## Binding Kinetics — Basic Principles

Direct measurement of biomolecular interactions is important in biotherapeutic drug discovery and development. The affinity of interaction directly affects the dose at which a biopharmaceutical is effective, and **understanding the mechanism of binding** has implications for the efficacy and desirability of a therapeutic candidate. Real-time data on specificity, affinity, and kinetics of binding interactions can benefit every stage of biopharmaceutical development, from early discovery to the manufacturing process.

**Kinetic analysis** is used to determine the **affinity of an interaction** and measure **association** and **dissociation** rate constants for **reversible, non-covalent binding**. Non-covalent binding is typically comprised of a combination of ionic/electrostatic interactions, hydrogen bonds, van der Waals forces, and hydrophobic effects. The specific recognition and binding of biological molecules by antibodies and other proteins is fundamental to many processes in biology.

**Kinetics** refers to how fast an interaction occurs.

**Association** measures how fast one molecule binds to another, and **dissociation** measures how fast a complex falls apart. After a certain period of time, **equilibrium** will be established, and complexes will associate at the same rate as they dissociate. Hence, the number of bound and unbound molecules remains constant.

**Affinity** measures how strong the complex is, specifically, how much complex is formed when binding reaches equilibrium. The simplest model used to describe this interaction between two biomolecules is represented by the equation below:

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

**A** represents the ligand molecule immobilized on the biosensor's surface, and **B** is the analyte in the solution.
The ratio between $\mathbf{k_d/k_a}$ is called $\boldsymbol{K_D}$, the **equilibrium dissociation constant**.
$\boldsymbol{K_D}$ is expressed in molar units (M).

The $K_D$ corresponds to the concentration of analyte at which 50% of ligand binding sites are occupied at equilibrium or the concentration at which the number of ligand molecules with analyte bound equals the number of ligand molecules without analyte bound.

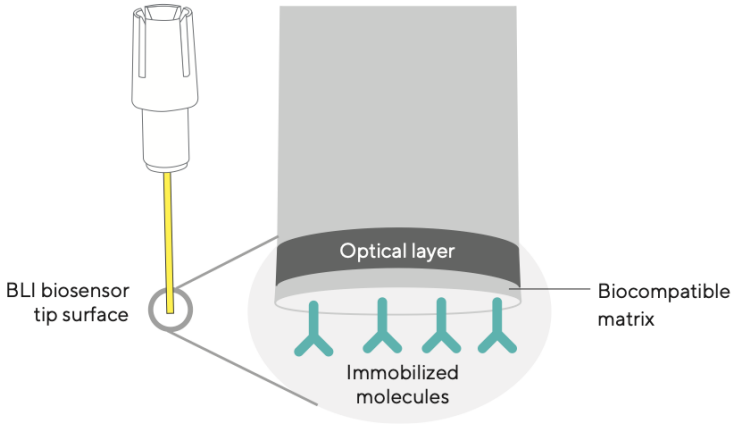
An inverse relationship **exists between $\boldsymbol{K_D}$ and affinity**—a smaller affinity constant indicates a tighter interaction or greater affinity of the analyte to the ligand.

A variety of analytical methods have been established to determine the $K_D$ values since the 1960s, including:
- radioligand binding assay (RBA),
- surface plasmon resonance (SPR),
- fluorescence energy resonance transfer method (FRET),
- affinity chromatography,
- **isothermal titration calorimetry (ITC)**
- **Bio-layer interferometry (BLI)**.

**Label-free analytical technologies** provide a powerful means to obtain accurate information about the rate of biomolecular complex formation and complex stability, key components of a drug-target interaction. The affinity of interaction directly affects the dose at which a biopharmaceutical is effective, and understanding the mechanism of binding has implications in the efficacy and desirability of a therapeutic candidate. Real-time data on binding interactions' specificity, affinity, and kinetics can benefit every stage of biopharmaceutical development, from early discovery to manufacturing.

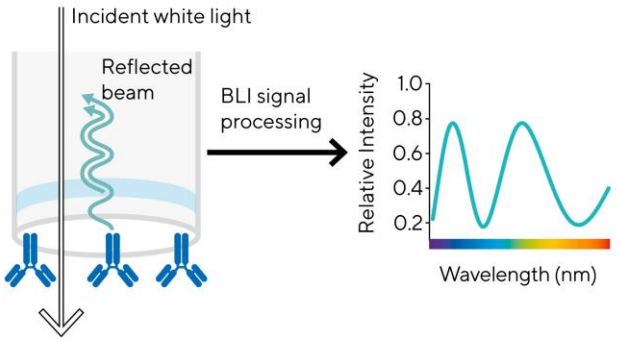
## Bio layer-interferometry

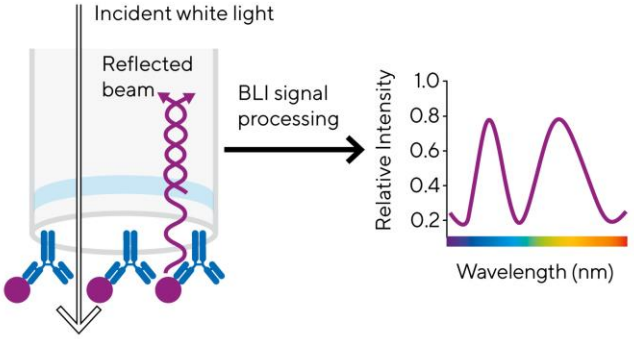
Bio-Layer Interferometry (BLI) is a **label-free** technology for **measuring biomolecular interactions**.
BLI is an **optical analytical technique** that measures **interference patterns** between light waves. **White light** is directed down the fiber optic biosensor towards two interfaces separated by a thin layer at the tip of the fiber: a **biocompatible layer** on the surface of the tip and an i**nternal reference laye**r.



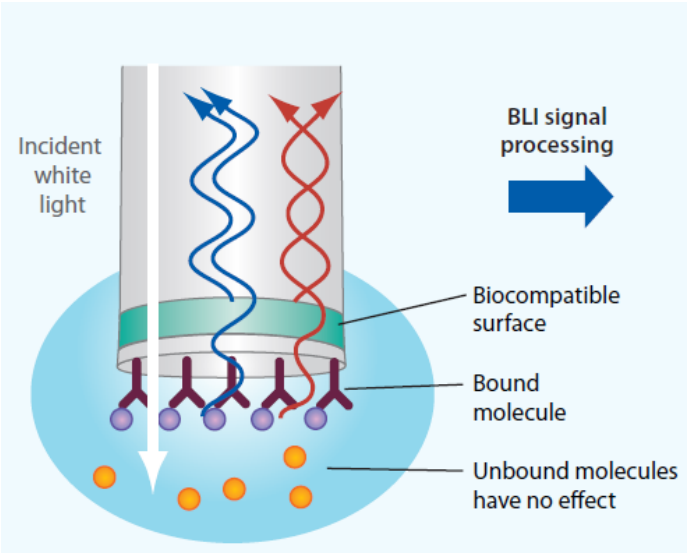

Light reflects from each of the two layers, and the reflected beams interfere constructively or destructively at different wavelengths in the spectrum. **This interference pattern is detected by the instrument**.

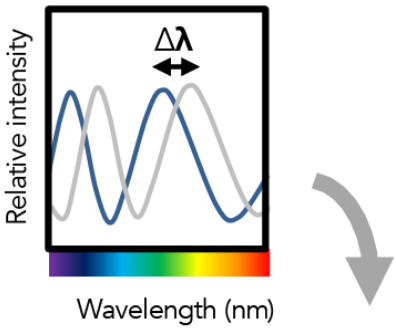
Any change in the number of **molecules bound to the biosensor tip** causes **a shift in the interference pattern** that can be measured in real-time.

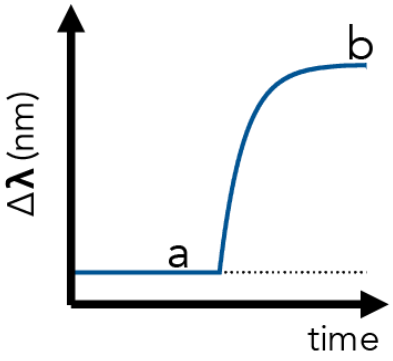



When the tip of a biosensor is dipped into a sample, **target molecules bind to the 2-dimensional coated surface**. This binding forms a molecular layer <u>that **increases in thickness**</u> as more target molecules bind to the surface. As the thickness at the tip increases, the effective distance between the two reflective layers increases, creating a shift in the interference pattern of the reflected light.



Therefore, the reflected light's spectral pattern **changes as a function of the optical thickness of the molecular layer**, *i.e.*, the number of molecules bound to the biosensor surface. This spectral shift is monitored at the detector and reported on a sensorgram as a change in wavelength (nm shift). Monitoring the interference pattern in real-time provides kinetic data on molecular interactions.

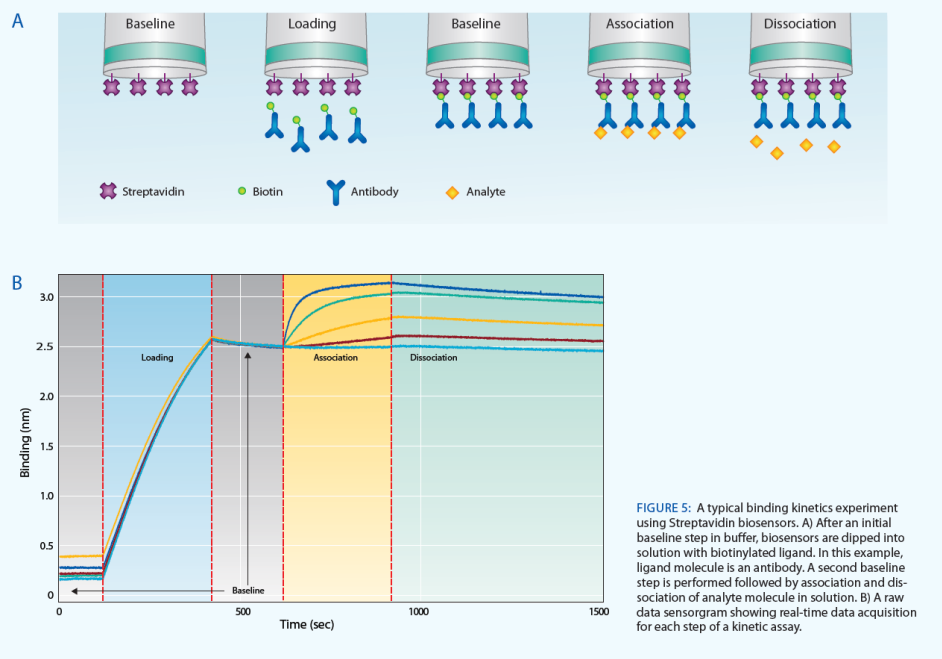



FIGURE 5: A typical binding kinetics experiment using Streptavidin biosensors. A) After an initial baseline step in buffer, biosensors are dipped into solution with biotinylated ligand. In this example, ligand molecule is an antibody. A second baseline step is performed followed by association and dissociation of analyte molecule in solution. B) A raw data sensorgram showing real-time data acquisition for each step of a kinetic assay.

Setting up a kinetic assay using BLI and the Octet platform is simple and straightforward. However, the appropriate choice of optimized experimental conditions is critical to determining accurate affinity and kinetic constants. The use of unstable or inactive proteins, improperly characterized reagents, or inappropriate buffer conditions will negatively impact results.

Analysis of biomolecular interactions starts with the immobilization of a ligand onto the surface of the biosensor. Biosensors come ready to use with standard binding agents such as streptavidin and amine-reactive groups, allowing for irreversible attachment of proteins to essentially create custom biosensor surfaces. Alternatively, capture agents such as anti-mouse IgG Fc capture or Ni-NTA provide a means for highly specific capture of antibodies or recombinant proteins, even from unpurified samples. The most important consideration for biosensor selection is choosing a format that best maintains the structure and activity of the immobilized ligand.

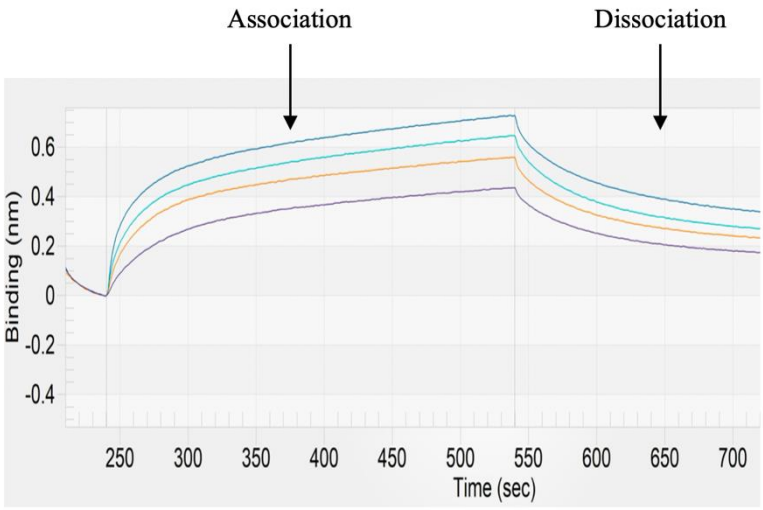


Figure 3

Measuring binding curves for a single analyte concentration is often sufficient for screening purposes or qualitative analyses. However, when reliable, accurate kinetic constants are required, a dilution series of at least four analyte concentrations should be measured in the association step. The analyte dilution series measured should ideally range from a concentration of about 10–20*$K$d down to about 0.1*$K$d, using 2-fold or 3-fold dilutions.

Figure 3 shows kinetic data (sensorgram) from an experiment using an optimal range of analyte concentrations. The binding signals span the dynamic range of the assay from near the detection limit to just below saturation. In the dissociation step, the biosensor is dipped into a buffer solution that does not contain the analyte. With no analyte present, the free concentration of analyte in the solution drops to zero, and the bound complex on the surface of the biosensor dissociates.

Kinetic data are finally interpreted based on a mathematical model of the interaction, from which kinetic equilibrium binding constants can be calculated based on association and dissociation rates.

# Isothermal titration calorimetry

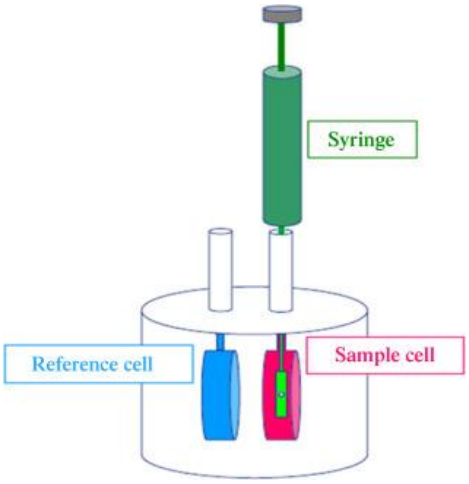
Isothermal titration calorimetry (**ITC**) is one of the physical techniques that directly measures the **heat discharged** or **consumed** along a **bimolecular reaction**. It is an analytical method where the ligand comes in contact with a macromolecule under **constant temperature**. It works on the basic principle of thermodynamics where contact between two molecules results in either heat generation or absorption, depending on the type of binding, that is, exothermic or endothermic.

ITC is the only technique that can simultaneously determine all binding parameters in a single experiment. Requiring no modification of binding partners, either with fluorescent tags or through immobilization, ITC measures the affinity of binding partners in their native states.

**How it works**

**The thermal core**

In the microcalorimeter, there are **two cells**, one containing water and acts as a reference cell, and the other containing **the sample**. The microcalorimeter needs to keep these two cells at **exactly the same temperature**. The heat sensing devices detect temperature differences between the cells when binding occurs and give feedback to the heaters, compensating for this difference and returning the cells to equal temperature.



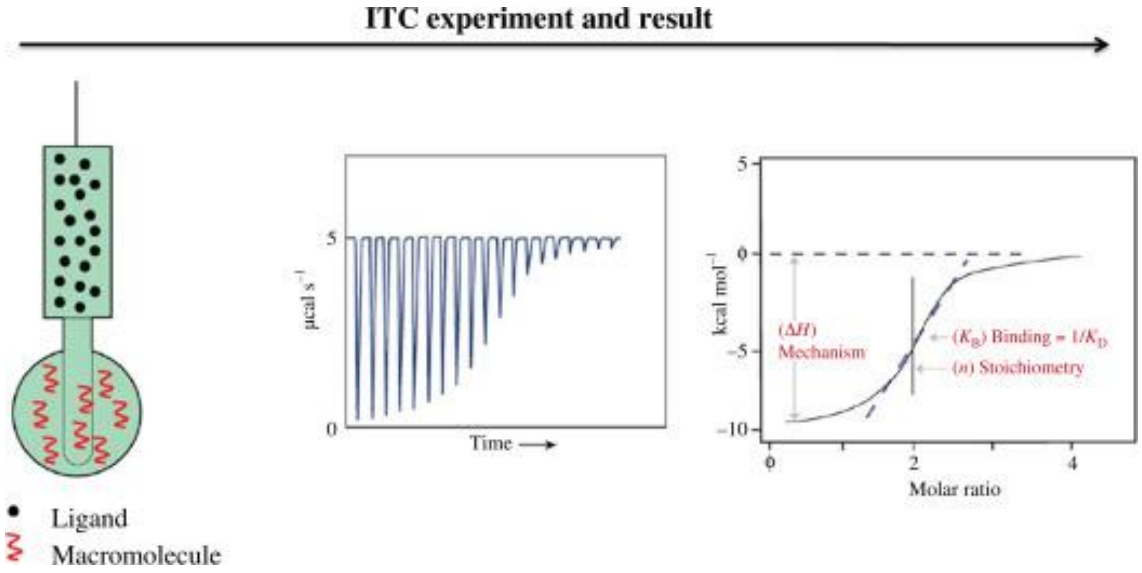

**Instrumentation of the typical isothermal titration calorimetry.**

**Making a measurement**

The reference cell and the sample cell are set to the desired experimental temperature. The ligand is loaded into a syringe which sits in a very accurate injection device. The injection device is inserted into the sample cell containing the protein of interest. A series of small aliquots of ligand are injected into the protein solution. If there is a binding of the ligand to the protein, heat changes of a few millionths of a degree Celsius are detected and measured.

As the first injection is made, the microcalorimeter measures all heat released until the binding reaction has reached equilibrium. The quantity of heat measured is in direct proportion to the amount of binding.

The **heat change is then simply calculated** by integrating the power over the time (seconds), which gives us the enthalpy of the reaction. The heat discharged or consumed all along the calorimetric reaction corresponds to the fraction of bound ligands, and increased ligand concentration leads to saturation of substrate, and finally, less heat is discharged or consumed.




As the temperature of the two cells comes back to being equal, the signal returns to its starting position. The second small aliquot of the ligand is injected into the sample cell, and once again, the microcalorimeter compensates for the small heat change detected. The molar ratio between the ligand and protein is gradually increased through a series of ligand injections. The protein gets more and more saturated, less binding of the ligand occurs, and the heat change starts to decrease until, ultimately, the sample cell contains an excess of ligand versus protein, bringing the reaction towards saturation.

Accurate measurement of released/absorbed heat is then used to determine:
- the **binding constants** (**Ka**);
- enthalpy (ΔH);
- entropy (ΔS);
- he reaction stoichiometry (n).

Thereby an entire thermodynamic parameter of the molecular binding can be obtained in an individual analysis by applying this technique.