wang, L., Bailly, C., Kumar, A., Ding, D., Bajic, M., Boykin, D. W., and Wilson, W. D. (2000). Specific molecular recognition of mixed nucleic acid sequences: An aromatic dication that binds in the DNA minor groove as a dimer. *Proc. Natl. Acad. Sci. USA* **97,** 12–16.

Weisblum, B., and Haenssler, E. (1974). Fluorometric properties of the bibenzimidazole derivative Hoechst 33258, a fluorescent probe specific for AT concentration in chromosomal DNA. *Chromosoma* **46,** 255–260.