Kinetic Characterization of Antibody/Antigen Interactions

Summary

The SKi Pro System was utilized to characterize several monoclonal antibodies. Serial dilutions of antigens specific for the antibodies were analyzed in real time in the SKi Pro flow cell, in order to determine the on rate (k_{on}), off rate (k_{off}) and affinity (K_D) of the antibody/antigen interaction. In addition, the limit of detection (LOD) of antigen concentration was calculated for EGF.

Introduction

A leading application of biomolecular interaction analysis technology is the characterization of antibody-antigen interactions. By measuring the association and dissociation rates of the interaction, antibodies with desired binding characteristics can be identified. A rapid association rate and a slow dissociation rate may be indicative of therapeutic efficacy. Affinity maturation programs exist to develop antibodies with improved binding affinity, which can be quantitatively ranked using biomolecular interaction analysis technology.

Antibodies are useful diagnostic reagents, and are frequently utilized as tandem pairs in a sandwich assay format. In a common sandwich assay format, one antibody serves to capture analyte, and the second antibody, binding to a different epitope on the analyte, serves to directly or indirectly carry the detection label. One advantage to sandwich assays is increased sensitivity via signal amplification by the second binding antibody. While a sandwich assay format may be desirable, a second antibody partner is not always available. A single ligand assay design can be utilized in cases where a sandwich assay design is not feasible. In a single ligand assay, one antibody captures analyte, and detection can occur by labeling the analyte, or by using label-free detection technologies.[1]

Conventional biomolecular interaction analysis technologies such as surface plasmon resonance (SPR) can enable a single ligand assay format, but may not deliver adequate analyte detection sensitivity. Antibodies immobilized in the 100–200 nm deep hydrogel biosurface are typically immobilized as a monolayer, limiting the analyte capture capacity of this technology.

Nanopore optical interferometry (NPOI), on the other hand, utilizes a 1.5–2.0 μm deep nanoporous silicon biosurface, increasing the surface area available for interaction analysis by 100 fold over SPR. Ligands are immobilized in multiple layers in the nanoporous silicon biosurface, increasing the analyte capture capacity and the sensitivity of this technology.

NPOI is used to measure antibody–antigen interactions in real time in a label–free format. In addition to determining antigen concentration, SKi Pro can characterize the kinetics of the antibody–antigen binding interaction, quantitating the on rate (k_{on}), off rate (k_{off}) and affinity (K_D) of the antibody to its antigen.

This application note demonstrates the use of SKi Pro to characterize the binding of whole molecule antibodies to their protein antigens. Antigens of molecular weights from 6–83 kilodaltons were analyzed. The sensitivity of this methodology is further illustrated by measuring the limit of detection (LOD) of the antibodies to concentrations of the smallest of these analytes.

Method

The work below describes four antibody/antigen pairs whose antigens are Fas Associated protein with Death Domain (FADD), Epidermal Growth Factor (EGF), Human Growth Hormone (HGH) and leptin. The antibod-
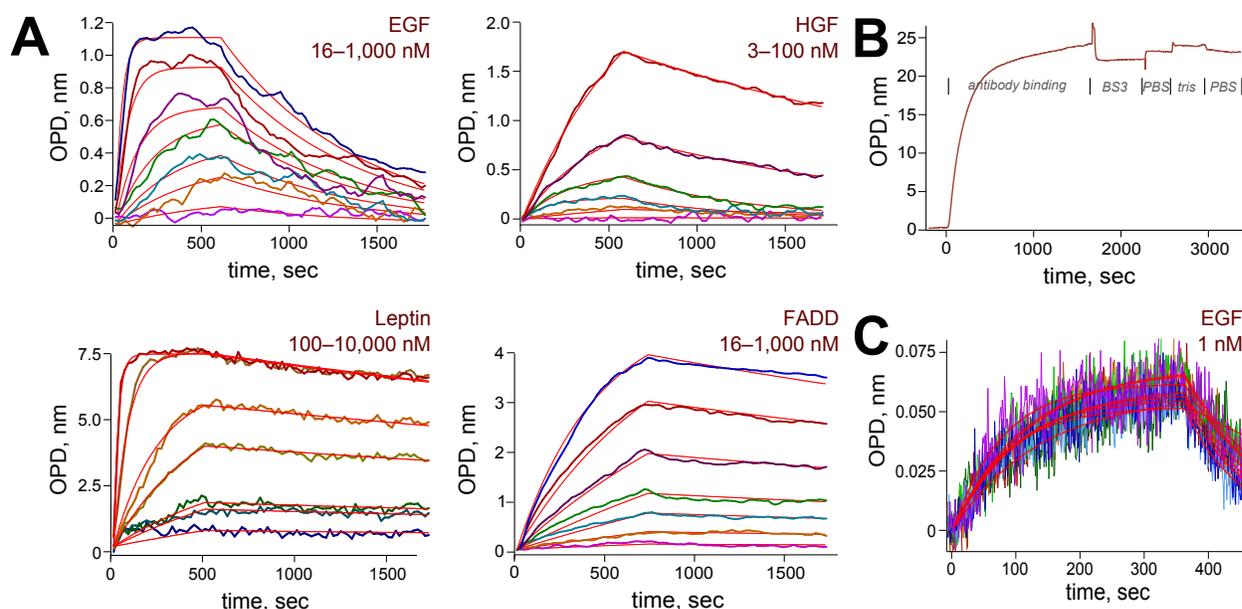
Figure 1: A Sensorgrams of the four antibodies studied here. Solid red lines are the two state fit which resulted from analysis with SKi Report, no refractive index term was included in this fit. **B** Immobilization of a-EGF using protein G chips with BS3 cross linking. **C** Limit of detection level data quality shown for EGF.

ies and protein antigens were obtained from the Sigma–Aldrich Corporation (St. Louis, MO). Except for Leptin, the antibodies were captured on the SKi Sensor Protein G biosurface, at 100 μg/mL in PBS and covalently immobilized utilizing BS3 (Pierce Biotechnology, Inc., Rockford, IL) cross-linking at 5 mM in water for 400 seconds, rinsing with PBS, quenching with 50 mM tris and again washing with PBS. This gave, on average, OPD=20 nm of surface coverage for the antibodies which corresponds to 1 ng/mm^2. Cross-linking prevents clone-specific dissociation of the antibody from the Protein G and allows multiple regenerations of the binding surface. Anti-leptin antibody was directly immobilized on carboxyl chips using EDC/sNHS activation (200 mM/50 mM in water) at 100 μg/mL in pH 4.5 acetate giving a OPD=40 nm surface (2 ng/mm^2).

Serial dilutions of the analytes were injected under flow rates of 10 μL/min and binding interactions to the immobilized antibodies recorded in real time. Kinetics of protein binding and dissociation were measured in the low nM to 1 μM concentration range, in PBS/0.05% BSA. For the EGF antibody, dissociation of the antigen was spontaneous and complete within the allowed 1,200 seconds, whereas for HGF, FADD and leptin antibodies dissociation proceeded spontaneously for 1,200 seconds and then chips were regenerated for 60 seconds with 10 mM glycine (pH 2.0). Binding parameters were determined by a global fit of all curves using a simple two state fit without any refractive in-

dex correction or diffusion terms using SKi Report, the software package which comes with the SKi Pro system. Relative uncertainties on the fitting parameters were 2–3%.

Results

Data from the several runs are shown in Figure **1A**, where both the raw data and two state fits ($Ab + Ag \rightleftharpoons AbAg$) are shown. Of course antibodies are expected to be bivalent, however as one does not anticipate allostery in the interaction of the antigen and antibody, the fact that the interaction is bivalent need not be directly considered. During the fit, it as if there is simply twice as much protein on the surface.

The stabilizing effect of BS3 crosslinking is illustrated in Figure **1B**. Here the binding of the antibody to protein G is shown to occur but the antibody is also shown to dissociate from the capture protein quickly enough that it would prevent regeneration during the binding runs. Crosslinking this complex with BS3 (Bis(Sulfosuccinimidyl) suberate) renders the surface stable, even under the low pH conditions used here for progressive regenerations.

For the a-EGF interaction with 6 kDa analyte, 8 separate runs were performed at low, 1 nM (6 ng/mL) concentration, which is a concentration > 50× below the affinity of the antibody. At this concentration the

signal level is still three times above the noise without any smoothing, and the signal amplitude has a CV of 8%.

Discussion

The current work illustrates the utility of the SKi Pro system in characterizing the binding of antibodies to protein antigens. Four monoclonal mouse antibodies were characterized, and k_{on}, k_{off} and K_D data obtained. Repeat analyses demonstrated excellent repeatability of measurements using the SKi Pro system. SKi Pro was shown to detect small protein molecules to the 6 ng/mL level with an antibody of only moderate affinity, showing its potential for single ligand immunoassay applications. The results of the characterization are summarized in Table 1

The aEGF-EGF system was measured once a month for three months using a different lot of anti-body from the manufacturer for each time. Throughout this set of data kinetic parameters varied by a factor of two, whereas the affinity constant varied by <10%.

Table 1: Results of the two state kinetic fit to the Rituxan and Campath experiments here.

	k_{on}, M^{-1} sec^{-1}	k_{off}, sec^{-1}	K_D, nM
a-TSH	9.0×10^4	1.1×10^{-3}	12
a-HGF	9.5×10^3	4.5×10^{-4}	48
a-EGF	2.6×10^4	1.3×10^{-3}	51
a-Leptin	3.9×10^3	1.3×10^{-4}	33
a-FADD	2.1×10^3	1.7×10^{-4}	80

References

[1] J.M. Berg, J.L Tymoczko, and L. Stryer. *Biochemistry*. W. H. Freeman, 2002.

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