

Kinetic Characterization of Ultra-High Affinity Antibodies

Summary

Directly characterizing the affinity of clinical antibody candidates poses a challenge, as the rate of dissociation is often very slow. In this application note, we demonstrate that the SKi Pro label-free biomolecule interaction platform is uniquely suited for accurate characterization of ultra-high affinity interactions in flow cell mode. In particular the real-time measurement of k_{off} in the range of 10^{-5} – 10^{-6} s^{-1} is demonstrated. We show examples of successful measurements and provide assay development guidelines based on numerous in-house and field-completed projects, first for a polyclonal antibody and then for four monoclonal antibodies.

Introduction

Biologics have quickly become a focus area in drug discovery and development. New biotherapeutics are often designed and optimized for very tight binding to their molecular targets. Accurate measurement of these ultra-high affinity interactions, specifically of very slow dissociation rates, is a challenge. Historically, Surface Plasmon Resonance (SPR) has been the most used technology in kinetic characterization of biomolecular interaction. However, one of the inherent drawbacks of SPR is that its gold-hydrogel surface is not stable enough for long measurements of slow dissociation. SKi Pro technology uses a silanized and PEGylated nanoporous silicon biosurface that is more stable and allows for the industry's lowest signal drift of <0.1 nm/hr^a. In addition, SKi Pro flow cell instruments are equipped with two continuous-flow pumps, as opposed to the syringe pumps gener-

ally included in competitive instruments. This enables smooth data acquisition without artifacts caused by syringe pump switchover and restroke. Here we show examples of accurate kinetic characterization for high-affinity, antibody-antigen binding and provide guidelines for successful measurement of slow dissociation rates.

Method

All experiments were done on a SKi Pro, X10 flow cell instrument, using SKi Sensor Carboxyl chips. Antibodies were directly immobilized on chip using EDC/sulfo-NHS chemistry by a standard protocol, at pH 4.5, with 100 μ g/mL of antibody. Appropriate control immunoglobulins, generally non-binding antibodies of the same iso-type, were immobilized at the same concentrations on the reference channels of the flow cartridges. Antigen binding and dissociations were carried out in PBS (pH 7.4) at 5 μ L/min flow rate per channel. This is performed at the concentrations shown.

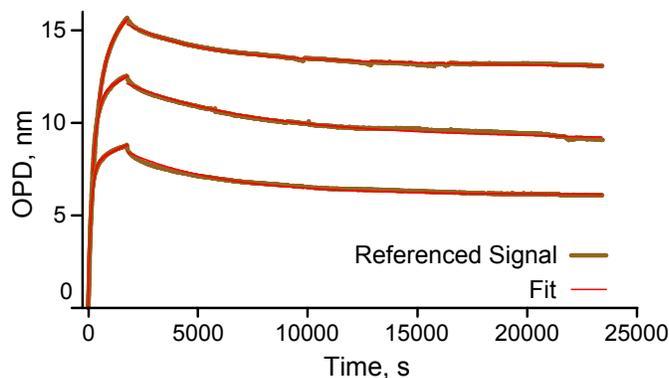


Figure 1: Kinetic characterization of a goat anti-human IgG Fc-specific antibody. Results of 3 separate runs are shown.

^aIn SPR terms this is equivalent to less than <0.08 RU/min

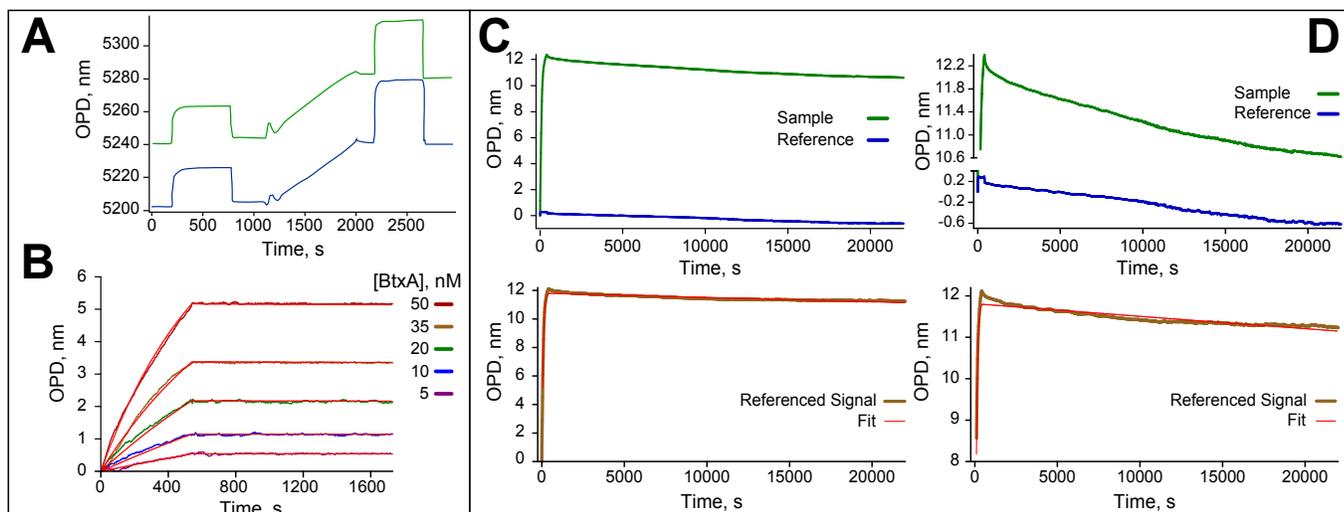


Figure 2: **A.** Anti-BtxA and control antibody immobilization. **B.** A multi-concentration binding experiment with 20 minutes dissociation times. **C-D.** A single concentration binding experiment with 6 hours dissociation time. Whole range traces are shown on **C** and narrow range dissociation traces on **D**. Sample and reference traces are shown on the upper panels and referenced signal on lower panels.

Anti-botulinum neurotoxin A (Btx) and anti-anthrax protective antigen (PA) recombinant antibodies are provided by Planet Biotechnology Inc., Hayward, CA, Anti-staphylococcal enterotoxin B (SEB) single chain Fv antibody fragment is a gift from Dr. Eric Sundberg of the Boston Biomedical Research Institute, Watertown, MA. Anti-human Angiopoietin-2 antibody is provided by CovX Research, San Diego, CA. Carboxyl coupling reagents (P/N SUPPLY-0309) and sensor chips (SKI-SENSOR-FC-8PACK-C) are from Silicon Kinetics. All other reagents were purchased from Sigma-Aldrich, St. Louis, MO.

Results

High affinity polyclonal antibody

While monoclonal antibodies are capable of high affinity interactions and are homogenous binders, the development of a ‘good’ monoclonal antibody can be a tedious process with no guarantee of success. In some cases, a polyclonal antibody could be a better choice for analytical applications. Affinity purification and pre-absorption can result in a tight-binding and highly specific polyclonal antibody batch. Affinity and apparent off-rate measurements are necessary to qualify such an antibody for its intended use.

Here we show an example of kinetic characterization of a goat anti-human, IgG Fc-specific antibody (Fig. 1). A different goat polyclonal antibody is immo-

bilized in the reference channel (immobilization data not shown).

The binding-dissociation curves in this case look different from that of monoclonal antibodies. This reflects the fact that there is a number of different binders in a polyclonal antibody preparation. Fitting these curves using a single binding site model, while possible, results in an uninformative low fidelity fit. Use of a two-binding sites model provides for much better fit shown in Fig. 1.

Results in the Table 1 show that the slow dissociation component, k_{2off} , is very low and is similar to the off-rates of high affinity monoclonal antibodies. These results demonstrate that this antibody would be a good choice for most analytical applications, especially as a primary antibody for capture of a human antibody of interest in a proper orientation before antigen binding.

Table 1: Results of a two exponential fit to the off rate section of Fig. 1. Uncertainties shown are 3σ .

	k_{1off}, s^{-1}	k_{2off}, s^{-1}
Run 1	2.92×10^{-4} (6)	1.23×10^{-6} (9)
Run 2	1.77×10^{-4} (6)	1.20×10^{-6} (15)
Run 3	2.34×10^{-4} (6)	1.26×10^{-6} (12)

Kinetics of monoclonal antibodies

A recombinant anti- Anti-botulinum neurotoxin A (Btx) antibody is immobilized in the sample channel of a car-

Table 2: Kinetic parameters derived from non-linear least squared, global fits to the sensorgrams for the four antigens studies. In the case of Botulinum Toxin, the results are the average of four separate runs.

Antigen	Antibody Type	Immobilized	$k_{on}, M^{-1}s^{-1}$	k_{off}, s^{-1}	K_D, pM
Botulinum neurotoxin A	Modified human IgG	Antibody	4.28×10^4	2.47×10^{-6}	58
Anthrax protective antigen	Modified human IgG	Antibody	1.93×10^4	1.63×10^{-5}	850
Staphylococcal enterotoxin	Single chain Fv	Antigen	5.61×10^4	5.90×10^{-6}	110
Human Angiopoietin-2	Humanized IgG	Antibody	8.64×10^4	1.10×10^{-5}	130

boxyl SKi Sensor chip using EDC/sulfo-NHS chemistry (Fig. 2A). A very similar recombinant antibody against a different antigen is immobilized in the reference channel. A typical multi-concentration binding experiment shows strong antibody-antigen interaction (Fig. 2B) with no measurable dissociation during the 20 minute periods used in this experiment.

Alternatively, a binding experiment is set up at a single concentration with a long, six hour dissociation (Fig. 2C–D). While the reference channel trace shows some low signal drift (upper panels), the referenced signal (lower panels) allows for accurate calculation of dissociation rate k_{off} .

Four antibodies are characterized in this way, supplied by organizations actively involved in their manufacture. These monoclonal antibodies were analyzed similarly, using covalent immobilization followed by sensorgram characterization. In Table 2, we show the results of kinetic measurements for a number of high affinity antibodies that were characterized at SKI in a similar fashion. In the case of anti-BtxA/BtxA the experiment is repeated four times and the results are shown in Table 3 below.

Table 3: Kinetic fit results for 4 separate long runs of anti-BtxA/BtxA as shown in Fig. 2. k_{on} value of $4.23 \times 10^5 M^{-1}s^{-1}$ is a result of a global fit for the multi-concentration run experiment. Uncertainties shown are 3σ .

	k_{off}, s^{-1}	K_D, pM
Run 1	2.46×10^{-6} (12)	57 (24)
Run 2	2.23×10^{-6} (9)	55 (18)
Run 3	2.47×10^{-6} (18)	58 (33)
Run 4	2.61×10^{-6} (24)	61 (42)

Discussion

By providing a stable surface with no flow rate aberrations over hours, the SKi Pro X10 allows for high qual-

ity measurement of slow off rates for both monoclonal and polyclonal antibodies, using direct immobilization. In addition to allowing rate quantification, the dissociative phase's form also gives information about the heterogeneity of the interaction as the biphasic dissociation for polyclonal antibodies shows.

However, when these off rates $\leq 1 \times 10^{-5} s^{-1}$ are to be characterized, care needs to be taken during both the setup and the measurement. Generally the most important guidelines are:

- Clean/sanitize the system before the experiment. That eliminates possible artifacts caused by proteins non-specifically bound to fluidic system components.
- Immobilization of a proper control protein in the reference channel is critical for accurate referencing. Immunoglobulins of the same specie and subtype are recommended for regular antibodies. For recombinant antibodies it would be better to use a different antibody with the same framework. For non-antibody binding partners it is recommended to use a non-binding mutant, if available, or, at least, a same family protein with no cross-reactivity toward the second binding partner.
- If indirect capture of the first binding partner is preferable, run a control experiment to measure dissociation of the captured protein. It is recommended to do covalent cross-linking of the captured binding partner in order to stabilize binding surface (see Application Note 9).
- Equilibrate new chip for 30 minutes before starting immobilization.
- If your binding buffer contains carrier protein (typically 0.05–0.1% BSA) or detergent, equilibrate the chip for 60 minutes in binding buffer

after immobilization. Otherwise, do 5 blank injections of sample dilution buffer.

- Do at least 3 binding-regeneration cycles before starting a long-dissociation experiment.
- Use flow rate within 5–10 $\mu\text{L}/\text{min}$ per channel rate for the long dissociation step.
- Use temperature control feature for the whole run.

Make sure that the ambient temperature is fairly constant during the run and is too far from the set flow cell temperature. Fast changes in ambient temperature or strong drafts can lead to artifacts due to slow compensation.

When these guidelines are followed high quality, slow dissociation data may then be obtained for a wide variety of antibody/antigen systems.

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