Label-free characterization of a cancer-associated transmembrane protein using Alto™ digital surface plasmon resonance

Summary

Many human diseases are known to be associated with transmembrane proteins (TPs), making them ideal candidates for drug development. Multi-pass transmembrane proteins are not stable outside of the cell membrane environment, and are therefore difficult to purify and to express in large quantities. This has resulted in the under-characterization of a critical group of biomolecules. One strategy for overcoming these challenges is to use vectors that closely mimic cell membranes, such as nanodiscs. In this application note, Alto Digital SPR was used to characterize binding kinetics of Claudin-18.2 in nanodiscs from ACROBiosystems, demonstrating its ability to provide high-quality data while significantly reducing precious sample consumption and time to results.

Introduction

Claudins and their link to cancer

Claudins are a family of small transmembrane proteins with a highly conserved structure, consisting of four transmembrane domains. They are important structural and functional components of tight junctions, which are responsible for cell-cell adhesion. Claudins regulate paracellular transport and are also involved in intracellular signaling. The upregulation, downregulation and mislocalization of Claudins have been associated with numerous types of cancers.[1] Due to their role in tight junctions and in cell signaling, dysfunction of these proteins can result in unregulated cell movement and proliferation. In cancer cells, Claudins are also known to be involved in signaling pathways directly related to metastasis and tumor progression.

Claudin-18 is one of the least understood out of the 27 known Claudins.[2] It has two splice variants, Claudin-18.1 which is found in the lungs, and Claudin-18.2 which is found in the stomach, specifically the gastric mucosa. In gastric cancers, Claudin-18.2 is upregulated, while in pancreatic, esophageal, ovarian, and lung cancers, it is present in tissues it would not normally be present in. The overexpression and inappropriate localization of Claudin 18.2 make it an attractive target for therapeutic intervention. Zolbetuximab, a monoclonal antibody against Claudin-18.2, is currently in Phase III clinical trials for treating gastrointestinal and pancreatic tumors. Claudin-18.2 is one of only two Claudins which have had antibody therapeutics go to clinical trials.[3,4] The success of zolbetuximab in treating cancer illustrates the importance of characterizing these proteins to optimize drug discovery.

Challenges in the characterization of transmembrane proteins

Transmembrane proteins (TPs) are involved in vital cellular functions including energy production, signal transduction and ion transportation. Because of their role in these important processes, TPs are the most common target for commercially available therapeutics.[5] Despite this, they are considerably less well characterized than their globular counterparts. This is because the hydrophobic nature of TPs makes them notoriously difficult to express, purify, and analyze. TPs are amphipathic in nature, and the hydrophobic domains result in them not being highly stable outside of their natural environment. This results in a protein that is highly prone to conformational shifts that can affect subsequent characterization and binding kinetics. Furthemore, when expressing TPs, finding an appropriate carrier form to extract TPs from cells is imperative to ensure preservation of structure and function for meaningful downstream analysis.

Nanodiscs

There are several strategies available to scientists for overcoming the challenges outlined above, one of the most successful being nanodiscs. Nanodiscs are discoidal lipid bilayers that range from about 7 nm to 50 nm in diameter (Figure 1). They are stabilized by amphipathic scaffolding proteins wrapped around the disc like a belt. Nanodiscs are self assembling, soluble, and create a native-like bilayer environment that maintains both structure and fucntion of TPs. These qualities make nanodiscs extremely powerful tools for studying TPs.

Using nanodiscs, ACROBiosystems has developed a full range of high-quality, full-length TPs with stabilized structure and high activity. Here we study kinetics using surface plasmon resonance (SPR) of human Claudin-18.2 stabilized in a nanodisc (Cat# CL2-H5586), and a human monoclonal anti-chimeric Claudin-18.2 antibody (Cat# CL2-M34).

Advantages of Alto

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 difficult to express proteins. Alto provides a label-free method that eliminates the need for a protien tag or label that could affect structure. 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.

DMF technology enables all fluidics to be disposable, allowing for precise handling of 2 µL sample volumes, crude sample compatibility, automated on-board serial dilution of analyte for multi-cycle titration, single cycle kinetics and zero fluidics maintenance. 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. Alto also minimizes operation time with simple design & analysis software. Acquisition of real-time binding data enables deeper insight into the binding kinetics of desired targets rather than just the affinity. Alto's sensors provide a versatile surface for capturing targets with protein A, streptavidin and others, allowing for characterization of a wide range of biomolecular systems from discovery to design.

In this application note, we demonstrate the applicability of Alto in measuring binding affinities and kinetics of human Claudin 18.2 in nanodiscs to a human monoclonal anti-Claudin 18.2 antibody.

Materials & Equipment

- Nicoya Alto 16-Channel Instrument (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KIN-CART-CBX-16)
- Running Buffer: HBS-TE (HBS with 0.1% Tween 20 and 3 mM EDTA), pH 7.4
- Alto CBX Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Regen Buffer: Gly-HCl pH 1.5: (ALTO-R-GLYHCl-1.5)
- Immobilization Buffer: Sodium Acetate pH 5.5 (ALTO-R-IMB-5.5)
- Capture Molecule: R&D Systems Inc. Human IgG Antibody, Cat# MAB11013-100
- ACROBiosystems Reagents:
  - Monoclonal Anti-Chimeric Claudin-18.2 Antibody, Human IgG1, Cat# CL2-M34
  - Human Claudin-18.2, His,Twin-Strep Tag (Nanodisc), Cat# CL2-H5586

Method

This label-free SPR assay was performed using Alto, the first and only 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 using the direct screening application on Alto's user portal, which was automatically uploaded to the instrument.

First, a 16-channel carboxyl cartridge was loaded into Alto followed by dispensing of the cartridge fluid into the cartridge. Reagents were pipetted into the cartridge wells following software-directed prompts. The experiment was then initiated by selecting the "Run Method" command on Alto. All subsequent steps were automated by Alto. The contents of each well are shown in Table 1.

Normalization of sensors with high and low RI droplets was performed first. The sensors were then cleaned with 10 mM HCl for 60 s, followed by a 5 min activation of the 16 carboxyl sensors with a drop consisting of EDC/NHS prepared from Nicoya's Surfacing Kit.

For creation of the capture surface, each sensor was exposed to 2 µL of the anti-IgG antibody at 50 µg/mL in sodium acetate pH 5.5 for 3 min. All sensors were then blocked with 1 M Ethanolamine for 5 min to quench remaining active carboxyl groups.

The ligand, monoclonal anti-Claudin 18.2 antibody, was passed over each of the 8 active channels for 3 min at 1 µg/mL in HBS-TE, pH 7.4. For the Claudin-18.2 nanodisc analyte, 2 µL of 600 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 Claudin-18.2 nanodisc used for binding were 2.47 nM, 7.41 nM, 22.2 nM, 66.7 nM and 200 nM. The 8 analytes were introduced in a multi-cycle kinetics (MCK) format with an association time of 200 s, followed by a dissociation time of 600 s after each concentration. The sensor surface was regenerated with a 60 s exposure of 10 mM Glycine-HCl pH 1.5 after each analyte concentration which resulted in complete removal of the analyte. The entirety of the test was completed in less than 8 h.

Upon completion of the test, binding curves were automatically fitted to a 1:1 binding model in the Nicosystem analysis software to determine kinetic and affinity constants.

Results & Discussion

Antibody capture with an anti-IgG antibody was chosen to ensure the anti-Claudin-18.2 antibody on the sensor surface was in an optimal orientation to bind to the Claudin-18.2 nanodiscs. Figure 2 shows the anti-IgG antibody capture of about 2000 RU.

Approximately 100 RU of the anti-Claudin-18.2 antibody was captured in each cycle of each lane. A small amount of bulk shift and NSB were observed in the reference channel, which was subtracted from the active channel for kinetic analysis. Full regeneration was achieved with 10 mM glycine-HCl, pH 1.5. A representative example of raw data from a single lane is shown in Figure 3.

The binding and kinetic fits of the Claudin-18.2 nanodiscs to the captured anti-Claudin-18.2 antibody resulted in high quality binding curves and kinetic parameter evaluations. Examples of the resulting MCK overlays are presented in Figure 4. The data were fit to a 1:1 Langmuir binding model and the calculated kinetic constants are presented in Table 2. The affinity constant of 14.7 nM that was determined on Alto closely matches with the value of 12.6 nM determined by ACROBiosystems on a conventional SPR instrument (Figure 4).

Conclusion

Alto was able to successfully characterize ACROBiosystems' Claudin 18.2 nanodisc assembly, providing kinetic parameters that were closely comparable to those determined by traditional SPR. This supports the use of the Alto for transmembrane protein applications such as cancer therapeutics and illustrates the ability of Alto to facilitate kinetic data that are both accurate and precise. Compared to traditional SPR, Alto's unique and fully automated digital microfluidic platform enabled analysis of up to 48 data points in a single cartridge, significantly reducing the time, sample volume and overall cost of kinetic analysis. By leveraging DMF technology, Alto streamlines SPR analysis by automating sample dilutions, eliminating fluidic maintenance, and reducing sample requirements by up to 200X.

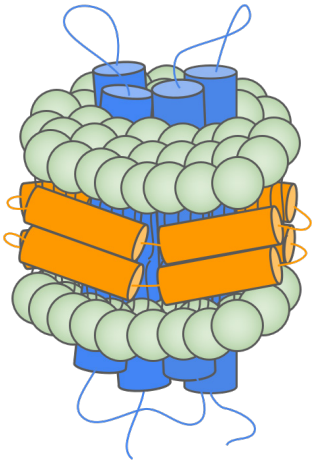

Figure 1: *Schematic of nanodisc structure. A four-pass transmembrane protein, representative of a Claudin, is shown in blue. The protein is surrounded by the lipid bilayer, shown in green. Amphipathic scaffolding proteins (orange) are wrapped around the lipid bilayer like a belt.*

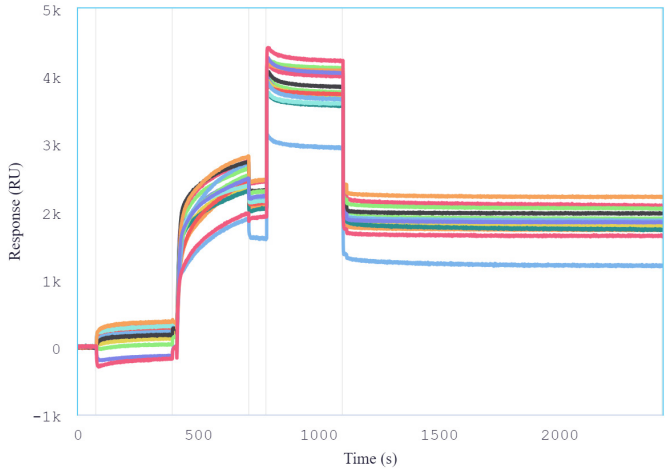

Figure 2: *Activation of response channels with EDC/NHS from Nicoya's Surfacing Kit, followed by immobilization of 50 µg/mL of anti-IgG antibody in sodium acetate pH 5.5 and blocking of sensors with 1 M Ethanolamine. Image was generated by Nicosystem analysis software.*

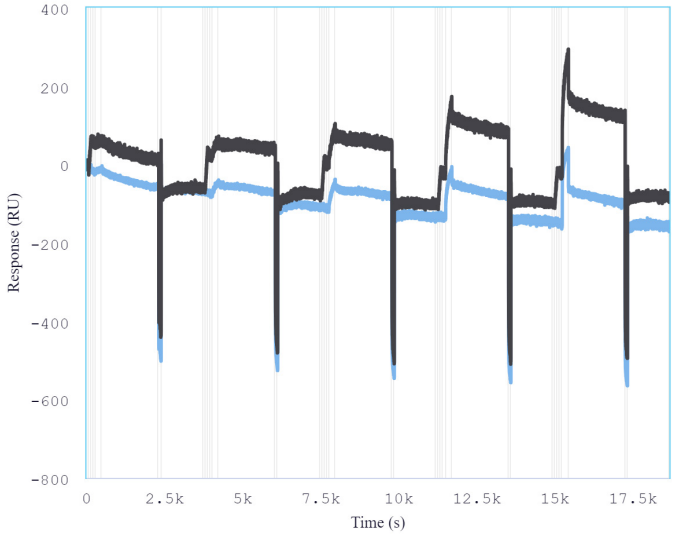

Figure 3: *Raw data collected from a single lane of the experiment. The active channel is shown in black and the reference channel is shown in blue. Ligand is captured on the surface, followed by analyte binding and a regeneration which removes the captured ligand from the surface. This is repeated with four more analyte concentrations. Image was generated by Nicosystem analysis software.*

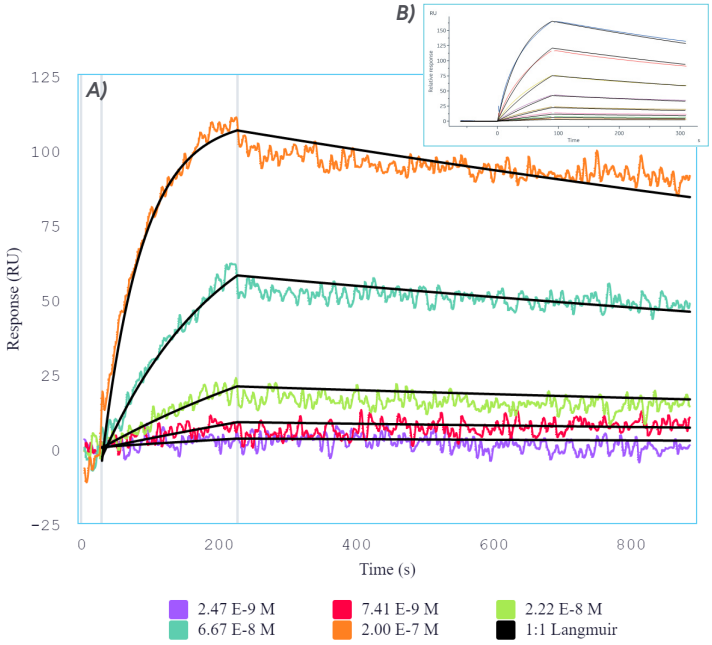

Figure 4: A) *Sample sensorgrams showing the binding of Claudin-18.2 to anti-Claudin 18.2. Image was generated by Nicosystem analysis software.* ***B)*** *Monoclonal Anti-Chimeric Claudin-18.2 Antibody, Human IgG1 captured on Protein A Chip can bind Human Claudin-18.2, His, Twin-Strep Tag (Cat. No. CL2-H5586) with an affinity constant of 12.6 nM as determined in conventional SPR assay (routinely tested).*

Table 1: Well layout with reagents and concentrations for multi-cycle capture kinetics on Alto 16-channel carboxyl cartridge.

	1	2	3	4	5	6	7	8	Vol. (µL)
R	Regeneration, Gly-HCl pH 1.5				HBS-TE	1 µg/mL Anti-Claudin-18.2 Ab in HBS-TE	HCl	HBS-TE	65
A	4% Glycerol				EDC		NHS		4
B	32% Glycerol				Quench (1 M Ethanolamine)				4
C	50 µg/mL Anti-IgG Ab in Acetate pH 5.5	50 µg/mL Anti-IgG Ab in Acetate pH 5.5	50 µg/mL Anti-IgG Ab in Acetate pH 5.5	50 µg/mL Anti-IgG Ab in Acetate pH 5.5	50 µg/mL Anti-IgG Ab in Acetate pH 5.5	50 µg/mL Anti-IgG Ab in Acetate pH 5.5	50 µg/mL Anti-IgG Ab in Acetate pH 5.5	50 µg/mL Anti-IgG Ab in Acetate pH 5.5	2
D	600 nM Claudin 18.2 nanodiscs	600 nM Claudin 18.2 nanodiscs	600 nM Claudin 18.2 nanodiscs	600 nM Claudin 18.2 nanodiscs	600 nM Claudin 18.2 nanodiscs	600 nM Claudin 18.2 nanodiscs	600 nM Claudin 18.2 nanodiscs	600 nM Claudin 18.2 nanodiscs	2
E	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	N/A
F	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	N/A
G	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	N/A
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	N/A
I	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	N/A
BF	HBS-TE	HBS-TE	HBS-TE	HBS-TE	HBS-TE	HBS-TE	HBS-TE	HBS-TE	180

Table 2: Kinetic parameters measured for Claudin 18.2 in nanodiscs.

	k_a (1/M*s)	k_d (1/s)	K_D (nM)
Alto	3.48×10^{4}	4.29×10^{-4}	14.7

References

1. Singh A.B., Sharma A., Dhawan P. Claudin family of proteins and cancer: an overview. J Oncol. 2010; 2010:541957. doi: 10.1155/2010/541957. Epub 2010 Jul 8.
2. Mineta K., Yamamoto Y., Yamazaki Y., Tanaka H., Tada Y., Saito K., et al. Predicted expansion of the Claudin multigene family. FEBS Lett. 2011; 585:606–12. doi: 10.1016/j.febslet.2011.01.028
3. Sahin U., Türeci Ö., Manikhas G., Lordick F., Rusyn A., Vynnychenko I., Dudov A., Bazin I., Bondarenko I., Melichar B., Dhaene K., Wiechen K., Huber C., Maurus D., Arozullah A., Park J.W., Schuler M., Al-Batran S.E. FAST: a randomised phase II study of zolbetuximab (IMAB362) plus EOX versus EOX alone for first-line treatment of advanced CLDN18.2-positive gastric and gastro-oesophageal adenocarcinoma. Ann. Oncol. 2021 May;32(5):609-619. doi: 10.1016/j.annonc.2021.02.005.
4. Adra N., Vaughn D.J., Einhorn L., Hanna N.H., Rosales M., Arozullah A., Feldman D.R., et al. A phase II study assessing the safety and efficacy of ASP1650 in male patients with incurable platinum refractory germ cell tumors. J. Clin. Oncol. 2020; 38:424. doi: 10.1200/JCO.2020.38.6_suppl.TPS424.
5. Overington J.P., Al-Lazikani B., Hopkins A.L. How many drug targets are there? Nat. Rev. Drug Discov. 2006; 5:993–96.
6. Gubellini F., Verdon G., Karpowich N.K., Luff J.D., Boël G., Gauthier N., Handelman S.K., Ades S.E., Hunt J.F. Physiological response to membrane protein overexpression in E. coli. Mol. Cell Proteomics. 2011; 10(10):M111.007930. doi: 10.1074/mcp.M111.007930.