



Kinetic & Affinity Characterization - Monoclonal Antibodies

OBJECTIVE

Determine on/off rates and equilibrium dissociation constants of myoglobin–antimyoglobin interactions.

CONCLUSIONS

- Real time, high quality measurements provide more detailed kinetic data for the binding event compared to other technologies such as RIA and ELISA.
- Attana A100® is a robust and accurate tool for determination of kinetic and affinity data.
- The developed method is in good agreement with literature data.

To further the understanding of molecular interactions it is useful to study their kinetic properties. For example, in the development process of new pharmaceuticals, kinetic experiments provide insights into potential drug candidates as well as to define lead targets. The Attana system can be used for real-time analysis of association and dissociation phases of a complex, enabling the study of kinetics and affinity.

ATTANA A100 BIOSENSOR

The Attana A100 biosensor utilizes the Quartz Crystal Microbalance (QCM) technique for real time, label-free measurements of molecular interactions. When molecules are added to, or removed from the sensor surface, the change in the resonance frequency corresponds to the change in mass on the surface. By immobilizing a target molecule to the sensor surface, and flowing an interacting molecule over the surface, the interaction can be studied in real time. The real-time information can provide kinetic, affinity and specificity data on the interaction.

METHOD

The antimyoglobin antibody was immobilized on an Attana carboxyl chip using an amine coupling run in a 1xHBST (10 mM HEPES, 150 mM NaCl and 0.005% Tween®) as running buffer, at a 10 µl/min flow rate. The carboxyl groups were activated for 3 min. with a 1:1 mixture of EDC and sNHS. The antimyoglobin antibody was then injected at 25 µg/ml in a 10 mM acetic acid solution, pH 4.5. The remaining active groups were deactivated by a 3 min. injection of 1 M ethanolamine. The immobilization process is displayed in **Fig. 1** and resulted in a frequency shift of 241 Hz. Subsequently a reference surface was prepared by activating (3 min. EDC-sNHS injection) and deactivating (3 min. ethanolamine injection) an Attana Carboxyl Sensor Chip.

In the kinetic experiment binding of myoglobin was monitored in a 1xHBST containing 0.005% Tween® 20, at a flow rate of 25 µl/min. Myoglobin was diluted in the running buffer and injected over the surface at different concentrations. The binding of myoglobin was registered, monitoring the association for 85 sec. and the dissociation phase for 300 sec. The surface was further regenerated with 1 pulse of 10 mM Glycine pH 2.5. A new binding curve at a different myoglobin concentration was then recorded. Data enabling a complete kinetic analysis were registered in this way. Reproducibility was tested by monitoring the binding curve for the same concentration, several times, to verify that the binding curves were similar. Non-specific binding was verified on the reference surface by injecting myoglobin at the highest concentration.

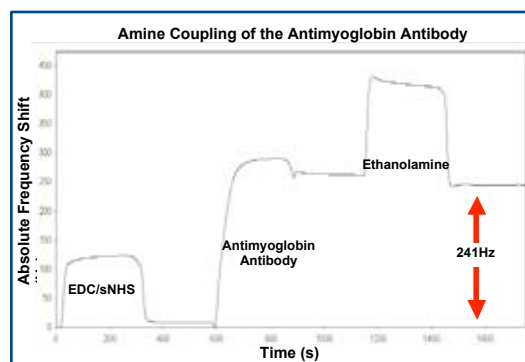


Figure 1: Immobilization of the antimyoglobin antibody.



Kinetic & Affinity Characterization - Monoclonal Antibodies

The equilibrium analysis was performed at a low flow rate, in order to achieve a long association time to reach equilibrium. Association was monitored for 300 seconds at a flow rate of 10 $\mu\text{l}/\text{min}$. As shown in **Fig. 2**, the model used for data analysis assumed a 1:1 binding between the antibody and the myoglobin molecules (Langmuir model). Kinetic data were globally fitted using the software Clamp XP*.

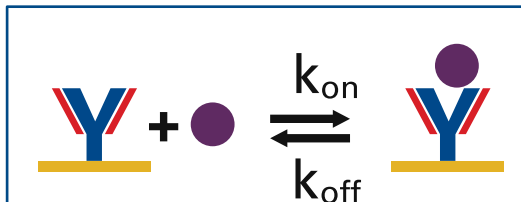


Figure 2: Schematic of the myoglobin–antimyoglobin interaction. The immobilized antibody interacts with the myoglobin forming a surface bound complex which is monitored in real-time by the sensor. The complex is formed at the association rate constant, k_{on} , and dissociates at the rate constant, k_{off} . The affinity of this binding is $K_A = 1/K_D = k_{\text{on}} / k_{\text{off}}$.

If equilibrium is reached, and Δf_{eq} are the frequency values obtained at equilibrium for each concentration C . The equation below describes their relationship with the affinity K_A :

$$\Delta f_{\text{eq}} / C_A = K_A (\Delta f_{\text{max}} - \Delta f_{\text{eq}})$$

The Scatchard plot, displaying $\Delta f_{\text{eq}} / C$ as a function of Δf_{eq} , if fitted to a linear curve, enables the determination of the affinity constant K_A , which is the slope of the line. Equilibrium data was fitted using the Attester™ Evaluation software.

RESULTS

Kinetic results: At a flow rate of 25 $\mu\text{l}/\text{min}$, no mass transport effects were observed. No nonspecific binding was detected on the reference surface. The highest concentration used, 18 nM, gave a frequency shift of 44 Hz, whereas the lowest concentration, 0.7 nM, resulted in a 4 Hz shift. Reproducibility between runs gave deviations of less than 10%.

A global analysis of the binding curves gave an association rate constant: $k_{\text{on}} = 5.7 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and a dissociation rate constant: $k_{\text{off}} = 6.4 \times 10^{-4} \text{ s}^{-1}$.

The affinity derived from this analysis was: $K_D = 1.1 \text{ nM}$ or $K_A = 9 \times 10^8 \text{ M}^{-1}$. Comparison with literature data derived in a RIA (1 nM) assay shows that the Attana instrument generates high quality data with good agreement to other methods.

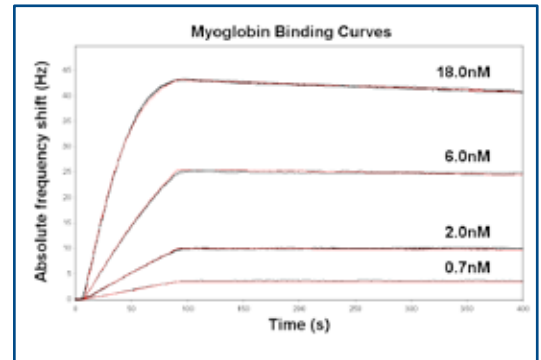


Figure 3: Myoglobin binding curves (black curves) and fit of the binding curves (red curves), using a simple 1:1 binding model.

Equilibrium analysis: The Scatchard plot in **Fig. 4** displays binding of myoglobin to the antimyoglobin antibody with an affinity constant of $K_D = 1.3 \text{ nM}$ or $K_A = 7.6 \times 10^8 \text{ M}^{-1}$. The affinity value derived from the equilibrium measurement is consistent with the value from the kinetics method, proving the robustness and accuracy of the method.

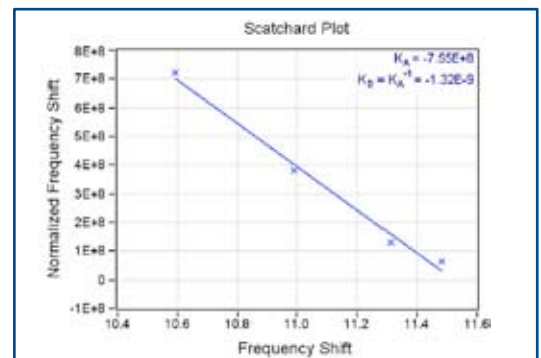


Figure 4: Scatchard plot of the equilibrium values using the Attester Evaluation software.

Attana Materials Used	Item Code
Attana Carboxyl Sensor Chip	3616-3033 (pack of 3)
	3616-3103 (pack of 10)
Amine Coupling Kit	3501-3001
HBS-T 10X (250 ml)	3506-3001
C-Fast: 3.1	3420-3001
Attester™: 3.1	3410-3001
Attester™ Evaluation: 3.1	3430-3001

Attana AB

Björnnäsv. 21. SE-113 47 Stockholm, Sweden
Telephone: +46 8 410 200 00, E-mail: info@attana.com
www.attana.com