Manual injection vs. Pump-assisted SPR experiments

Surface plasmon resonance instruments provide a wealth of information such as kinetics, affinity, and concentration. However, one must be mindful that the type of an SPR experimental setup can affect the type of data that one can obtain. There are two types of setup which are designed to perform either *steady-state* or *kinetic* measurements. Steady-state measurements can be achieved via *manual injection* to obtain affinity data, whereas kinetic analysis requires a *pump* to determine both kinetic and affinity data (**Fig. 1**). Thus, the resulting sensorgrams would appear differently as well (See Blog on Sensorgram for a review). This tech note will explain the differences in manual injection mode vs. a pump-assisted mode to obtain affinity and kinetic data.

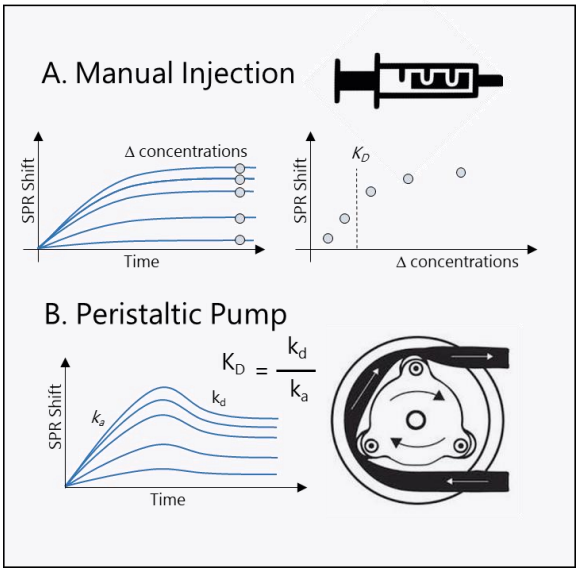

Fig. 1. A. Affinity data can be obtained from steady-state measurements via manual injections. B. Kinetic and affinity data can be obtained from kinetic analysis via a pump-assisted setup (e.g. peristaltic pump). Note the difference in sensorgram shape between A and B.

Basic principles of kinetics and affinity

Let us start with the general idea that ligands (*L*) are immobilized onto a SPR sensing surface and are introduced to an analyte (*A*). If *A* has an affinity for *L* at a 1:1 ratio, then one can assume a Langmuir binding model:

$$A + L \rightleftharpoons AL \qquad (1)$$

with k_a ($M^{-1}s^{-1}$) and k_d (s^{-1}) as rate constants (also known as k_{on} and k_{off}, respectively) in the forward (association) and backward (dissociation) reactions, respectively. Then, the dissociation equilibrium constant, K_D, which is the point at which half of the surface-immobilized ligands are bound to analytes, can be expressed in terms of the free analyte [A], ligand [L], and analyte-ligand complex [AL] concentrations (**Eq. 2**). Furthermore, K_D can also be defined in terms of the rate constants, k_a and k_d (**Eq. 2**):

$$K_D = \frac{[A][L]}{[AL]} = \frac{k_d}{k_a} \qquad (2)$$

K_D is often a preferred parameter to solve for over K_A (association equilibrium constant) as it is expressed in molarity (M), whereas K_A is in M^{-1}.

Fig. 2 is an illustrated sensorgram that represents the 3 main regions used in both steady-state and kinetic experiments. Steady-state measurements involve the association and steady-state phases (regions A and B) to obtain only affinity information, whereas kinetic analysis involves the association and dissociation phases (regions A and C) to obtain affinity and kinetic data.

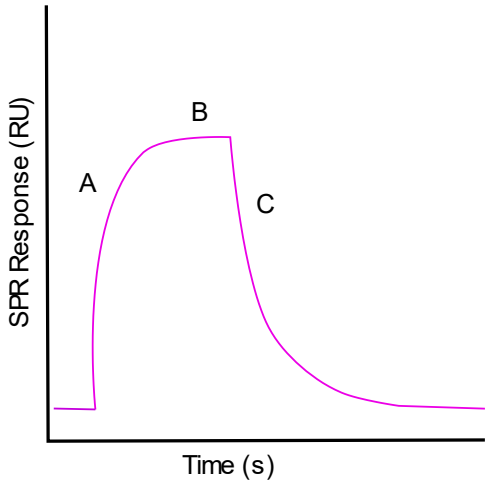

Fig. 2. The association (A), steady-state (B), and dissociation (C) regions of the sensorgram.

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During association or sample injection (**Fig. 2,** region A), the net rate of complex formation of *AL* is given by [1]:

$$\frac{d[AL]}{dt} = k_a[A][L] - k_d[AL] \quad (3)$$

Eq. 3 can be re-written in terms of SPR response values, *R*:

$$\frac{dR}{dt} = k_a[A](R_{max} - R_t) - k_d R_t \quad (4)$$

where *[AL]* (concentration of AL at any given time, t) is proportional to R_t, R_{max} is the total surface binding capacity or maximum SPR response in RU, and $R_{max} - R_t$ is the free *[L]*.

Upon integration, one obtains a single exponential curve [1]:

$$R_t = \frac{[A]R_{max}}{[A] + K_D}\left(1 - \frac{1}{e^{([k_a[A]+k_d)t}}\right) \quad (5)$$

where the first part of the above equation represents the response at steady-state [1] (**Fig. 2**, region B):

$$R_{eq} = \frac{[A]R_{max}}{[A]+K_D} + RI \quad (6)$$

Eq. 6 is obtained by setting Eq. 4 to zero (the net rate of *LA* formation is zero) and replacing R_t to R_{eq} for equilibrium conditions (**Eq. 7**). Following some rearrangements and replacing k_d/k_a for K_D, one would obtain **Eq. 6** when solving for R_{eq}. *RI* represents an offset value for bulk refractive index, which is assumed to be the same for all samples. It is used as an offset for the R_{eq}-axis [2].

$$\frac{dR}{dt} = k_a[A](R_{max} - R_{eq}) - k_d R_{eq} = 0 \quad (7)$$

The rate of dissociation after the end of injection (**Fig. 2**, region C) is given by **Eq. 8** since [A] = 0 in **Eq. 3**:

$$\frac{d[AL]}{dt} = -k_d[AL] \quad (8)$$

Upon integration of **Eq. 8** and relating response to it, one obtains a single exponential decay function that describes the dissociation phase, where t-t_0 is the time interval of the interaction, R_0 is the response at the start of dissociation, R_t is the response at time t [1]:

$$R_t = R_0 e^{-k_d(t-t_0)} + R_{t\to\infty} \quad (9)$$

As one can see, the dissociation curve only depends on k_d. $R_{t\to\infty}$ serves as an offset value if the dissociation part of the sensorgram does not reach zero when t→ ∞ [1].

Manual injection mode for steady-state measurements

Fig. 3 is a photo of a setup used for steady-state measurements via manual injection where samples are manually injected into an SPR instrument via a syringe. Steady-state measurements involve the observation of equilibrium binding (net rate of binding is zero, **Eq. 7**) as a function of analyte concentrations to determine the K_D value, which is a measure of the strength of binding.

A typical experiment involves injecting a series of increasing analyte concentrations (at least 5 concentrations). It is crucial to generate sensorgrams from a wide range of concentrations (0.1 to 10 x K_D)[1], otherwise the K_D value would not be accurate (see Technote on Sensorgrams). Furthermore, samples must be in contact with the sensor surface for a reasonable time to reach steady-state (where the binding curve levels out) (**Fig. 1A**). Notice that there would not be a dissociation phase since steady-state conditions need to be met and there is no flow.

Then, the SPR response at steady-state can be plotted against analyte concentration to obtain a calibration curve. K_D can be determined by fitting that curve into the steady-state equation (**Eq. 6**), which relates the SPR response to analyte concentration and K_D. The values for K_D and R_{eq} are best determined from a non-linear curve fitting of **Eq. 6** to the data using a suitable fitting software.

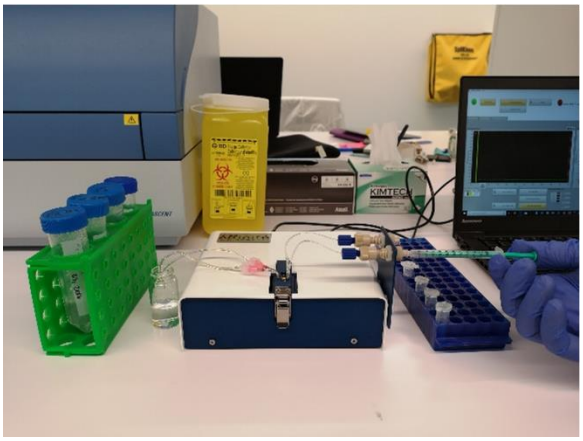

Fig. 3. Photo of Affinité's P4SPR™ with manual injection.

Eq. 6, which is based on the Langmuir binding isotherm, assumes that the analyte is monovalent and homogeneous, the ligand is homogeneous, and all binding events are independent [3].

Furthermore, when [A] << K_D, **Eq. 6** becomes **Eq. 11**:

$$R_{eq} = \frac{[A]R_{\max}}{K_D} \quad (11)$$

And when [A] >> K_D, $R_{eq} = R_{max}$.

The time to reach steady-state can be pre-determined by setting up some preliminary experiments under the same conditions such as flow rate, analyte concentration, and ligand density [3]. This value can also be approximated by using **Eq. 10**, where θ represents the fraction of equilibrium [1]. If the time to reach 90% of equilibrium is desired, use $\theta = 0.90$.

$$t_\theta = \frac{-\ln(1-\theta)}{k_a[A]+k_d} \quad (10)$$

When the analyte concentration is equal to K_D (k_d/k_a) and $\theta = 0.90$, **Eq. 10** can be simplified to [1]:

$$\frac{2.3}{2k_d} \sim \frac{1}{k_d} (11)$$

Steady-state measurements are not limited to injection volume and are unaffected by mass transport limitations due to longer contact time. [1]. However, it is not very practical to perform steady-state measurements for interactions with low K_D (<10 nM), i.e. high affinity, since the k_d values are low and dissociation time is often very long [3]. In contrast, interactions that have high K_D (>100 μM) can be easily investigated [3].

Pump-assisted SPR experiments for kinetic and affinity data

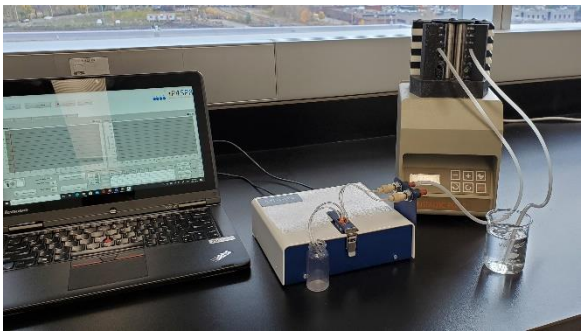

Fig. 4. Photo of Affinité's P4SPR™ connected to a peristaltic pump.

Pumps are required for kinetic measurements since a continuous flow must be provided to observe the dissociation phase with time following sample injection (association phase) (**Fig. 1**). **Fig. 4** shows how a pump is connected to an SPR instrument (Affinité's P4SPR) to collect kinetic data. Once the sensorgrams are collected, the association phase is generally fitted with a suitable binding model (usually 1:1 interaction Langmuir model, e.g. **Eq. 5**) to obtain the k_a value, and the dissociation phase is then fitted with the single exponential decay model to find k_d. Thus, K_D can then be determined from **Eq. 9**. The steady-state phase is not observed in kinetic analysis because the wash buffer is flowed before the analyte in contact with the ligand-immobilized surface reaches equilibrium, forcing the analyte to dissociate from the ligands.

Kinetic analysis can be performed in two separate ways. One is called multi-cycle kinetics and the other is called single-cycle kinetics [2]. In multi-cycle kinetics, one analyte concentration is injected to provide one complete sensorgram, followed by a regeneration step. Then, a new analyte concentration is injected to collect another sensorgram. This is repeated until all the analyte concentrations are run. A single-cycle measurement runs multiple analyte concentrations (usually from low to high) within the same cycle with no regeneration steps in between. It is recommended that various concentrations (at least 5-8 for multi-cycle and

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up to 5 for single-cycle kinetics [2]) of the analyte sample should also be used to eliminate any possibilities of artifacts due to concentration dependency when fitting into a binding model [3]. The selection of concentrations should be centered around the K_D (from 0.1 to 10 x K_D). Another point that sets kinetic measurements apart from steady-state analysis is that they require more sample volume (due to the use of a pump) and are subject to mass transfer effects.

Mass transfer limitation is the reality of kinetic experiments due to the presence of a flowing solution of analyte in a fluidic cell containing the surface-immobilized ligand layer. Before any analytes interact with the ligands, the analytes must be transported by diffusion from the bulk solution to the surface layer (**Eq. 12**) [2].

$$A_{bulk} \rightleftharpoons A_{surface} \qquad (12)$$

where k_m is mass transfer coefficient for both directions, which describes the reversible diffusion-controlled transfer of the analyte from the bulk solution to the unstirred surface layer and vice versa. It is dependent on the flow rate, flow cell dimensions, and diffusion properties of the analyte molecule [3]. Mass transfer limitation occurs when the analyte from the bulk solution is not being diffused into the sensing layer as fast as analytes are binding to ligands.

If the diffusion is slow compared to the association rate of the analyte-ligand complex, the observed K_D would be lower than the actual K_D (because it reflects more on the diffusion rate). One way to circumvent this is to immobilize a lower ligand density and increase flow (>30 µL/min) as it would require a lower rate of mass transfer [2, 3]. To determine if the binding interaction is limited by mass transfer, one can keep the analyte concentration constant but inject it at different flow rates [2, 3]. An indication of a mass transfer limitation is if the binding curves are different, i.e. do not overlay [2]. Thus, the association curve data can be fitted with a Langmuir interaction model with mass transport or change experimental parameters (as mentioned above) to eliminate the mass transport limitation issue [2].

Molecular masses of analytes also play a strong role in kinetic analysis. Molecules with faster k_a values can be measured if their molecular masses are high [3]. This is because larger molecules can produce larger signals, allowing for lower ligand densities. In turn, lower ligand densities mean lower rates of transport [3].

Summary of manual vs. pump-assisted modes and Affinité's P4SPR™ mode duality

Although both types of measurements can provide affinity data, one cannot just do a sole experiment to obtain both steady-state and kinetic data sets to calculate two independent dissociation equilibrium constant (K_D) values. It is just not technically possible as the sample volume is limited in the manual injection mode. Conversely, when using a pump, not enough contact time is available to allow steady-state to occur when kinetic data are being collected. Nevertheless, it is often a good practice to validate a K_D value obtained from either method first and then compare it to the second method [3].

Table 1 summarizes the features of performing a manual injection vs. a pump-assisted SPR experiment. Besides considering the type of data one wants to collect, factors such as costs, amount of sample volume, time, and portability can also determine the type of experiment that one can perform.

Table 1. Comparison of features between manual vs. pump-delivered modes of SPR experiments.

	Manual	**Pump**
Type of data	Obtain affinity data via steady-state measurements using a calibration curve	Get kinetic and affinity data using k_a and k_d
Cost	Low	High (dependent on pump specs)
Mass transfer limitations	Unaffected by mass transfer	Subject to mass transfer
Type of interaction	For interactions with fast on and slow off rates	For interactions with fast on and off rates
Sample volume	Low volume	Requires more sample

Length of experiment	10-20 min per injection	10-15 min per injection
Other considerations	More interactive for educational purposes and can be portable	Less portable

Affinité Instruments has anticipated the need for researchers to do either steady-state and/or kinetic measurements. Our portable P4SPR™ instrument can do both. Syringes can easily be used to inject samples directly into the P4SPR (**Fig. 3**). Alternatively, a pump, such as a peristaltic pump, can be integrated into the P4SPR to deliver samples (**Fig. 4**).

About Affinité Instruments

Established in 2015 as a spin-off of the Université de Montréal, Affinité instruments' foundation is built on deep knowledge accrued throughout more than a decade of research in SPR. The commercialization of promising innovations is spearheaded by a leadership team experienced in business, science and engineering.

About the Author

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