

## Analyte Interaction and Kinetics

### Introduction

A simple binding interaction analysis by surface plasma resonance (SPR) starts with the immobilization of ligand to the sensor chip surface, as described in bulletin 5821. This is followed by the addition of the analyte of interest to the buffer flowing over the ligand surface. The interaction of the ligand and analyte is measured by the SPR instrument as a change in refractive index over time. From this, the association ( $k_a$  or  $k_{on}$ ), dissociation ( $k_d$  or  $k_{off}$ ), and equilibrium ( $K_D$ ) constants can be derived. This data is valuable to those studying biomolecular interactions in many applications from binding site interface analysis and concentration determination to thermodynamic analysis.

### Full Kinetic Profile

To generate a full kinetic profile for the interaction of an analyte with a ligand and obtain the binding constants above, one must measure the interactions at multiple analyte concentrations. Typically, multiple analyte concentrations are required for good model fitting. The benefit to using the ProteOn™ protein interaction array system for kinetic analysis is that its 6 x 6 array lends itself perfectly to the simultaneous injection and analysis of up to 6 analyte concentrations at once. One of these analyte concentrations can be sacrificed for use as a real-time double reference.

### Determination of Analyte Concentrations

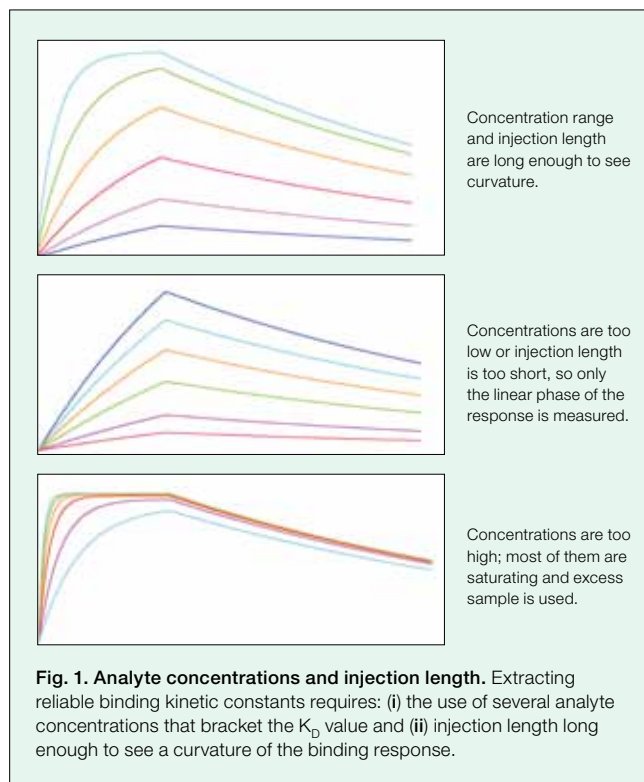
As a rough guide, the range of concentrations needed for an analyte injection should span 10x greater than and 10x less than the expected  $K_D$ . If you are starting with an unknown system and you have no prior knowledge of the  $K_D$ , search the literature to discover if a similar interaction system has been previously studied to obtain guidance for where to start. If this is not possible, then consider what type of biomolecules you are working with. For example, if it is an antibody-antigen interaction, you would expect something within the nanomolar to subnanomolar  $K_D$  range for a tight interaction. If this is a completely novel system, then choose a very large range of concentrations for an initial scouting experiment. This allows you to hone in on the concentration range, gradually decreasing the concentration range to span 10x above and below the  $K_D$ .

### Analyte Preparation

Knowing your analyte concentration is key as it directly affects  $k_a$  and  $k_d$ . Analyte samples should be created using serial dilution into running buffer to minimize bulk effects. Take care to avoid vortexing as this will cause bubbles and destroy proteins. Samples may be centrifuged for about 15 seconds to ensure that all of the solution is at the bottom of the tubes prior to loading into the instrument.

### Analyte Injection Parameters

Typically, analyte injections are performed at a high flow rate of about 50–100  $\mu\text{l}/\text{min}$ ; this helps to reduce any mass transport effects that may be present if a lower flow rate is used. Injections should be performed once the baseline is stable. A stabilization step may be needed in some cases (see bulletin 5821). In order to calculate correct binding constants, the amount of time allotted to the association phase should be enough to observe a curvature of the binding response, and the dissociation phase should be long enough to observe decay in the response (Figure 1).



Initial analyte injection times can be guided by the strength of the interaction that you are examining. If you are working with a small molecule that has fast binding and dissociation from the ligand surface then you can keep the times short, between 1–2 min association and 1–2 min dissociation. See the table below for guidance on analyte injection times.

Analyte Injection Time		
Type of interaction	Association, min	Dissociation, min
Fast binding/fast dissociation	1–2	1–2
Slow binding/slow dissociation	5	10–60
Fast binding/slow dissociation	1–2	10–60

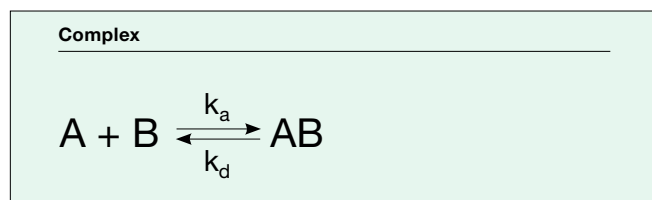
### Analysis of Binding Data

Once you have optimized the analyte injection parameters and concentrations to generate good quality reproducible data, you are ready to perform data analysis. This analysis allows you to determine the type of interaction you are dealing with and to obtain binding kinetics, concentration, or thermodynamic parameters.

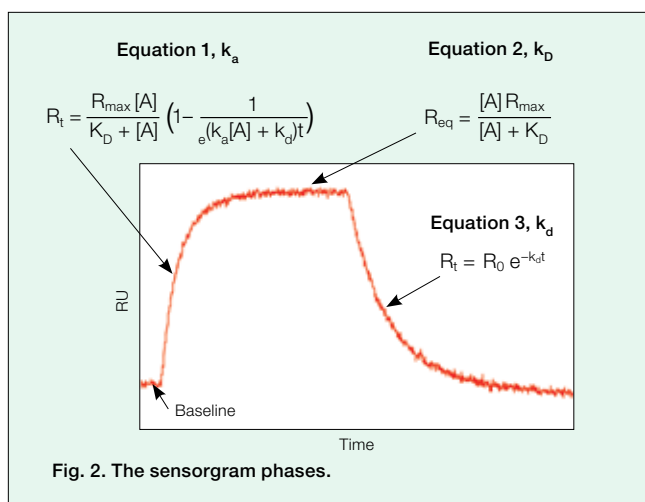
### Kinetic Analysis

#### Binding Models – Langmuir

If you are using the ProteOn protein interaction system, you have a choice of seven different binding models for performing your interaction analysis. However, it is recommended that you try to fit your SPR interactions to the simplest model. The most commonly used model is the Langmuir (O'Shannessy et al. 1993), which describes a 1:1 interaction where one ligand molecule interacts with one analyte molecule. The complex that forms follows pseudo-first-order kinetics and it is assumed that the binding is equivalent and independent for all binding sites. It is also assumed that the reaction is not limited by mass transport. Many interactions will adhere to this model, which can be described by a basic equation shown below, where B represents the ligand and A is the analyte. The rate of formation of the complex is represented as the association constant  $k_a$  and the rate of complex decay is represented by the dissociation constant  $k_d$ .

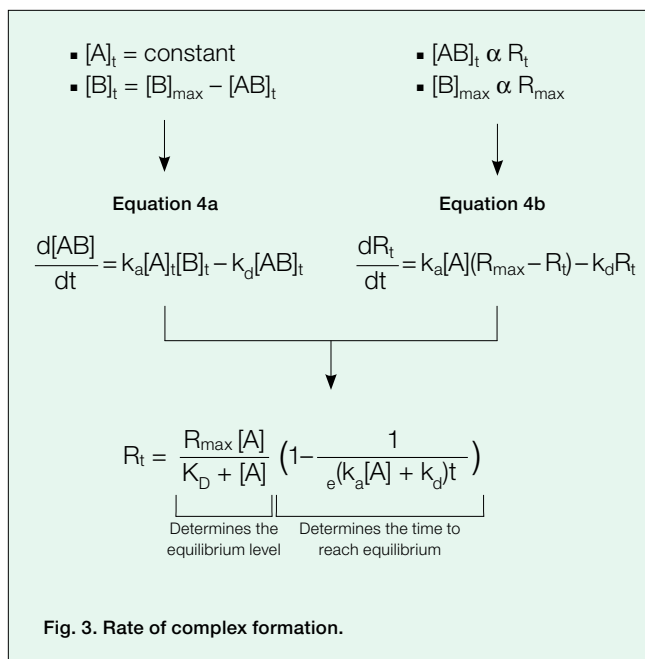


Relating this equation to the SPR sensorgram is simple (shown in Figure 2), where the relevant equations that describe each part of the sensorgram are displayed.



### Association Phase

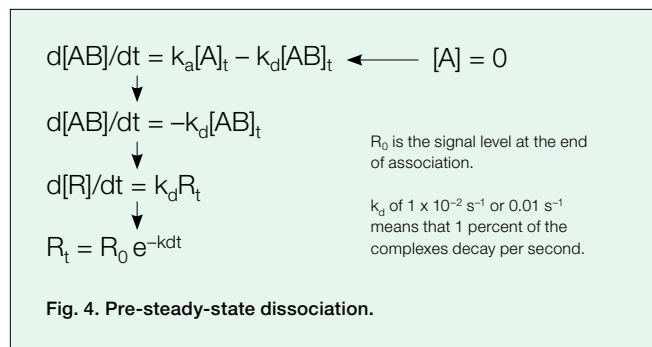
The association phase, where the analyte is flowed across the ligand surface and binding is measured, allows the determination of the rate of formation of the complex over time. There is an associated increase in response units over time as the complex forms at the chip surface. Figure 3 provides an explanation of how Equation 1 was derived.



As can be inferred from Equation 4a, the change in the amount of complex forming over time is proportional to  $k_a$  and  $k_d$  with the amount of analyte (A) in excess. The complex formation can be further described in terms of response units, where the change in response units over time is again proportional to  $k_a$  and  $k_d$  with the amount of analyte (A) in excess. Upon integration these equations become Equation 1 (Figure 2), which describes the level of response at equilibrium and also the time taken to reach equilibrium.

## Dissociation Phase

In the dissociation phase the analyte is removed from the flow and comes to a concentration of zero. The rate of complex dissociation follows simple exponential decay kinetics (Figure 4).



## Langmuir with Drift or Mass Transport

There are two kinetic interaction models based on the Langmuir equations: Langmuir with drift and Langmuir with mass transport. Langmuir with drift is commonly used in experiments that use a capture surface, such as antibody screening (see bulletin 5821) or His-tag capture. In such cases the captured ligand may leach from the capturing agent surface, leading to baseline drift before the analyte injection and during the association and dissociation phases. This model calculates only a linear drift that is constant with time.

The second model is Langmuir with mass transport. Mass transport is the process of an analyte diffusing from the bulk solution to the biosensor chip surface. To determine if your experiment is mass transport-limited, and whether to use this model, inject a fixed analyte concentration at different flow rates. If the binding curves are different, then this interaction is mass transport-limited.

In contrast, if the binding curves are independent of the flow rate (all binding curves overlay) then the diffusion is not limiting and the simple Langmuir model can be applied.

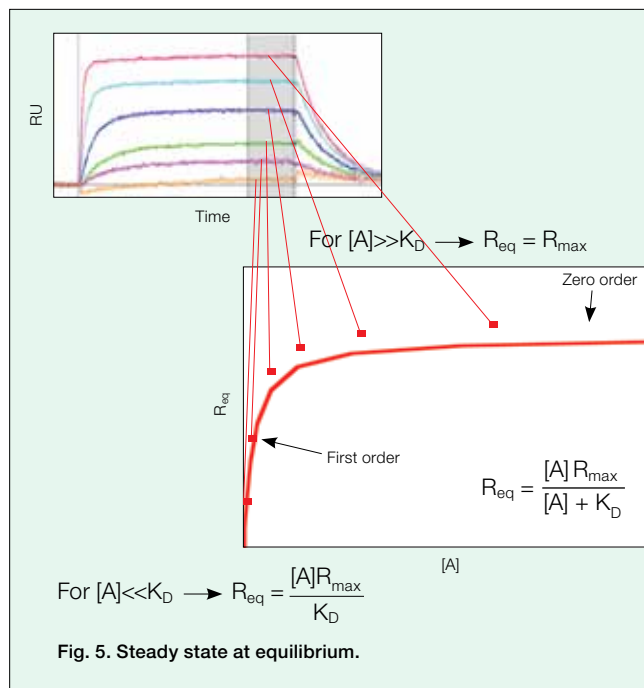
## Other Binding Models

When choosing a method to determine binding kinetics for your interactions, keep it simple for SPR analysis. If you are dealing with more complex binding models, SPR may not be the best option. The ProteOn system offers four complex binding models for analyzing non-Langmuir interactions: heterogeneous analyte, heterogeneous ligand, two state, and bivalent analyte.

## Equilibrium Analysis

Equation 2 (Figure 2) describes the steady state or equilibrium phase of the interaction, the equilibrium constant,  $K_D$ . This is when the rate of association equals the rate of dissociation. To use SPR to determine the  $K_D$ , determine and plot the  $R_{max}$  at a given range of concentrations of analyte (see Figure 5). Equation 2 is a first order reaction but as the concentration of the analyte becomes greater than the  $K_D$ , the response at equilibrium ( $R_{eq}$ ) is close to  $R_{max}$  and Equation 2 becomes

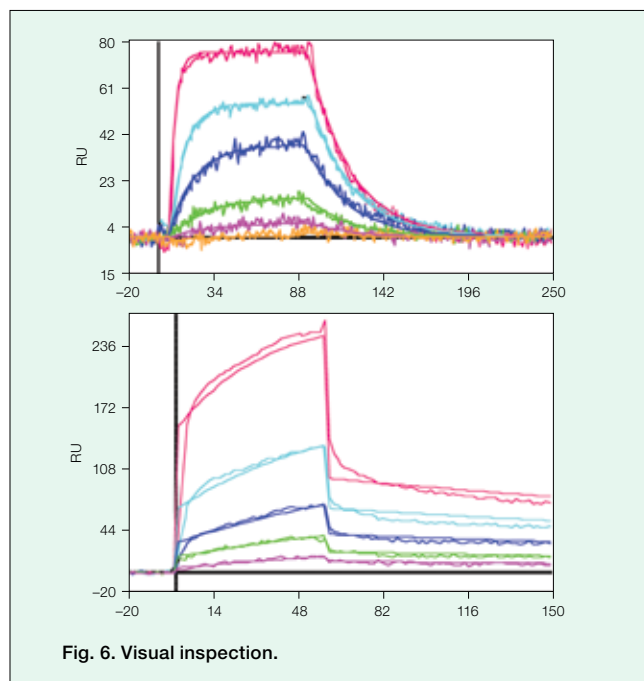
zero order. When performing equilibrium analysis it is important to use data in which the responses of all analyte concentrations have reached equilibrium, and to confine the fit region to the areas where the signals are flat.



## Evaluation and Presentation of Data

When evaluating your analyses and preparing data for publication, here are a few things to keep in mind.

- Visual inspection — the lines of the resulting fit should pass through the experimental data (Figure 6). Both the fitted and original data should be displayed for publication



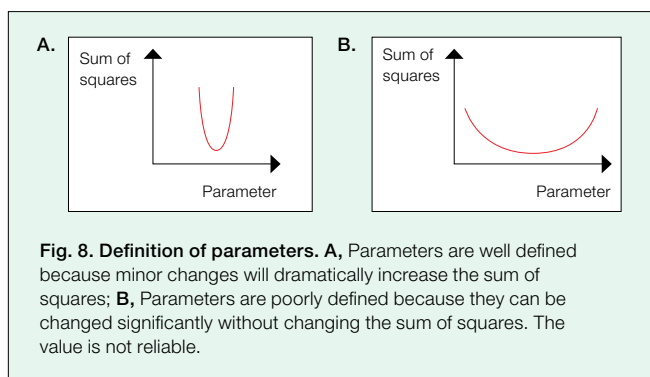
- Parameter results — fitted parameters should be within an expected and reasonable range. The example shown in Figure 7 is for the same ligand on all 5 vertical channels with the same analyte concentration series injected in the horizontal direction. This data can be analyzed in global, grouped, or local analysis. Global and grouped analysis are recommended and the results can be compared to see how reliable the data are. If there is a close association of the data from the two analyses then you can be confident of the results. The calculated  $K_D$  value obtained from equilibrium analysis must be similar to the  $K_D$  calculated from the individual  $k_a$  and  $k_d$  values from kinetic analysis. The fitting parameters must be recorded when publishing data

$k_a$ , 1/Ms	$k_d$ , 1/s	$K_D$	$R_{max}$ , RU	$\chi^2$ , RU
<b>Global</b>	<b>Global</b>	<b>Auto Defined</b>	<b>Grouped</b>	
$7.37 \times 10^5$	$1.47 \times 10^{-4}$	$1.99 \times 10^{-1}$	179	5.4
			176	
			179	
			172	
<b>Grouped</b>	<b>Grouped</b>	<b>Auto Defined</b>	<b>Grouped</b>	
$7.61 \times 10^5$	$1.46 \times 10^{-4}$	$1.92 \times 10^{-1}$	177	5.4
$7.31 \times 10^5$	$1.65 \times 10^{-4}$	$2.26 \times 10^{-1}$	178	4.6
$7.52 \times 10^5$	$1.40 \times 10^{-4}$	$1.86 \times 10^{-1}$	177	4.7
$7.04 \times 10^5$	$1.37 \times 10^{-4}$	$1.94 \times 10^{-1}$	174	4.6

**Global:** Parameters are identical for all sensorgrams.  
**Grouped:** Parameters are identical only for a certain ligand.  
**Local:** Parameters are independent for each sensorgram.

Fig. 7. Parameter results.

- $\chi^2$  — is the average of squared residuals (the average of the squared differences between the measured data point and the fit). The lowest value that can be expected is the signal noise (~1–2 RU) (Figure 8). These values should also be published as they show how confident the fit is. Typically values should be less than 10% of  $R_{max}$



- Standard errors — determine the shape of minima and how sensitive the fit is. High values indicate that the parameter value may be changed significantly without seriously affecting the quality of fit (Figure 8) and should be included in publications
- Residuals — the residuals in the residuals plot should form a random scatter with the magnitude of the noise level (Figure 9). It is helpful to display the residual data along with the fitted data when publishing your work

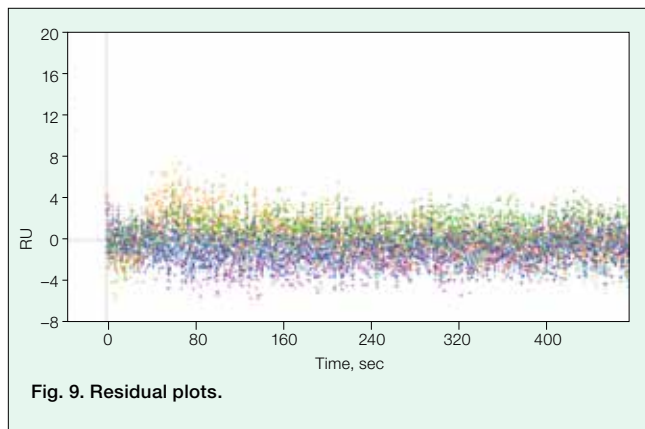


Fig. 9. Residual plots.

## Conclusions

Understanding the general theory and applying the experimental procedures covered in this bulletin can facilitate the production of high-quality, robust SPR data. Correct evaluation and presentation of data are important considerations when conveying SPR findings to others. Care should be taken to ensure experimental repeatability and data analysis transparency. Follow the suggestions discussed in this bulletin to improve the overall quality of both your data and your publications.

## Reference

O'Shannessy DJ et al. (1993). Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: use of nonlinear least squares analysis methods. *Anal Biochem* 212, 457–468.



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