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Octet® BLI Workflows in Small-Molecule Interaction Discovery and Characterization

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Small-molecule drugs comprised a majority of all new drug approvals in recent years. Small-molecule drug development, however, remains a challenging and time-consuming process. Low molecular weight leads, low solubility, non-specific binding propensities highlight a few challenges researchers must circumvent in the small-molecule drug discovery workflows. Label-free binding technologies such as Bio-Layer Interferometry (BLI) on the Octet® platform serve as an invaluable tool in drug development workflows as it provides a direct confirmation of small-molecule binding to drug targets that is useful in hit-identification. Octet® BLI assays further help characterize interactions by measuring binding affinity and kinetics that serve as critical attributes in lead selection. In this short review, we describe a few selected examples of how Octet® BLI binding kinetic and affinity data can be utilized in small-molecule drug discovery programs from hit identification, characterization, and structure-based drug design and optimization workflows.

Introduction

The discovery of small-molecule therapeutics from target identification to clinical evaluation is a complex, challenging yet an important area of drug discovery. Small molecules continue to be the primary modalities of new drugs, accounting for more than 50% of new drug approvals in the US in 2021.[2] Usual advantages of small molecules over biologics such as ease of manufacturability, cell permeability, reversible inhibition, resistance to proteolysis and non-immunogenic behaviors drive new small-molecule drug development programs. However, delivering small-molecule candidate drugs for clinical evaluation is time-and resource-intensive and typically takes years to discover and launch as a new drug to the market.

The discovery process typically involves target identification and validation, hit identification, lead identification and lead optimization before candidate drugs are moved to clinical evaluation. One main challenge in establishing small-molecule binding interactions *in vitro* is the low molecular weight of small molecule analytes and their inherent weaker affinities that demand higher sensitivity instrumentation and careful assay development for confident data generation. Small-molecules also suffer from low solubility in aqueous buffers and often require the use of organic solvents such as DMSO or DMF in buffers to improve solubility. Additionally, small molecules tend to non-specifically bind to targets that require additional experimental assessments to make sure they are excluded early in the discovery process. Therefore, it is common for small-molecule drug development programs to utilize a range of biophysical technologies in-tandem to screen, verify and characterize small-molecule binding interactions. Octet® Bio-Layer Interferometry (BLI) label-free binding technology is an ideal tool for small-molecule drug development research that helps accelerate candidate discovery and selection timelines. High-throughput binding screens can quickly identify hits and characterize target binding affinities, kinetics, specificity and selectivity during hit validation and optimization. Some of the key advantages of utilizing Octet® BLI platforms in small-molecule workflows include:

- Fast time to results: Data processing from parallel biosensors provides fast time to results. Depending on the Octet® system, data from 1–16 biosensors can be processed simultaneously, enabling you to generate data or optimize experiments faster while also allowing assay flexibility to perform a multitude of biosensor assays.
- Crude sample compatibility: A fluidic-free design, together with dip-and-read biosensors enable crude sample compatibility where you can analyze interactions from complex mixtures, lysates, or extracts
- Ease-of-use: The microplate-based design eliminates microfluidic clogging, extensive sample preparation practices, and instrument maintenance and downtimes.

In this brief review, we highlight a few examples of how Octet® BLI assays can be utilized in small-molecule hit selection and optimization workflows and share how orthogonal methods such as SPR, isothermal titration calorimetry (ITC), nuclear magnetic resonance (NMR) and differential scanning fluorimetry (DSF) can be used in-tandem to supplement and confirm findings.

Small-Molecule Binding Assays Utilizing Octet® BLI Systems

Small molecule binding affinity and kinetics can be measured on Octet® R2, R4, R8, RH16, and RH96 BLI systems. Biosensor selection is a critical assay consideration since small-molecule binding detection requires biosensors with higher ligand loading capacities to realize higher detection sensitivities. Stable protein immobilization is also critical in high sensitivity biosensor assays. Sartorius offers a specialized streptavidin-based biosensor for small-molecule applications. This Super Streptavidin (SSA) Biosensor enables higher ligand immobilization levels compared to other Streptavidin-based biosensors. Streptavidin-biotin ligand immobilizations provide an extremely stable capture surface that also helps maintain a stable assay baseline. A typical small-molecule binding characterization assay workflow using a 96-well plate assay format with an 8-channel Octet® R8 BLI system is depicted in Figure 1. In this example, the binding characterization of seven compounds is depicted with a five-point titration for each compound. The sample plate carries reagents for protein immobilization, assay buffers and serially diluted compounds. Steps in a typical small-molecule binding characterization assay include:

- Pre-immobilization baseline: This step should be performed once to condition and equilibrate biosensors in the ligand loading buffer to establish a stable pre-loading baseline. Typical step-times are 30–60 seconds.
- Protein loading: This is performed once in a typical small-molecule binding assay. The loading step immobilizes protein targets onto biosensors. For example, biotin-labeled proteins are captured onto SSA biosensors until the signal reaches saturation. Higher loading densities are recommended for small-molecule binding applications to improve assay sensitivity. Typical loading times are 10–15 minutes or until saturation.
- Post-immobilization baseline: This step removes unbound proteins from the biosensors and equilibrates the protein-loaded biosensor in the binding buffer. While not shown in this figure, a separate post-immobilization

wash step may be required if the protein requires a longer equilibration time to achieve a stable baseline when exposed to DMSO-based buffers. Typical equilibration times can range from 3–5 minutes depending on the protein target.

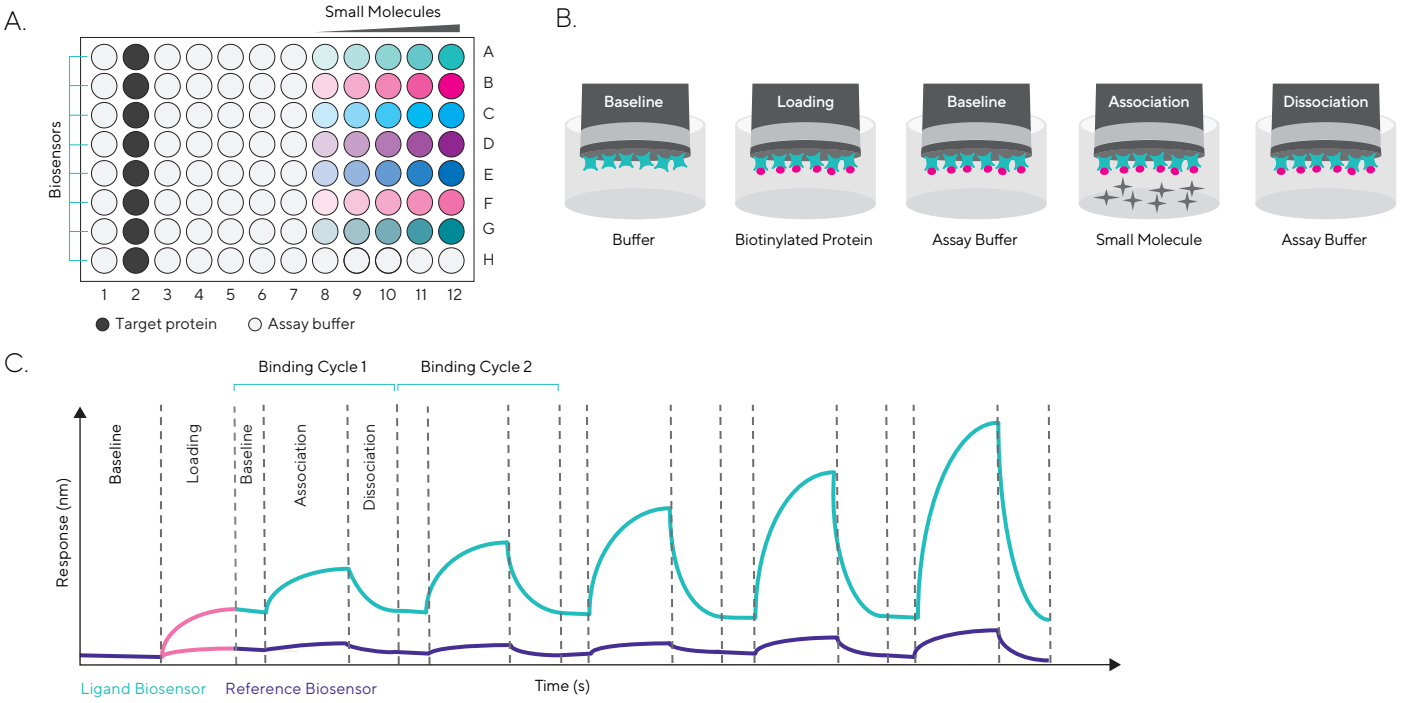
- Pre-association baseline: This baseline step establishes a stable baseline before analyte binding. Typical baseline step times are 30–60 seconds for small-molecule applications.
- Association step: Protein loaded biosensors dip-and-read binding responses for small-molecule binding assessment. Typical step times are 1–3 minutes or until signals approach equilibrium.
- Dissociation step: Biosensors dip into the assay buffer where bound small molecules dissociate into the buffer. The same assay buffer-well that was utilized for the pre-association baseline is recommended for dissociation analysis. Typical dissociation step times are 1–3 minutes or until responses reach close to the pre-association baseline.

In general, small molecule-protein interactions exhibit fast association and dissociation kinetics that allows users to observe complete dissociation of the bound small molecule from the biosensor during the dissociation phase. Therefore, the protein-loaded biosensor can be re-utilized to assay the entire small-molecule analyte titration series, cycling between assay steps pre-association baseline, association, dissociation, until the entire titration is performed.

After the ligand (protein) immobilized biosensor binding to small molecules has been assayed, it is required to perform a second identical assay with reference biosensors that are immobilized with reference protein or un-immobilized biosensors to capture small-molecule non-specific binding at each small-molecule analyte concentration. Reference biosensor data can also be used to capture and negate baseline drifts, bulk effects and systematic data artifacts from the experiment. For a detailed review on small-molecule-protein binding assay development on the Octet® BLI systems, please refer to Sartorius Technical Note, *Small Molecule Binding Kinetics*.

Figure 1

Typical Small-Molecule Characterization Assay Workflow Utilizing an 8-channel Octet® R8 BLI System.



Note. (A) An example plate map for characterizing seven small-molecule binding interactions. The sample plate contains reagents for protein loading (immobilization), assay buffer and seven small molecules with 5-point dilutions. Alternatively, one could also utilize protein loading in a separate experiment. In that case, the plate would only carry small molecule(s) and assay buffers. Octet® RH16 and RH96 BLI systems can accommodate two sample plate positions that can either be two 96- or 384-well plates. (B) Typical assay steps for a kinetic characterization assay. Steps include pre-immobilization baseline, protein loading step, pre-association baseline, association and dissociation steps. (C) An example small-molecule kinetic assay where a single biosensor is used to measure the binding of all analyte dilutions, from lowest to highest concentrations, for a small-molecule analyte. Following the ligand biosensor binding assessment, a second biosensor loaded with a negative control protein or unloaded biosensor will assay non-specific binding responses for all analyte concentrations which will be subtracted during data analysis.

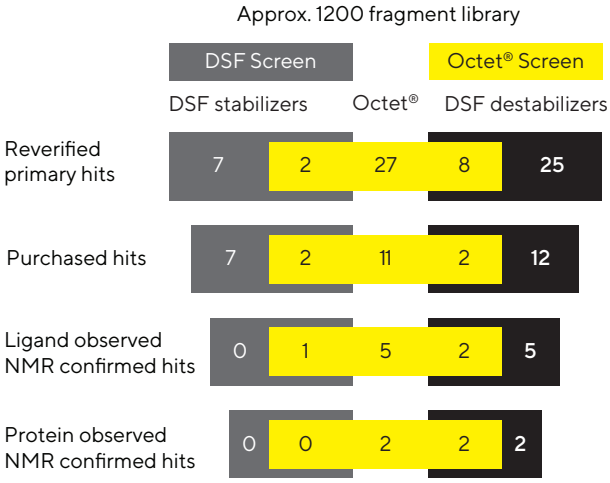
Small-Molecule Binding Screens

Wartchow et al., carried out small-molecule binding screens against protein-protein interactions involving BCL-2, JNK-1, and eIF4E with a library of 6500 compounds using an Octet® RH16 BLI system.[3] To summarize, proteins at 0.5 µM were loaded onto SSA Biosensors overnight at 4°C. The remaining streptavidin binding sites in the biosensor were then blocked with biocytin. Similarly, reference biosensors were prepared by loading biotinylated streptavidin protein that was biotin-labeled in the presence of biocytin. The authors claim that preparing the reference biosensor in this way, compared to biocytin blocked SSA Biosensors, led to reduced standard deviations. For the eIF4E screen, a primary hit-rate of 3.5% was observed before removal of 0.4% of the hits due to showing unusually high signals and 0.6% for unreasonably slow off-rates. The hits were validated with a second screen using a single point 200 µM concentration yielding a 50% confirmation rate to bring the final hit rate to 1.3%. This hit rate was reported to be similar to the biochemical assay hit rate of 1.4%. Overall, the Wartchow group found that the results obtained with BLI hit assessments and screens confirmed results from biochemical assays and identified compounds with atypical binding profiles and novel hits that were missed with biochemical screens.

Similarly, Morreale et al., utilized an small-molecule binding screen to identify inhibitors of Ube2T (E2 ubiquitin-conjugating enzyme).[4] A fragment library of approximately 1200 candidates was screened using the Octet® BLI system and DSF, followed by a one-dimensional 1H NMR spectroscopy secondary screen. The binding screen identified 90 hits. The binding assay was performed by immobilizing biotinylated Ube2T on SSA Biosensors and screening fragments at a 200 µM concentration. Out of the 90 fragment hits from the initial screen, 37 hits were reconfirmed using a second Octet® BLI assay that re-evaluated the small-molecules using a 6-point titration curve. The parallel DSF screen, identified 42 hits (9 protein stabilizers and 33 protein destabilizers). Between the Octet® screen and the DSF screen, a total of 69 hits were identified, of which 10 hits were common between the two methods (Figure 2). Out of these 69 hits, 34 were chosen based on scaffold diversity and commercial availability, of which 11 were unique Octet® hits and 4 were common to both methods, for further evaluation. Ligand-observed and protein-observed NMR spectroscopy assays reduced the number of hits to 6, which included 4 hits identified using Octet® BLI screens. 3 compounds showed enzymatic inhibition and one hit yielded a crystal structure that identified a novel inhibitor binding pocket in Ube2T.

Figure 2

Ube2T Small Molecules Hits Identified by the Octet® BLI System and DSF Binding Screens.



Note. The number of selected hits is depicted at each step of the biophysical cascade implemented in this study. Figure adapted from Morreale et al.[4]

Octet® Kinetic Assays to Determine Structure-Kinetic Relationships

Chaikuad et al., utilized Octet® BLI binding assays to evaluate the binding mechanism and selectivity of SCH772984; a potent kinase inhibitor towards ERK1/2.[5] A novel induced allosteric binding pocket adjacent to the ATP binding site was discovered using crystallographic evidence where SCH772984 bound to both ERK1 and ERK2. Octet® binding analysis of ERK1 to SCH772984 exhibited slow association and dissociation rates attributing to kinetic constraints caused by structural rearrangements by SCH772984 as an allosteric inhibitor. However, SCH772984 showed fast binding kinetics to Haspin; a kinase related to ERK1/2 kinase. Slow inhibitor off-rates are considered as an important parameter for sustained efficacies of kinase inhibitors and other target families, but the structural mechanisms that influence inhibitor binding kinetics are poorly understood. Binding kinetics were also assessed with other ERK/Haspin inhibitors (VTX-11e, FR180204, and 5-iTU) where VTX-11e also exhibited slow off-rate kinetics binding to ERK while FR180204 and 5-iTU binding was associated with fast kinetics. However, both FR180204 and 5-iTU showed similar slow off-rates binding to its natural binding target Haspin. In this study, inhibitors with slow off-rate kinetics correlated with prolonged inhibitory activity in cells, implying mechanistic consequences from binding kinetics. To identify structural requirements for slow off-rates and the new binding mode, a series of crystal structures were examined for the conservation of key residues lining the ERK1/2-SCH772984

interface, where it was hypothesized that two aromatic tyrosine residues located within the P-loop and αC could be critical for stabilization of SCH772984 binding mode in ERK1/2 and slow binding kinetics. However, slow association and dissociation kinetics were retained, albeit with reduced binding affinities, when mutations to these tyrosine residues were tested (Y36Q, Y36A, Y64H and Y64K) using Octet® BLI kinetic assays. The double mutants Y36A Y64H and Y36Q Y64H also retained slow binding kinetics. However, substituting Y64 to lysine or aspartate strongly reduced inhibitor off-rates, identifying the origins of slow off-rates in the binding pocket. Similarly, these tyrosine residues were important in slow off-rates with Haspin binding as well, elucidating structure-kinetics relationships of SCH772984 inhibitor binding to ERK1/2.

Characterizing *In Vitro* Mechanism of Action of Small-Molecule Modulators of Capsid Assembly

Lad et al., utilized a suite of label-free assays to characterize the mechanism of action of two small-molecule inhibitors of HIV-1/2 capsid (CA) assembly that had been identified by high-throughput screening campaigns.[6] Both inhibitors (PF74 and BI64) were known to target the CA by modulating capsid polymerization. Crystal structure data indicated that the small molecules bound to distinct binding sites in the N-terminal regions of CA, and biochemical assays confirmed opposite inhibitory effects for the two molecules, with PF74 increasing CA polymerization and BI64 exerting an opposite effect in reducing polymerization. Binding analysis of PF74 and BI64 to monomeric and hexameric CA was measured using an SPR, ITC and Octet® BLI assays. Biotinylated monomeric and hexameric CAs were immobilized onto streptavidin (Octet® BLI system) and neutravidin (SPR) biosensors respectively to assess binding of the two inhibitors (Table 1). The Octet® BLI system and SPR data produced similar results for both PF74 and BI64 binding to CA hexameric and monomeric conformations. PF74 interacted with CA hexameric conformations with significantly higher affinities compared to monomeric forms, confirming its preference for hexameric CA, to accelerate polymerization. BI64, on the other hand, displayed a different binding profile compared to PF74, with < 100 nM affinities for monomeric conformation and negligible binding to hexamer CA protein with an estimated affinity of > 10 µM (SPR) and > 30 µM (Octet® BLI system), which were the highest concentrations of the compound tested due to limited compound solubility. Biosensor assay data were well in agreement with ITC data (Table 1). Data indicated that PF74 preferentially binds to the assembled CA hexamer whereas BI64 only appears to bind CA monomers. These conclusions correlated well with Epic-based functional assembly assays and were consistent with structural data related to the inhibitor-binding sites on CA.

Structure-Based Design of Slimmer BIRB-796 Analogs to Inhibit MAPK13

Alevy et al., used Octet® BLI binding assays to develop a series of MAPK13 inhibitors that regulated IL-13 driven mucus production as a therapeutic strategy for inflammatory airway diseases.[7] The inhibitor discovery was based on MAPK13 homology to MAPK14, where MAPK14 inhibitor scaffolds were selected and optimized towards MAPK13. Crystal structural data of BIRB-796, a potent MAPK14 inhibitor that weakly binds to MAPK13 revealed structural elements that enhanced binding interactions with MAPK14 but not with MAPK13. Crystal structure information was then used to synthesize several analogs that were optimized for MPAK13 binding. MAPK13 binding kinetics to these analogs were tested using Octet® BLI assays where it was revealed that inhibitors binding to an Asp-Phe-Gly-out mode (DFG-out) led to slow dissociation rates while complexes formed by compounds that bind in the DFG-in mode were mostly short-lived. These kinetic behaviors also translated into MAPK13 inhibitor potencies, where it was observed that compounds that bound in the DFG-out mode consistently showed higher inhibitory potencies than DFG-in mode binders, drawing structure-kinetic relationships. The Alevy lab further realized that DFG-out mode binders not only potently inhibited MAPK14 activity but also suppressed IL-13 induced MUC5AC production in primary-culture human airway epithelial cells. Taken together, this work describes a workflow for the rational design of inhibitors using a scaffold hopping strategy.

Table 1

Label-free Analysis of HIV Capsid (CA) Protein Binding to the Small-Molecule Compounds PF74 and BI64.

	Octet® BLI		SPR		ITC	
	wt CA	Hexamer CA	wt CA	Hexamer CA	wt CA	Hexamer CA
PF74	K_D = 1.9 µM (k_{on}= 5.87 x 10^4, k_{off} = 0.11)	K_D = 78 nM (k_{on}= 1.57 x 10^5, k_{off} = 0.012)	K_D = 1.3 µM (k_{on}= 4.64 x 10^5, k_{off} = 0.62)	K_D = 21 nM (k_{on}= 5.59 x 10^6, k_{off} = 0.12)	2.8 µM	120 nM
BI64	K_D = 83 nM (k_{on}= 2.98 x 10^4, k_{off} = 0.009)	N.B (30 µM)	K_D = 25 nM (k_{on}= 4.5 x 10^5, k_{off} = 0.011)	N.B (10 µM)	97 nM	N.B

Note. Here, k_{on} is reported in $M^{-1}s^{-1}$ and k_{off} is reported in s^{-1}.

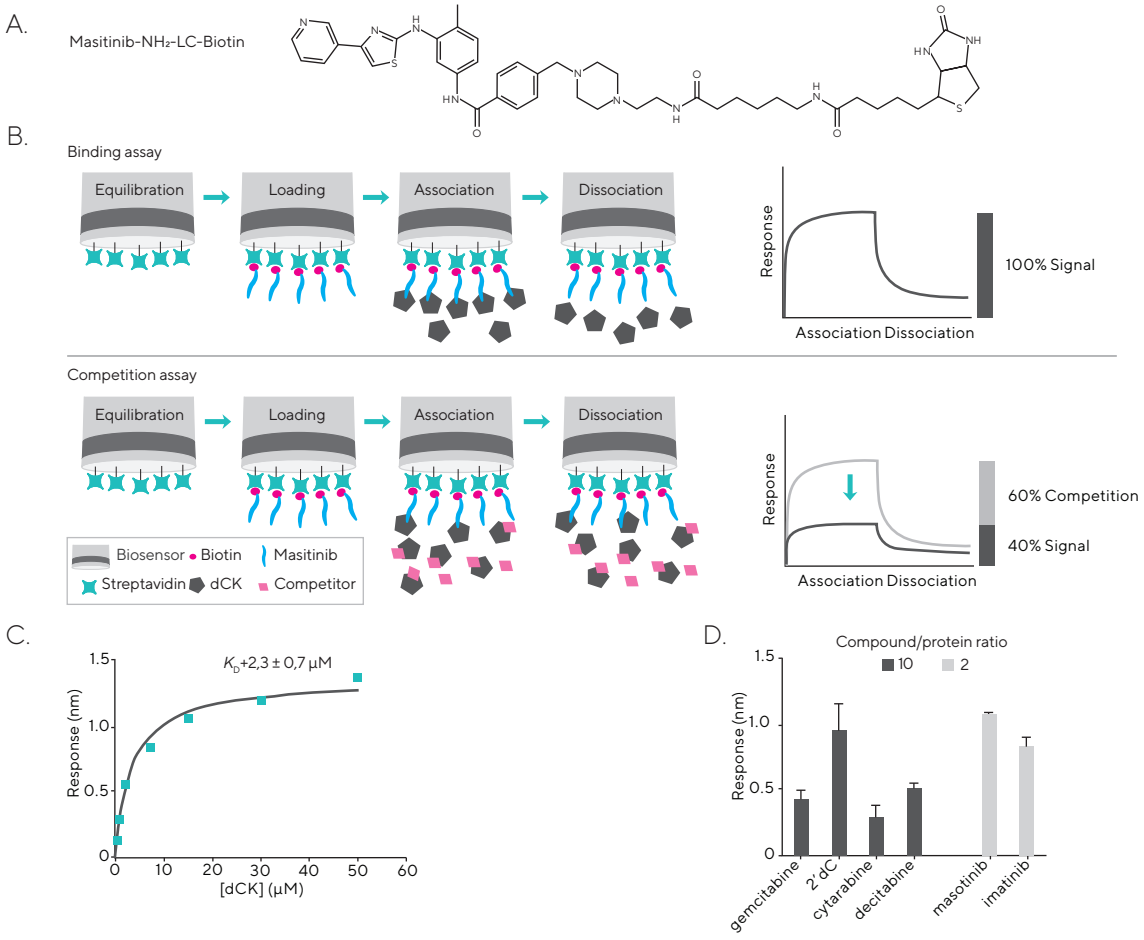
Small Molecules Immobilized as Ligands for Target Validation Studies

One of the main challenges to using small molecules as the ligand in biosensor assays is the need for a viable immobilization motif within the small-molecule scaffold that will not adversely affect the target binding site. However, this assay orientation is used as a hit validation strategy when the binding scaffold is known. One key assay development tip to this assay orientation is small-molecule loading density optimization to prevent overcrowding the surface with the smaller molecule ligand, especially when the analyte is much larger and would hinder access to the ligand-binding site. Additionally, the solubility of the small molecule should also be considered, the use of high immobilization concentrations should be avoided to circumvent small-molecule aggregation in the biosensor and buffer components that would retain small-molecule activity in the biosensor should be utilized.

Hammam et al., used an Octet® BLI kinetic assay to validate deoxycytidine kinase (dCK) as a target for masitinib[7]. The mechanisms of masitinib synergy with gemcitabine in inhibiting the growth of human pancreatic adenocarcinoma *in vitro* and *in vivo* were identified using biophysical, biochemical and crystallography evidence. Masitinib interaction with dCK led to nucleoside kinase activation that enhanced physiological and pro-drug phosphorylation of dCK substrates. As part of the mechanistic validation, the binding of dCK to an immobilized biotinylated-masitinib probe was measured together with competition assays to compare substrate affinities (Figure 3). A masitinib-NH2-LC-biotin conjugate containing a glycol spacer moiety was immobilized on Super Streptavidin (SSA) Biosensors at 1 µM and assessed for dCKbinding with varying concentrations (Figure 3B). Steady-state analysis was used to calculate an affinity of 2.3 µM between masitinib and dCK (Figure 3C).

Figure 3

Octet® BLI Systems Can Be Used to Determine dCK Binding to Substrates and Kinase Inhibitors.



Note. (A) Structure of the biotin-modified version of masitinib. (B) A schematic representation of binding experiments using SSA Biosensors. (C) dCK (0.1–50 µM) binding to streptavidin-coated biosensors loaded with a conjugated-biotin version of masitinib. The dissociation constant (K_D) was determined using data at equilibrium (response at the end of the association step) using steady state analysis. (D) Competitions assays were used to assess binding of dCK (10 µM) to fixed masitinib in the presence or the absence of competitors (100 µM for substrates such as gemcitabine, 2'dC, decitabine, aracytine and 20 µM for kinase inhibitors such as masitinib and imatinib). The data were standardized to a competitor-free control. Figure reproduced from Hammam et al.[8]

This interaction was also assessed in the reverse orientation, where biotinylated dCK was immobilized onto SSA Biosensors and masitinib was used as the analyte. Single site binding affinities of 1.6 µM were observed, with data correlating well between both assay orientations. This implies that modifying the masitinib molecule did not significantly impact dCK binding. Relative substrate binding levels compared to masitinib for several tyrosine kinase inhibitors were estimated using a competition assay (Figure 3B and 3D). Binding of 10 µM dCK to immobilized masitinib was measured in the presence of substrates or pro-drug gemcitabine, 2'-dC, cytarabine and decitabine. The data generated supports the findings that masitinib, and other TKIs such as imatinib, enhance the dCK dependent phosphorylation of the pro-drug gemcitabine and cytarabine, potentiating the activity of these drugs.

Similarly, Choby et al., utilized Octet® BLI to validate their previously identified small molecule using a phenotypic screen to its proposed binding target.[9] The small molecule was known to activate heme biosynthesis in *S. aureus* and exert toxicity when grown anaerobically (IC_{50} = 162 µM) and aerobically (IC_{50} = 5 µM). A series of genetic and proteomic approaches uncovered that the Suf Fe-S cluster biogenesis machinery that is likely the small-molecule binding target. To identify the small-molecule binding target, presumably within the Suf Fe-S complex, a pull-down assay was performed with a biotinylated small molecule. Subjecting the pull-down complexes to LC-MS/MS analysis identified SufC and PdxS, among others, and further pull-down experiments enriched proteins from the SufC machinery (SufB, SufD, SufC, SufS). An Octet® BLI binding assay was used to validate findings from the pull-down and LC-MS/MS analysis. Biotinylated small molecule 882 was immobilized onto Streptavidin (SA) Biosensors and tested for purified SufC binding. Data indicated a binding affinity of 2 µM. For further validation of the binding result, the Octet® assay orientation was reversed so that a biotinylated SufC was immobilized to the SA Biosensors and assayed for small molecule 882 binding to SufC. This reverse assay produced a similar binding affinity of 2 µM. While in most cases it is not necessary to validate a binding result is both orientations, the Choby lab in this case opted to do so to confirm that the binding interaction observed was not a byproduct of the biotin-tag that was introduced to the small molecule. Additionally, the binding assays eliminated other proteins that were pulled down in co-IP experiments validating SufC as a small-molecule binding target that complemented a plethora of genetic, proteomic and molecular biology observations.

Summary

Detecting and characterizing small-molecule binding interactions with target proteins is an important step in drug discovery and development programs. However, small molecule related applications are challenging and require sensitive instrumentation to confidently analyze small-molecule interactions. The Octet® BLI platform has been widely used to measure binding affinities and kinetics of small-molecule targets. They offer fast time-to-results and are easy-to-use due to non-fluidics-based sample analysis and dip-and-read biosensors. Octet® BLI can be easily scaled on throughput and can process up to 96 biosensors simultaneously. In this review, several selected publications highlight the use of Octet® BLI platform in small molecule related workflows, from target identification, validation and structure-based molecule development.

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