APPLICATION NOTE

nicoya

Label-Free Characterization of the Interleukin-6 Receptor and Anti-IL-6R Antibody Drug Tocilizumab Using Alto™ Digital SPR

Overview

Therapeutic monoclonal antibodies are one of the fastest growing classes of drugs, and interleukins and their signal pathways are a preeminent target for treatments due to the vast number of problems their improper regulation present. Surface plasmon resonance (SPR) is a powerful tool that is well-suited for analyzing the binding interactions associated with signaling proteins, their receptors and related effectors. In this application note, we demonstrate Alto's applicability to the development of monoclonal antibody therapeutics, with a focus on interleukin and interleukin-related therapies.

Introduction

Interleukins are a group of proteins involved in critical immune cell functions such as proliferation, differentiation, and adhesion. They are of particular importance in coordinating immune-related stresses such as inflammation. Interleukins are a type of cytokine, which is a broad term encompassing small signaling proteins involved in modulating various types of cell communication. Once secreted by cells, they travel to their target cell where they bind to their receptor, triggering their respective downstream functions. Playing such critical roles in immune and stress responses, dysregulation of interleukins can result in debilitating conditions such as autoimmune diseases. In some cases, interleukins themselves are even used as therapeutics. For example, analogues of interleukin 2 are used to treat malignant melanoma and renal cell cancer, and interleukin 7 is used in antiretroviral therapies and for immune recovery from stem cell transplants. This application note will focus specifically on interleukin-6 and the related drug tocilizumab, which is used to treat chronic inflammation conditions.

Interleukin-6 (IL-6)

IL-6 is expressed in response to various environmental stress factors such as infection by a pathogen or damage to cell tissue. IL-6 essentially acts as an alarm system, warning the body of stress so that it can activate the appropriate defense mechanisms. There are a number of transcription factors responsible for IL-6 expression and its subsequent downregulation upon sequestering of the stress. The improper regulation of IL-6 and its receptor, IL-6R, by these transcription factors can result in the development of several diseases including multiple myeloma and prostate cancer. Inflammation is a common reaction to environmental stress, therefore this behavior is particularly common in autoimmune diseases characterized by chronic inflammation such as rheumatoid arthritis and systemic juvenile rheumatoid arthritis.

Mechanism of Action (MoA) and Treatment

The IL-6/IL-6R receptor–signaling system is made up of two receptor chains and two downstream signaling molecules. The IL-6R constitutes the receptor domain, and occurs in both a soluble, cytoplasmic form and a transmembrane form. The signal transducing domain is the glycoprotein gp130. After binding of IL-6 to IL-6R, the resulting complex induces homodimerization of complexed gp130 and triggers a downstream signal cascade.

With this MoA in mind, one method scientists have used to treat chronic inflammatory conditions where IL-6 is implicated is to block IL-6 from binding IL-6R. Tocilizumab is an FDA approved, monoclonal anti-IL-6R antibody with high binding affinity, allowing it to outcompete IL-6 to bind IL-6R (Figure 1). It is commonly used to treat rheumatoid arthritis and systemic juvenile rheumatoid arthritis. Therapeutic monoclonal antibodies are one of the fastest growing classes of therapeutics for a wide range of diseases and cancers, highlighting the importance of developing and characterizing these types of systems.

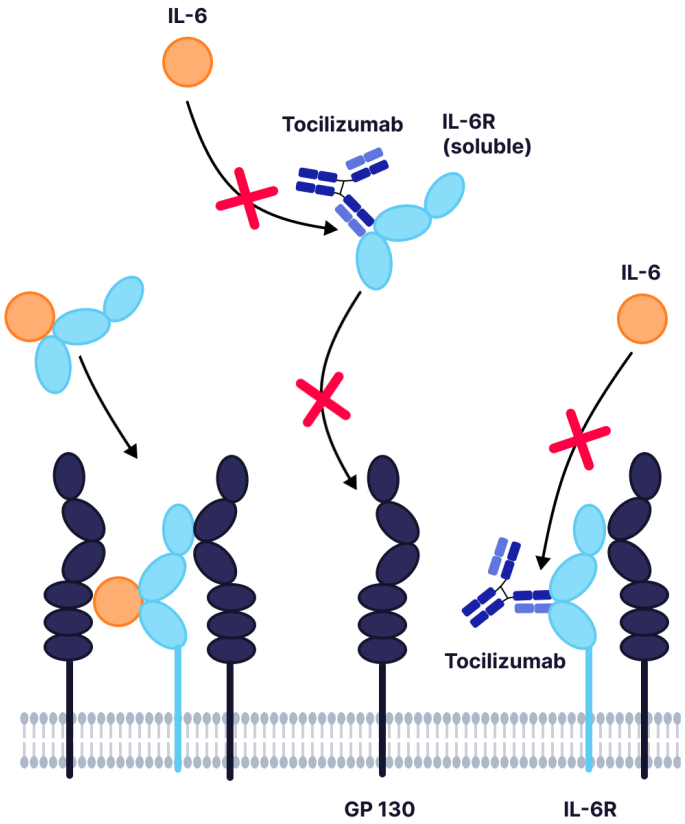

Figure 1: *Inhibition of IL-6 Signaling by Tocilizumab. IL-6 binding to IL-6R or the soluble IL-6R (sIL-6R) causes the dimerization of gp130 and triggers a downstream signal cascade. Tocilizumab binds to the IL-6R (or sIL-6R), preventing the binding of IL-6, which mediates IL-6 trans-signaling and reduces the chance of a cytokine storm.*

Characterizing Interleukins and Antibody Drugs with SPR

To facilitate the development of interleukin therapeutics, therapeutics targeting the dysregulation of interleukins, and therapeutic monoclonal antibodies in general, highly selective and specific bioassays that adequately reflect the proposed MoA are needed from discovery, through development, and into production. Surface plasmon resonance (SPR) is a powerful tool that meets these needs and is well-suited for analyzing the binding interactions associated with signaling proteins, their receptors and related effectors. In particular, therapeutic monoclonal antibodies are well suited to SPR due to their large size and high affinity binding. Traditional techniques such as ELISA and western blot (WB) require time-consuming washing and incubation steps, and depend on the use of tags for analysis. Compared to such traditional techniques, the information content provided by SPR enables increased efficiency in the screening and the characterization of potentially life-saving drugs.

Advantages of SPR:

- No need for additional labels, eliminating the introduction of another irrelevant binding event to account for and design around.
- Acquisition of real-time binding data that enables deeper insight into the binding kinetics of monoclonal antibody drugs or other targets rather than just the affinity.

Accelerate mAb Discovery with Digital SPR

Alto, a high-throughput SPR platform enabled with digital microfluidic (DMF) technology, has a number of advantages that make it ideal for SPR characterization of monoclonal antibody drugs and targets. With the ability to discreetly control nano-liter sized droplets of each ligand and analyte, binding assays are simple to implement and provide novel insights into therapeutic performance, while reducing consumption of precious samples by up to 200X. In addition, Alto's 16 independent channels provide the ability to simultaneously analyze multiple targets in many different assay formats, while significantly reducing hands-on time with complete assay automation.

In this application note, we demonstrate the applicability of Alto in measuring binding affinities and kinetics of IL-6 and the antibody drug tocilizumab through several experimental approaches.

Materials & Equipment

- Nicoya Alto 16-Channel Instrument
- Alto 16-Channel Carboxyl Cartridge (KIN-CART-CBX-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Ligands, analytes and capture molecules:
    - IL-6 (Sino Biological, Cat: 10395-HNAE) and
    - IL-6R (Sino Biological, Cat: 10398-H49H-B),
    - Tocilizumab (Selleckchem, Cat: A2012),
    - ChromoTek Nano-CaptureLigand™ human IgG/rabbit IgG, Fc-specific VHH, biotinylated (Proteintech, Cat:. shurbGB-1-100)

Method

Instrumentation

This label-free SPR assay was performed using Alto, the first and only digital microfluidic (DMF) powered SPR. Alto uses a cartridge-based, gold nanostructure sensor with 16 channels (8 reference channels and 8 active channels). The experimental method was designed remotely using Alto's cloud-based user portal, the Nicosystem, which was pushed to the instrument with a click of a button.

Method

First, a 16-Channel Carboxyl Cartridge was loaded into Alto followed by dispensing of the cartridge fluid into the cartridge. All the reagents were pipetted into the cartridge wells following software-directed prompts, and since Alto is an automated SPR instrument, only a single top concentration of each analyte sample was pipetted.

After all the reagents were loaded into the cartridge, the experiment was initiated by selecting the "Run Method" command on Alto. All subsequent steps were automated by Alto.

In all experiments, normalization of sensors with high and low RI droplets was first performed. The sensors were then cleaned with 10 mM HCl for 60 s, followed by a 5 minute activation of the 16 carboxyl sensors with a drop consisting of a 1:1 ratio of 25 mM EDC/NHS mixed on cartridge.

For IL-6/IL-6R kinetics with IL-6 as ligand, the IL-6 ligand at 100 µg/mL in acetate pH 5.5 was passed over the 8 active channels for 5 minutes and was immobilized on the activated carboxyl surface.

For the IL-6R analyte, 2 µL of 450 nM solution was loaded into each analyte well, and the mixing of five 3-fold serial dilutions per analyte was automated by Alto. The concentrations of IL-6R used for binding were 1.85 nM, 5.56 nM, 16.7 nM, 50 nM and 150 nM. Replicates of the IL-6R analyte were introduced in a single cycle kinetics (SCK) format with an association time of 200 s, without dissociation or regeneration between each sample, starting from the lowest to highest concentration. A dissociation time of 600s was set to run after the highest concentration. The sensor surface was regenerated with a 60 s exposure of 10 mM Glycine-HCl pH 1.5, which resulted in complete removal of the analyte.

For IL-6/IL-6R kinetics with IL-6R as ligand, streptavidin in acetate pH 5.0 was passed over all 16 channels for 5 minutes and was immobilized on the activated carboxyl surface. Next biotinylated IL-6R at 10 µg/mL was passed over the 8 active channels for 5 minutes. The same protocol for the analyte and regeneration were used as above, with the same concentrations used for the IL-6 analyte.

When studying tocilizumab binding IL-6R, a capture protocol was used. This follows the same normalization and cleaning steps as above, but begins with the building of a capture surface that is used to immobilize the ligand. The capture molecule, bt-NanoLink Nano-CaptureLigand™ human IgG/rabbit IgG, Fc-specific VHH, was passed over all 16 channels for 5 minutes and was immobilized on the activated carboxyl surface. The ligand, tocilizumab, was passed over the 8 active channels for 60 s and was immobilized on the capture molecule.

The same protocol for the analyte and regeneration were used as above, except concentrations of 1.23 nM, 3.70 nM, 11.1 nM, 33.3 nM and 100 nM of tocilizumab were analyzed.

Results & Discussion

The interaction of IL-6R with IL-6 was investigated by SPR on the Alto. Single cycle kinetics (SCK) binding assays were acquired both for the binding of IL-6 to IL-6R immobilized on a streptavidin surface, as well as for IL-6R binding to directly immobilized IL-6. All of the data was fit to a one-to-one binding kinetic model. Kinetic rates and affinity values are presented in Table 1. When IL-6 was used as the analyte with IL-6R, association and dissociation rate constants of 7.46×10^4 M^1s^1 and 2.08×10^{-3} s^{-1}, respectively, were determined, resulting in a K_D of 32.2 nM (Figure 2). This is in close agreement to the previously determined SPR K_D value of 47 nM[1].

When IL-6R was used as the analyte, a higher affinity K_D value of 6.35 nM (k_a and k_d values of 1.09×10^5 M^1s^1 and 5.56×10^{-4} s^{-1}, respectively) was determined (Figure 3). This increase in affinity (due to the slower dissociation), could be due to closely packed IL-6 immobilized on the sensor surface, mimicking dimerization of the IL-6, which has previously been shown to have an increased affinity for the interaction of IL-6R with dimerized IL-6[1].

The interaction of tocilizumab binding to IL-6R was performed using a single cycle kinetics capture method

on Alto. Tocilizumab capture on a human IgG/rabbit IgG, Fc-specific VHH prepared surface was optimized for IL-6R binding kinetics by tuning the immobilization level of tocilizumab to ~200 RU (Figure 4a). Binding of automated dilutions of IL-6R determined association and dissociation rate constants of 1.41 x 10^5 $M^{-1}s^{-1}$ and 3.62 x 10^{-5} s^{-1}, respectively, resulting in a K_D of 293 pM (Figure 4b, Table 1), which was similar to the previously determined SPR K_D value of 612 pM[2]. The kinetic data shows that Alto was able to obtain accurate kinetics and affinity values by analyzing up to 48 data points in a single cartridge. The SCK experiments were completed in under 5 hours, requiring only 30 minutes of hands-on time for setup.

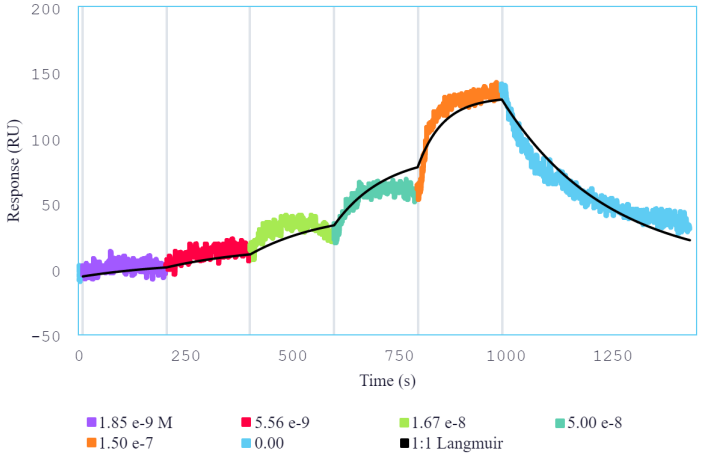

Figure 2: *Single-cycle kinetics of IL-6 (analyte) binding to immobilized IL-6R (ligand) on Alto. Analyte was titrated from 1.85 nM to 150 nM. Black curve is the Langmuir 1:1 binding fit model analyzed in the Nicoya Analysis Software.*

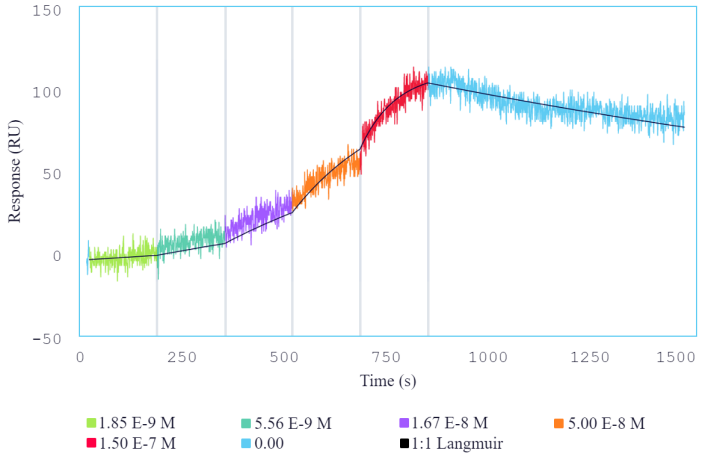

Figure 3: *Single-cycle kinetics of IL-6R (analyte) binding to immobilized IL-6 (ligand) on Alto. Analyte was titrated from 1.85 nM to 150 nM. Black curve is the Langmuir 1:1 binding fit model analyzed in the Nicoya Analysis Software.*

Table 1: Kinetic parameters measured for the interactions of the IL-6/IL-6R and IL-6R/tocilizumab systems using Alto.

Ligand	Analyte	K_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)
IL-6R	IL-6	7.46E+04	2.08E-03	32.2
IL-6	IL-6R	1.09E+05	5.56E-04	6.35
Tocilizumab	IL-6R	1.41E+05	3.62E-05	0.293

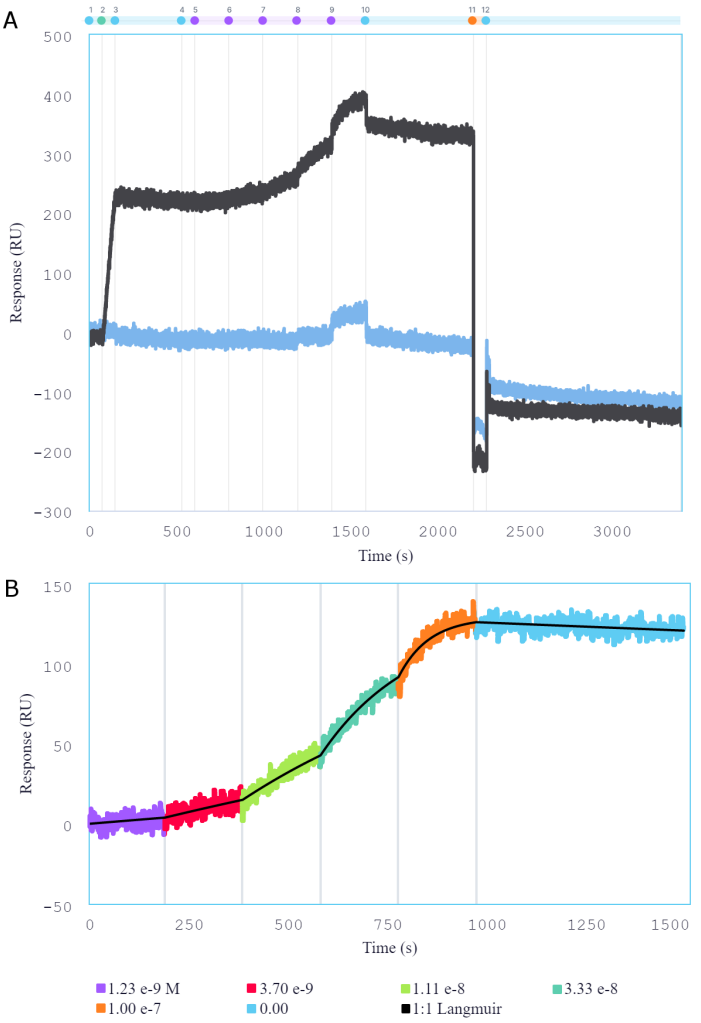

Figure 4: A. *Reference (blue trace) and active channel (black trace) binding data (with drop timeline above) showing capturing of tocilizumab (green drop 2) to the human IgG/rabbit IgG, Fc-specific VHH surface followed by binding of the IL-6R analyte (purple drops 5-9 - titrated from 1.23 nM to 100 nM). Full regeneration is achieved by 10 mM glycine HCl pH 2.0 (orange drop 11 returning the response to the baseline before tocilizumab capture).*
B. *Single-cycle kinetics of IL-6R (analyte) binding to tocilizumab (ligand) captured on a human IgG/rabbit IgG, Fc-specific VHH surface on Alto. Analyte was titrated from 1.23 nM to 100 nM. Black curve is the Langmuir 1:1 binding fit model analyzed in the Nicoya Analysis Software.*

Conclusion

An analysis of multiple interactions of different molecules with IL-6R with Alto demonstrated the platform's ability to characterize a wide range of affinities while providing comparable data to conventional SPR systems. All aspects of the experiment were automated by Alto using DMF technology, allowing all analysis to be conducted on a single cartridge with just a fraction of typical sample requirements, while requiring just 30 minutes of hand-on time with the instrument. The resulting data highlights Alto's ability to provide high-quality data while reducing time to answer, proving its ability to accelerate the development of therapeutic classes implicating interleukins and their related receptor molecules.

References

1. Ward LD, Hammacher A, Howlett GJ, Matthews JM, Fabri L, Moritz RL, Nice EC, Weinstock J, Simpson RJ. Influence of interleukin-6 (IL-6) dimerization on formation of the high affinity hexameric IL-6.receptor complex. J Biol Chem. 1996 Aug 16;271(33):20138-44. doi: 10.1074/jbc.271.33.20138. PMID: 8702737.
2. Lyman M, Lieuw V, Richardson R, Timmer A, Stewart C, Granger S, Woods R, Silacci M, Grabulovski D, Newman R. A bispecific antibody that targets IL-6 receptor and IL-17A for the potential therapy of patients with autoimmune and inflammatory diseases. J Biol Chem. 2018 Jun 15;293(24):9326-9334. doi: 10.1074/jbc.M117.818559. Epub 2018 Apr 20. PMID: 29678878; PMCID: PMC6005441.