Learn how Octet® BLI transforms our approach to drug discovery and development in the field of multispecific antibodies.

Improved antibody production and recombinant techniques have fueled the development of antibodies and antibody constructs. Bispecific antibodies (bsAbs) are a versatile class of targeted therapeutics designed to bind two different sites, which can be either two different epitopes on the same antigen or located on two antigens. The clinical therapeutic effects of bsAbs are superior to those of monoclonal antibodies (mAbs), with broad applications for tumor immunotherapy as well as for the treatment of other diseases. The high-throughput Octet® BLI platform allows for rapid screening of cell lines expressing the target bsAb – label-free, fluidics-free and in real-time. This collection of case studies provides insight into solutions from our customers working in the field of engineering bsAb as next generation therapeutics. We hope that this can stimulate new ideas for the development and improvement of current clinical strategies.
Introduction

Variability in the mechanism of action for many bsAbs is often linked to the construct designs currently used during development. Although the majority of bispecifics engage immune cells to destroy tumor cells, some are also used to deliver payloads to tumors or to block tumor signaling pathways. Future progress will involve the development and use of new targets, new biologics and structural combinations, new platforms, and new geometric configurations as well as combining treatments with traditional biologic drugs, other forms of immunotherapy, physical and chemical therapies. ¹

In order to accelerate the development process of such biologics, an easy quantitative approach to assess bispecific interactions in a versatile, label-free, and easy-to-use format is required. However, there is currently a limitation in technologies that allow for quantitative functional assessment of two interactions to one bispecific molecule. Many traditional technologies for characterizing bispecific antibodies efficiently are limited in their ability to provide quantitative functional assessment of two interactions. Furthermore, commonly used assay platforms like ELISA and SPR are labor and time intensive. The high-throughput Octet® BLI platform allows for rapid screening of cell lines expressing the target bsAb – label-free, fluidics-free and in real-time. This collection of case studies highlights the research of customers working in the field of engineering bispecific and multispecific antibodies as next generation therapeutics.

Case 1

A Novel T-Cell Engaging Bispecific Antibody Targeting the Leukemia Antigen PR1/HLA-A2 ²

T-cell engaging antibodies form a novel class of immunotherapies. The PR1 peptide, derived from the leukemia-associated antigens proteinase 3 and neutrophil elastase, is overexpressed on HLA-A2 in acute myeloid leukemia (AML). A high affinity T-cell receptor-like murine monoclonal antibody, 8F4, was developed previously by a group at University of Texas MD Anderson Cancer Center. The TCR-like antibody 8F4 binds to the PR1/HLA-A2 complex, mediates lysis of AML, which inhibits leukemia colony formation and eliminates AML xenografts by antibody-dependent cellular cytotoxicity (ADCC). Jeff Molldrem’s team improved the potency of 8F4, their prime candidate for a clinical trial linking T-cell cytotoxicity with a bispecific T-cell-engaging antibody. Utilizing flow cytometry and Bio-Layer Interferometry (BLI) exhibited high affinity and specific binding of the 8F4 bsAb, that binds PR1/HLA-A2 on leukemia and CD3 on neighboring T-cells. The antibody binding kinetics were assayed using the Octet® RED384 (Octet® RH16). The 8F4 bsAb, monovalent 8F4 Fab, and bivalent monoclonal 8F4 parent antibody were covalently coupled to the biosensor. To determine the binding to PR1/HLA-A2 a concentration series of PR1/HLA-A2 was measured and HLA-A2/pp65 used as control. A biotinylated recombinant human CD3εδ fusion proteins was captured on a streptavidin biosensor and 8F4 bsAb or parent anti-human CD3 antibody derived from OKT3 were evaluated. The data analysis was performed using the Octet® data analysis software.
This novel bispecific antibody targeting the PRI/HLA-A2 leukemia-associated antigen demonstrates therapeutic potential providing a novel immunotherapy to target PRI-presenting myeloid and solid tumor malignancies. To better define the antileukemia activity and toxicity further in vivo evaluation steps are ongoing to pave the way for an additional therapeutic option in a patient population that lacks effective therapies.

Case 2

A “Trojan Horse” Bispecific Antibody Strategy for Broad Protection Against Ebola Viruses

The interaction between the glycoprotein of filoviruses, such as Ebola virus (EBOV), and the entry receptor Niemann-Pick C1 second luminal doman (NPC1-C) is a desirable target for monoclonal antibody therapies. This is due to the receptor binding site being conserved amongst all filoviruses and necessary for cell entry. The group around Anna Wec built upon this by devising a strategy where MR72, a mAb specific to the NPC1 receptor, and mAb-548, a murine Ab that interacts with human NPC1-C was coupled to a mAb that targets a conserved glycoprotein (GP), FVM009. Thereby mAb-548 and MR72 were used as the backbone construct. MAb-548 was used as it was found to block the critical GPCL–NPC1-C association in vitro.

They used a design strategy, dual-variable domain immuno-globulin (DVD-Ig), to combine the heavy and light chain variable domains of FVM09 with mAb-548 and MR72. As FVM09 recognized a conserved linear epitope in the GP of Ebola viruses, the bispecific construct could be delivered to the target endosomes. Bio-Layer Interferometry was used to conduct affinity studies comparing the DVD-Ig constructs affinity to EBOV GP to the parent FMV09. No loss of affinity was found.

The DVD-Ig constructs were also tested for their neutralization ability in human cells. They were found to have a specific and potent response to cells infected with recombinant vesicular stomatitis viruses (rVSV) with an EBOV GP whereas the parent mAbs had minimal to no neutralizing potential. As the bispecific antibody construct targeted the GP-NPC1 interaction, a conserved feature amongst filoviruses, they successfully neutralized four other types of EBOV, indicating a broadly neutralizing response.

Their “Trojan-horse” mechanism illustrates the potential of targeting other viruses that use intracellular receptors or require structural changes in the endolysosomal pathway to gain entry.

Case 3

Bispecific Antibodies Targeting Different Epitopes on the HIV-1 Envelope Exhibit Broad and Potent Neutralization

HIV-1 envelope protein (ENV) is antigenically diverse, as such no single antibody is effective against all strains. HIV-1 antibodies are usually either highly potent or broad in their coverage, but the ability to combine both into one antibody would provide a significant advantage in neutralization potency. Furthermore, testing of two independent monoclonal antibodies for clinical use requires a lot of time and resources, thus combining these into one construct would be highly advantageous not only for therapeutics but also manufacturing.

“We hope that our ‘Trojan horse’ antibody strategy of targeting viruses in lysosomes might work against other disease-causing viruses like flu, dengue, or Lassa, which, like Ebola, also enter host-cell lysosomes as part of their life cycles.”

Kartik Chandran
Professor of Microbiology & Immunology
at Albert Einstein College of Medicine

Find out more about how the Octet® BLI platform could help you to accelerate your research.
Current studies show bsAbs have increased potency and breadth against HIV-1. Using four different parental HIV-1 broadly neutralizing antibodies (bNabs), Asokan and his colleagues at the NIH, used the CrossMab CH1-CL format to design bispecific IgGs, all sharing a common Fc region but each targeting a different vulnerable region on the HIV-1 ENV trimer: CD4bs, V3-glycan, V1V2-glycan, and MPER.

They used an Octet® RED384 (Octet® RH16) system to verify the bispecificity of the bsAbs. Streptavidin biosensors loaded with biotinylated RSC3 were dipped in the constructed antibodies. They then used a second ligand to probe the second arm of the Ab. Affinity measurements were also performed using Octet® BLI. Anti-human Fc biosensors were used and loaded with bsAbs or parental Abs. They were then dipped in different concentrations of ligand. Results from the BLI testing indicated all bsAbs were able to bind both ligands whilst the binding affinity, when compared to the parent Ab, was not affected. They also found that against 206 HIV-1, the bispecific antibodies neutralized 94-97% of strains. Their work highlights the promising role of bsAbs in HIV-1 infection, both prophylactically and post-infection.

Case 4

**Fab-Arm Exchange Combined with Selective Protein A Purification Results in a Platform for Rapid Preparation of Monovalent Bispecific Antibodies Directly from Culture Media**

The benefit of bsAbs has been established in literature and the clinical setting for quite some time. It would be highly advantageous to generate bsAbs in a high throughput manner, yet many first-generation manufacturing methods have structural limitations preventing high throughput screening.

A group from AstraZeneca designed a method whereby they were able to rapidly generate monovalent bsAbs. In order to repeat the physiological processes in human IgG4 Fab-arm exchange to generate monovalent bsAbs, the group engineered a point mutation in the CH3 domain. Within the CH3 domain of IgG1, they also incorporated the mutations H435R and Y436F which ablate protein A binding in one arm and facilitate pure bsAb production. The combination of mutations and improvement of the Fab-arm exchange allowed pure monovalent bsAbs to be cultivated straight from the culture media using protein A purification.

The group then used an Octet® RED96 (Octet® R8) to test bispecific binding. Using streptavidin biosensors, they captured biotin labelled HER2 and EGFR. These were submerged in either solution containing parental or bispecific antibodies, followed by another wash in solution containing EGFR or IGF1R. The dual binding assay showed that only the bsAbs was capable of binding both ligands. Their novel approach allows for high throughput generation of monovalent bsAbs, greatly improving the capacity of bsAbs in therapeutics.

Case 5

**Rational Design of a Tri-specific Antibody Targeting the HIV-1 Env with Elevated Anti-Viral Activity**

In another study performed by the group at AZ they combined single chain variable fragments (ScFv) domains of two broadly neutralizing antibodies (bNabs). The bNabs were specific to the CD4 binding site and the V3 glycan.
patch, both of which have been identified as points of conserved vulnerability on HIV-1 ENV. The anti-HIV-1 bispecific ScFvs, had a higher neutralization potential compared to parent bNabs. This was then combined with a third bNab specific to MPER of the HIV-1 envelope glycoprotein (Env), another widely recognized vulnerable site.

The group then used the Octet® BLI platform to test the specificity and affinity of the Tri-NAbs against the parent bNabs. The Tri-Nab showed positive binding to all three ligands tested whereas the parental bNab unsurprisingly bound the one target ligand. When tested against a panel of 208 viruses the Tri-NAb achieved a 99.5% neutralization coverage, proving an effective candidate for next-gen HIV-1 therapeutics.

Case 6

**Bispecific antibodies with Fab-arms featuring exchanged antigen-binding constant domains**

Monoclonal antibodies can acquire the property of engagement of a second antigen via fusion methods or modification of their CDR loops, but also by modification of their constant domains, such as in the mAb² format where a set of mutated amino acid residues in the CH3 domains enables a high-affinity specific interaction with the second antigen. The researchers tested the possibility of introducing multiple binding sites for the second antigen by replacing the Fab CH1/CL domain pair with a pair of antigen-binding CH3 domains in a model scaffold with trastuzumab (TRA) variable domains and VEGF-binding CH3 domains from the CT6 clone. Such bispecific molecules were produced in a Fab-like format and in a full-length antibody format. They were expressed at a high level and their high-affinity binding to both target antigens was retained. Binding to VEGF via CT6-domains was quantitatively evaluated using label-free BLI applying an Octet® RED96e (Octet® R8) instrument. TRA-CT6 mAb² and its heterodimerized counterpart showed similar binding affinities and so did the Fab-like construct. To exclude the negative impact of improper heterodimerization on antigen-binding properties of novel bispecific constructs, they examined the composition of bispecific (FabCabs) and full-length IgG-like bispecific antibodies and found that correct heterodimer formation could be confirmed for all analyzed molecules. When FabCabs were introduced into the whole-length antibody format, they could surprisingly show an improved binding to VEGF. This was due to about 10-fold slower off-rate than could be measured for full-length antibodies with C-terminally positioned VEGF-binding domains. The parameters of binding kinetics were also evaluated with immobilized full-length IgG-like molecules.

**Bispecific antibodies with Fabs featuring exchanged antigen binding CH3 domains** offer an alternative solution in positioning and valency of antigen binding sites. The novel symmetric bispecific format that features antigen-binding constant domains replacing the Fab constant domains to confer a second specificity to an antibody opens novel possibilities of multiple valency of antigen engagement and spatial positioning of its binding sites.

Case 7

**Biophysical and biochemical characterization of a VHH-based IgG-like bi- and tri-specific antibody platform**

A group from the Technical University Darmstadt, Germany in cooperation with Merck KGaA endeavored to

“One critical component of bsAb development is the identification of target pairs of antibodies with synergistic effect, necessitating the development of high-throughput approaches. To address this hurdle, we developed a platform to rapidly assemble and evaluate candidate bsAb for improved biological functionality in high-throughput format directly from culture media.”

**James Steinhardt**
Antibody Discovery and Protein Engineering, AstraZeneca
develop a generic platform approach for the facile generation of IgG-like bi- and tri-specific VHH-derived antibodies. This generic platform utilizes the replacement of the variable domains of a human antibody by two independent VHH-based paratopes. As a result, one VHH is grafted onto domain CH1 of IgG1 while the other VHH-based paratope is grafted on the constant region of the light chain, Cκ or Cλ, resulting in a tetravalent bispecific IgG-like molecule. Four different VHHs were used to gain an in-depth understanding of their platform approach. Octet® BLI technology was used to characterize the biomolecular interaction of these monovalent bispecific IgG-like VHH-based antibodies. Kinetic measurements were performed also for characterizing simultaneous binding, periodic binding, and epitope binning of the antibodies. For kinetic analysis, the antibody-like molecules were loaded on anti-human-Fc (AHC) biosensors. To demonstrate simultaneous antigen binding, respective antibody-like molecules were captured to the surface of anti-human IgG Fc (AHC) biosensors. For the kinetic measurements with EGFR bound in saturation or without prior EGFR binding the antibody was loaded on anti-Penta His biosensors. For the assessment of periodic antigen-binding antibodies were loaded on Anti-Human-Fc (AHC) biosensors and epitope binning analysis was performed by loading EGFR (ECD) via its histidine-tag on anti-Penta His (HIS1K) biosensors. This work demonstrates the successful combination of a heavy chain heterodimerization technique and the replacement of the variable domains of a human antibody by two independent VHH-based paratopes. This novel approach allows facile engineering of bi- and tri-specific antibodies with flexible valences. They further showed that the different described formats comprise acceptable properties and that early-stage developability is feasible. Due to issues with mis-assembly and aggregation, the BiTri series was not considered for further development. The new platform affords the benefit to tailor multi-specific entities with respect to their biophysical and biochemical properties.

**Case 8**

**Potent Bispecific Neutralizing Antibody Targeting Glycoprotein B and the gH/gL/pUL128/130/131 Complex of Human Cytomegalovirus**

Human cytomegalovirus (HCMV) is a ubiquitous pathogen which can infect a broad range of cell types. Following congenital infection among transplant patients, the virus can cause developmental disorders and life-threatening complications. Promising drug candidates against HCMV infection are neutralizing monoclonal antibodies (mAbs). Single neutralizing antibodies targeting one HCMV glycoprotein often lack either potency or broad cell-type coverage. The group of the University of Texas in cooperation with Merck Research Laboratories characterized two human derived HCMV neutralizing mAbs previously. To combine the strengths of both monoclonal antibodies, they developed an IgG–single-chain variable fragment (scFv). The resulting bispecific antibody showed high-affinity binding to both gB and gH/gL/pUL128/130/131 complex.

The Octet® BLI platform was used to characterize the binding avidities of parental and bispecific antibodies. Equilibrated protein A biosensors were loaded with either the parental or bispecific antibodies. To remove non-specifically bound material a baseline in buffer was run before an association step was performed in diluted gB or pentamer antigens. The dissociation was measured in...
By analyzing specific binding results in addition to IgG titer in a single high-throughput instrument, we are able to assess the binding activity of a bispecific molecule to both targets to rank the top pools/clones during cell line development.”

Eva Rubio-Marrero
Bristol-Myers Squibb

buffer again. To provide similar binding conditions, the sensors tips were regenerated between individual experiments. One blank biosensor was used to monitor any nonspecific binding of antigens onto the biosensor surface. One antibody-loaded sensor associated with buffer only served as a reference. Following the experiment all data were analyzed using Octet® Data Analysis software version 10.0. A 1:1 binding model fitting the binding curves was applied.

Functionally was tested with multiple cell lines. They could show that the bispecific antibody combined neutralization breadth and potency of the parental mAbs and inhibited post infection viral spreading. Furthermore, the bispecific antibody was easily produced in CHO cells and showed a single-dose pharmacokinetic profile comparable to that of parental antibodies. Furthermore, the bispecific antibody retained broadly and potent neutralizing activity after 21 days in circulation with the potential for developing bispecific neutralizing antibody therapies against HCMV infection.

Case 9

Bispecific VH/Fab antibodies targeting neutralizing and non-neutralizing Spike epitopes demonstrate enhanced potency against SARS-CoV-2

The emergence and rapid spread of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) makes effective therapeutics necessary to curtail the global pandemic. The Wells Lab at UCSF reports a promising protein engineering strategy to rapidly improve the potency of antibody therapeutics. A combination of neutralizing and non-neutralizing bispecific IgG binders against different epitopes on the receptor-binding domain (RBD) of the Spike protein were developed.

The group deliberately exploited non-neutralizing RBD antibodies and demonstrated a dramatic effect in neutralization when used in cocktails also containing neutralizing binders. The researchers used phage display for the identification of antigen-binding fragments (Fabs). These Fabs do not block ACE2 or neutralize the virus while binding the Spike RBD. A ~ 25-fold potency improvement was observed in neutralizing SARS-CoV-2 combining these non-neutralizing Fabs in bispecific IgGs containing a neutralizing VH domain as well. The improvement was epitope-dependent, as the geometry of newly developed bsAb plays a crucial role binding the Spike protein. The Octet® BLI platform was used to measure the analyte binding onto Spike-RBD-Fc or Secto, respectively. Their results on a promising and rapid engineering strategy show that the potency of SARS-CoV-2 antibodies can be improved by combining both binders against neutralizing and non-neutralizing epitopes on Spike RBD.

Case 10

Rational selection of building blocks for the assembly of bispecific antibodies

There are several ways to generate bispecific antibodies using variations on the heavy chains and/or the light chains. Over the years genetic engineering has reinforced the production of a variety of bsAbs for greater flexibility in size, valency, specificity, stability, and biodistribution. However, to meet industrial scale productivity, enforcing the correct quaternary assembly of these molecules remains critical. Here, a team at Amgen describes Chain Selectivity Assessment (CSA). This high-throughput
Method can efficiently screen and identify monoclonal antibodies (mAbs) that show a native preference toward assembling bispecifics without the need for further engineering. Octet® BLI technology was used to measure the binding affinities ($K_D$) of the purified antibodies (bispecific or monoclonal) to soluble antigens. All experiments were run on an Octet® HTX (Octet® RH96) instrument using the 96-tip mode. Dilution series of antigen were measured against both kinds of antibodies, which were captured onto streptavidin SAX biosensor tips. The chosen assay format allowed a monovalent 1:1 binding interaction model to be applied and direct comparison of kinetics and affinities.

Moreover, the group could apply CSA to also identify rare light chains (LCs) without engineering. They demonstrated that the success of developing multispecific molecules with optimal manufacturability properties depends on the selection of parental mAbs as the building blocks.

Conclusion

Bispecific antibodies (bsAb) represent a highly promising and emerging therapeutic area. Due to structural and biological differences from monospecific Abs, development of a bioassay strategy for bsAb poses unique challenges and considerations. Increasing numbers of bsAbs are making their way into market with their broad applications including targeting tumor cells, blocking signaling pathways, diagnosing infectious diseases and gene therapy. Many candidates differ in their composition, affecting valency, geometry, flexibility, size, and half-life. Not all members of this huge variety of bsAb formats qualify to become drugs. Strong emphasis is therefore on identifying candidates that exhibit drug-like properties and fulfill safety, developability, and manufacturability criteria. There is likely to be an exciting new wave of bsAb therapeutics available in the coming years. While their importance in therapeutics is cemented, limitations exist in their production with the lack of a high-throughput screening method. This in turn negatively impacts their potential in a clinical setting. The fluidics-free Octet® BLI platform provides label-free screening of bsAbs in high-throughput, as systems are capable of analyzing up to 96 samples in parallel. Target binding characterization is an essential analytical step for the selection of high affinity and high specificity therapeutics regardless of the molecule type. Kinetic analysis further describes the components of association and dissociation that comprise the overall affinity interaction. The examples in this guide demonstrate how Octet® systems are used to address real-world research and complete projects faster. The Octet® BLI platform exemplifies how this robust and easy-to-use assay approach can be leveraged to accelerate the timeline to discovery, development and production of potent biologics against a variety of diseases of global concern.

Bispecific antibodies poised to deliver wave of cancer therapies to propel the field of better clinical outcomes in oncology.
References
