Kinetic Binding Analysis on Agile R100

- Understanding interactions between small molecules, proteins, peptides, aptamers, and other molecules is critical for designing new pharmaceuticals and diagnosing human pathogens.

- The most effective form of kinetic binding analysis involves label-free, independent determination of association and dissociation binding rates describing biomolecular interactions.

- Herein are described methods of biomolecular analysis with rapid throughput, high sensitivity, and analytical flexibility, and Agile R100 uses these models to extract kinetic binding data.

ABSTRACT
Biosensors are used in a variety of fields to characterize biomolecular interactions in real-time. The results of these interaction studies inform the development of the next generation of diagnostics and therapeutics and provide insights into the mechanisms by which pathogens evolve and infect. Label-free assays are particularly useful in these endeavors since they eliminate both the chance of the added label interfering with the native binding chemistry and the additional chemistry steps to attach the label to the molecule under investigation. Data collected from a real-time, label-free biosensor can be analyzed using several models, and the optimal model depends on the prevalence of second order interactions in the system. Field-Effect Biosensing (FEB) technology collects biosensor data and can be analyzed using kinetic binding models developed over the last few decades. Independent extraction of association and dissociation binding rates with FEB allows for advanced molecular design for pharmaceutical applications.

INTRODUCTION
Data Analysis Theory
The mathematics behind the analysis of biosensor data begins with the 1:1 Langmuir model and gets increasingly more detailed as more potential convoluting effects are considered. In its simplest form, the Langmuir model describes the binding of one target to one analyte molecule to form a complex. Further, all binding sites are assumed to be equal and independent and unaffected by mass transport. Beyond the 1:1 Langmuir model, one may consider drift and mass transport effects in kinetics analysis without much additional computation. Second order effects that cannot be reduced to pseudo-first order kinetics represent the most complicated situation to accurately model.

$k_{on}$, $k_{off}$, $K_D$, and $k_{obs}$
In biochemistry and pharmacology, there are very few reactions that are truly irreversible or truly completely reversible. Analyte molecules are continuously binding and unbinding to the thousands or millions of targets bound to the sensor surface. The non-equilibrium reaction of target (T) and analyte (A) can be written as:

$$ T + A \rightleftharpoons TA $$

where TA is the complex of the target with bound analyte.

Therefore, the binding rate of target and analyte is:

$$ \frac{dT[A]}{dt} = k_{on}[T][A] - k_{off}[TA] $$

where [T] is the unoccupied target concentration, [A] is the free analyte concentration, [TA] is the complex of the target with bound analyte concentration, $k_{on}$ is the association binding rate, and $k_{off}$ is the dissociation binding rate.

When the system is in equilibrium, the concentrations of targets, analyte, and complex of the target with bound analyte are no longer time dependent.

$$ \frac{d[T[A]}{dt} = k_{on}[T][A] - k_{off}[TA] = 0 $$

Then, $K_D$ is defined as such and called the dissociation constant.

$$ K_D = \frac{k_{off}}{k_{on}} = [T][A]/[TA] $$

In Equation 2, the number of analyte molecules in each experiment tends to be several orders of magnitude higher than the number of targets bound to the sensor surface. Even at low analyte concentration [A], the concentration can be considered constant throughout association since the number of free molecules remains relatively unchanged. Based on this assumption,

$$ \frac{d\theta}{dt} = k_{on}(1 - \theta)[A] - k_{off} \cdot \theta $$

where $\theta$ is the fraction (time-dependent) of occupied targets on the sensor surface.

Solving this differential equation produces the relation:

$$ \theta = (1 - e^{-k_{obs}t}) \cdot \theta_{eq} $$
where $\theta_{eq}$ is the fraction of occupied targets once the binding reaction has equilibrated, and $k_{obs}$ is the observed binding rate at a given concentration is defined as:

$$k_{obs} = k_{on}[A] + k_{off}$$  

(7)

**Standard Curve and Hill’s Equation**

In biochemistry and pharmacology, the binding of a analyte to targets, such as on the Agile R100 sensor surface, is described by:

$$\theta = \frac{T^n}{T^n + K_D^n}$$  

(8)

where $A$ is the concentration of analyte and $n$ is the Hill coefficient (the degree of cooperativity), which represents the degree of cooperativity.

The fraction $\theta$ can be translated into sensor response $S$:

$$S = \theta S_{max} = \frac{n^n}{A^n + K_D^n} \cdot S_{max}$$  

(9)

where $S_{max}$ is the maximum sensor response when all targets have bound analyte.

When the substrate concentration is equal to $K_D$:

$$A = K_D$$  

(10)

Then,

$$S = \frac{1}{2} S_{max}$$  

(11)

The equilibrium dissociation constant $K_D$ is the concentration of the analyte that induces 50% of the saturated sensor response.

**Agile R100**

For a given target and analyte pair on Agile R100, the sensor response with respect to time $S(t)$ is linearly proportional to the fraction of targets that have analyte bound.

$$S(t) = S_{eq} \theta = S_{eq} (1 - e^{-k_{obs}t})$$  

(12)

where $S_{eq}$ is the sensor response when the binding chemistry is at equilibrium, which is also reported as the “Sensor Response” in Agile R100’s Data Analysis panel, and $k_{obs}$ is given by Equation 7. Sensor response $S$ can be either positive or negative depending on the binding chemistry’s influence on the Agile R100 biosensor chip. It is common for negative data to be reported as positive, and the “invert function” option can be selected in Agile Plus software to make the data positive.

**I-Response and C-Response**

During each experiment performed with an FEB system, there are several parameters that can be used to determine the sensing behavior of the system. For example, The Agile R100 analysis suite analyzes the electronic current through each sensor channel and monitors changes in the average current in response to dynamic biomolecular interactions. This is known as the sensor I-Response, while changes to the device capacitance are recorded as the C-Response. Advanced statistical analysis is performed on all sensor channels to calculate the average time response and standard deviation, from which one may extract $k_{on}$ and $k_{off}$.

**Data Analysis Methods**

**Association and Dissociation Kinetics**

During sensor functionalization, blocking and quenching steps are employed to reduce nonspecific binding; however, there are still small numbers of nonspecifically bound proteins and salts and other biomolecules that may contribute to sensor drift over time. To account for the sensor drift, the data analysis model consists of a single exponential response which corresponds to specific binding and a linear response $kt + c$ which corresponds to drift induced by nonspecific electrostatic interactions:

$$S(t) = S_{eq} \cdot e^{-k_{obs}t} + kt + c$$  

(13)

where $k$ and $c$ are constants.

The fist analysis step is to perform a linear regression on the calibration region of the experiment. The subsequent analyte association and dissociation sections subtract the linear drift from the total sensor response. For this reason, it is critical to perform a calibration step before every individual analyte concentration to determine a baseline for that specific interaction. This calibration step, also known as referencing or blank subtraction, consists of exposing the sensor to the same assay buffer the analyte is dissolved in but without any analyte present.

During analyte association, after the nonspecific electrostatic interactions are subtracted from the total response, a single exponential curve is fitted to the data to determine $k_{obs}$, as shown in Equation 13.

During analyte dissociation, the total sensor response is denoted as:

$$S(t) = S_{eq} \cdot e^{-k_{off}t} + kt + c$$  

(14)

After the sensor drift is removed from the total dissociation response, a single exponential curve is fitted to the data to determine $k_{off}$.

After these two coefficients $k_{obs}$ and $k_{off}$ are calculated, the association binding rate $k_{on}$ can be extracted using:

$$k_{on} = \frac{k_{obs} - k_{off}}{[A]}$$  

(15)

Finally, the equilibrium dissociation constant $K_D$ is given by (Equation 4).

**Regression to Hill’s Equation**

In addition to Equation 4, $K_D$ can be determined from a standard curve, a plot of the sensor response as a function of analyte concentration. From Equation 11, $K_D$ is the analyte
concentration at which 50% of the maximum sensor response is produced. The exact concentration at which this occurs is calculated by fitting to Equation 9, where $K_D$, $n$, and $S_{\text{max}}$ are unknown coefficients to be determined by the fit. With 3 unknown coefficients, at least 4 analyte concentrations are required to generate a standard curve, but more are suggested to improve accuracy.

This analysis complements the previous method of determining $K_D$, and both methods should produce values that agree within error. However, it is possible for the $K_D$ values to disagree between the standard curve and association and dissociation kinetics if there are other higher-order processes such as heterogeneous analyte, heterogeneous ligand, two-state, or bivalent analyte occurring during association or dissociation.

$k_{\text{obs}}$ Plot

From the definition of observed binding rate $k_{\text{obs}}$ in Equation 7, there is an additional method to calculate the dissociation binding rate $k_{\text{off}}$ and the association binding rate $k_{\text{on}}$ from a linear regression on a plot of $k_{\text{obs}}$ vs. analyte concentration $[A]$. After this linear regression, the slope of the fit is the association binding rate $k_{\text{on}}$, and the y-intercept is the dissociation binding rate $k_{\text{off}}$. With $k_{\text{on}}$ and $k_{\text{off}}$, the equilibrium dissociation constant $K_D$ can be found using Equation 4. This method is especially useful if only association binding experiments were performed without any dissociation experiments, as may be the case with strongly binding antibody and antigen pairs that do not unbind in any reasonable time scale.

CONCLUSIONS

There are several models available to characterize molecular binding. Several methods for obtaining the equilibrium dissociation constants and binding rates are discussed herein. The 1:1 Langmuir binding model is sufficient for many target and analyte pairs, but higher orders considerations are necessary when the interaction is affected by mass transport, rebinding of the analyte, systematic sensor drift, or heterogeneous analytes or targets. Generally, the simplest model that accurately describes the data is the correct one. The kinetic binding results obtained from this type of analysis can inform the design and development of a new class of biomolecules to improve human health.

REFERENCES


