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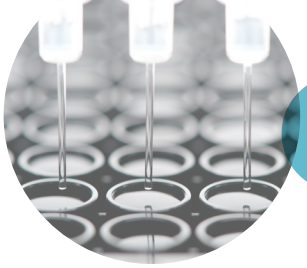

Label-free technologies for accurate determination of affinity and kinetics rate constants

Introduction

From target molecule identification to lead selection and optimization, equilibrium affinity and kinetics rate constants are critical parameters in the advancement of a potential therapeutic drug through the development cycle. These parameters enable researches to better understand mechanisms of action (MOA) of the drug candidate against its target as well as provide necessary information in the selection of optimal candidates. In addition to new drug lead molecules, kinetics characterization is also critical in comparing biosimilar molecules to their originator counter parts. For biosimilars, the evaluation of product quality attributes, especially functional equivalence which may be assessed through ligand binding studies is of primary importance in providing confidence for similar clinical safety and efficacy in patients. Full characterization of lead molecules is typically performed using purified molecules, however, lead selection can be done with non-purified samples as well through off-rate ranking in screening type of experiments. For antibody-based drugs, other characteristics including epitope binning may also be necessary for the classification and selection of optimal antibody clones.

Label-free surface-based analytical methods such as Bio-Layer Interferometry (BLI) and surface plasmon resonance (SPR) together with isothermal calorimetry (ITC) and ELISA are widely used for characterization studies. BLI and SPR techniques provide real-time dissociation and association rate constants, which are useful indicators in determining mechanisms of action (MOA). ELISA and ITC on the other hand do not provide these critical information; affinity constant (K_D) is determined by the ratio of the dissociation and association rate constants (k_d/k_a)[1]. However, a noted concern with solid-surface based methods for affinity characterization is surface chemistry and artifacts that may cause differences in the exhibited affinity constants between techniques; as a result, assay optimization is crucial in the determination of accurate

The Octet platform advantage

Octet® instruments utilize BLI technology for affinity characterization of biomolecular interactions. The instruments are a walk-away platform that requires minimal analyst interaction, hence is less prone to analyst mistakes that may lead to high data variability. Rate constant measurements are fast and are performed in parallel with sensors moving to samples rather than a microfluidics-based approach. In addition, the real-time detection mode speeds assay troubleshooting for rapid optimization. The sample plate format also leads to much faster assay development time on Octet instruments over SPR-based techniques as it provides real estate for design of experiments through which various assay conditions can be evaluated simultaneously. In addition, interactions can be measured in crude matrices or with non-purified samples unlike SPR where samples need to be purified to prevent the fluidic channels from clogging. Octet instruments also require lower sample volumes when compared to either SPR or ITC. ITC, while different from BLI and SPR in that it is a solutions chemistry-based technique, also does not provide rates-based information during binding analysis. Moreover, certain interactions chemistries may be difficult to analyze if the matrices in which the analytes are dissolved in are different, or if there's no heat exchange upon binding. Sample concentration limitations may also prevent the use of ITC for weak binders where high analyte concentration is required.

In this short communication, we list some examples from the literature where BLI, SPR or ITC-derived affinity constants are compared using similar biological model pairs while noting the prior-mentioned advantages of the BLI technique. These advantages, when combined with the ease of use and high-throughput capabilities of Octet instruments make the platform better suited for rapid assay optimization than any of the comparable techniques. The platform is also more versatile in that it can accommodate any sample type.

Accurate affinity characterization and antigen activity

An often ignored factor in biomolecular interactions, affinity characterization is the accuracy of the concentration of the analyte. While the observed off-rate is independent of the analyte concentration, the on-rate depends on an accurate input of this concentration. If the concentration is wrong, the derived affinity constant will also be inaccurate. As a result, it is likely that this parameter is a major contributor to the high variance sometimes observed between experiments in determining affinity constants. Even when accurate analyte concentrations are provided, the analyte may exist as heterogeneous populations, or in populations with conformations resulting in a lower active or effective concentration than the nominal value used in calculating the affinity constant.

In a study to explore the dynamic range of a KINEXA assay in characterizing antibody-antigen affinity, a group at Rinat, Pfizer[2] used the Dickkopf protein 1 (DKK1) and other members of the protein's family. They first used the Octet and Biacore instruments to determine the active concentration of the protein samples before subjecting them to an affinity analysis on KINEXA and Biacore. For human DKK1, they determined the activity to be 31–39% by SPR, compared to 39% on the Octet system. For human DKK4, the SPR assay yielded an activity of 37–39% while the Octet assay exhibited 45%. Due to the Octet system's well-known ability to accurately determine antigen concentration, analyte sample concentrations for the kinetics experiments were adjusted based on active concentration results from the Octet system. The novelty in this experimental design is that the analyte concentration is based on only the active components with the authors clearly demonstrating that accuracy in affinity constant determination is a function of analyte concentration.

In another study, Kamat *et al.*[3] at Regeneron also show that challenges in determining accurate and reproducible affinity constants of antibody-antigen interactions can be addressed through the evaluation of optimal experimental conditions. In their study, they found that with the experimental conditions properly evaluated, low and optimal immobilization ligand density on the biosensor surface reveals single-cycle kinetics experiments performed on the Octet HTX instrument to be reproducible and comparable to data obtained from the Biacore 4000 and MASS-1, both SPR instruments. In addition, when they evaluated the binding characteristics of 150 antibodies to 10 antigens, they found good concordance between the three instruments ($R^2 > 0.9$), suggesting that under optimal assay conditions, the three techniques produced comparable data. Their key finding, as has been suggested by others, is that ligand density is critical to the accuracy of high-affinity interactions on the Octet platform. This conclusion is likely true for SPR-based technologies as well. Another study with similar conclusions was carried out by Neuber *et al.*[4] in an FcRn-human IgG interac-

Technology	Instrument	Surface chemistry	Ligand	Analyte	Affinity constant (K_D), nM	Reference #
BLI	Octet HTX system	Anti-human Capture (AHC)	mAb-6	28 kDa Antigen	2.94	3
SPR	Biacore 4000	Anti-human Fc on CM5			1.80	
SPR	MASS-1	Anti-human Fc on CM5			2.98	
BLI	Octet HTX system	Anti-human Capture (AHC)	mAb-22	105 kDa Antigen	1.33	3
SPR	Biacore 4000	Anti-human Fc on CM5			1.36	
SPR	MASS-1	Anti-human Fc on CM5			0.93	
BLI	Octet QK system	Streptavidin	HypA	UreE	5.40	5
SPR	Biacore X100	Biotin-Capture on CM5			13.50	
BLI	Octet RED384 system	Streptavidin	hCA II	mICA	32.00	6
SPR	Biacore X	CM5	mICA	hCA II	9.00	
BLI	Octet RED system	Streptavidin	wt EGF-Fc	PCSK9	900.00	7
SPR	Biacore 3000	CM5			935.00	
BLI	Octet RED system	Streptavidin	EGF52-Fc	PCSK9	120.00	7
SPR	Biacore 3000	CM5			113.00	
BLI	Octet RED96 system	Anti-His	hu-TLR4	Antibody	1.35	8
SPR	Biacore 2000	CM5			3.20	
BLI	Octet RED96 system	Anti-His	Cyano-TLR4	Antibody	18.00	8
SPR	Biacore 2000	CM5			16.00	

Table 1: A select list of journal articles that directly compare affinity constants between the same biological molecules as derived by the BLI and SPR technologies.

tions study. This group noted that though FcRn binding typically varies widely due to the sensitivity of the receptor to pH changes, moderate differences observed in affinity constant analysis between SPR and BLI could be mainly attributed to experimental conditions especially where the immobilized ligand density was not properly controlled. In their study, the group however reports that their BLI method was robust and high-throughput, and allowed for fast sample analysis and evaluation of the results. They further stated that the BLI method is suitable for resolving even moderate differences in FcRn binding affinities by examining non-oxidized vs. oxidized IgG samples. In reviewing the literature, there are numerous reports that showcase comparison of affinity constants derived using both BLI and SPR with a clear indication that when the assay is well designed and developed, the affinity constants are comparable. Table 1 shows a listing of some examples.

A recent study by Simona de Franco *et al.*[9] compares affinity constants derived through the Octet platform and an ITC in a study of the binding of RNA to a variety of zinc fingers. In their work, Simona *et al.* postulate that since RNAs have proven to be major players in eukaryotic biology in many different ways, including in the implications of mRNA processing in diseases such myotonic dystrophy and cancer, functionalized RNA binding proteins (RBPs) that are able to target a chosen RNA sequence would be valuable tools for RNA manipulation. They chose to work with zinc-finger (ZF) proteins as they are an attractive alternative candidate for RBP engineering and have been successfully used to design DNA-binding proteins. They measured the binding affinities of the different ZFs for their selected RNA sequences and noted overall that BLI and ITC measurements were in good agreement with all dissociation constants in the low µM range. The BLI data, however, provided other kinetics information, allowing for an in-depth evaluation of other binding characteristics of the studied mutants.

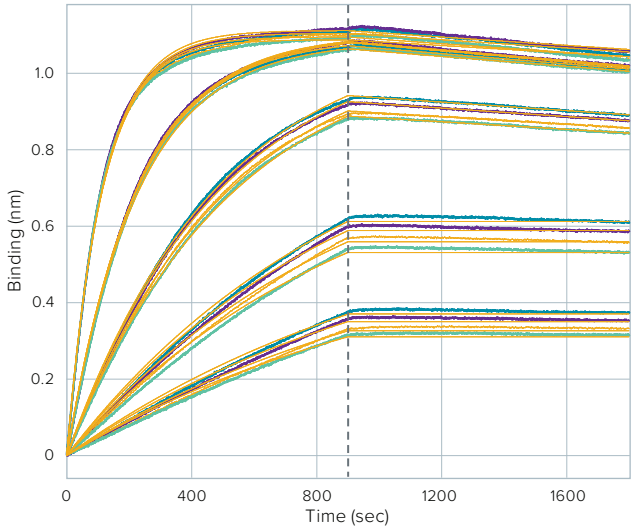

Figure 1: Human IgG binding to its receptor. Biotinylated receptor molecule was immobilized onto a High-Precision Streptavidin biosensor at low density. Human IgG was serially diluted 2-fold starting at 10 nM. The assay was run on an Octet RED96e system at 25°C.

In another comparison between BLI and ITC, Henry Maun *et al.*[10] investigated the biochemical and structural aspects of the anti-Shh monoclonal antibody 5E1 interaction with Shh. 5E1 is a pathway antagonist that is widely used to study Hh signaling in both developmental biology and cancer and is known to block binding of all three mammalian Hh ligands to Ptc1 with low nanomolar affinity, thereby inhibiting Hh signaling. Both BLI and ITC were used to characterize the binding of human Shh and rat Shh to 5E1 in the absence or presence of divalent metals (Ca^{2+} and Zn^{2+}). They found that in the absence of any divalent metal ions, ch5E1 Fab bound to Shh with a K_D of 7 nM. The addition of Ca^{2+} favored the interaction, reducing the K_D by 6-fold to 1.1 nM while the presence Zn^{2+} in the buffer did not substantially change the affinity (K_D 4.8 nM) indicating that Zn^{2+} by itself is not a major modulator of the interaction under the conditions of the study. However, a combinantion of both Zn2 and Ca^{2+} further reduced the K_D by 3.6-fold to 0.31 nM, resulting in an overall increase in affinity. Both BLI and ITC reported very similar affinity constant data.

In another study[11], Noah et al., investigated the ATP binding cassette (ABC) transporter p-glycoprotein in three conformational states to obtain a comprehensive view of transporter dynamics. These proteins comprise one of the largest families of membrane proteins and utilize ATP hydrolysis to drive substrate transport across a cell membrane. BLI was used to measure the affinity of wild type and mutant P-gp to ATP and AMPPNP. The BLI data revealed two binding affinities as had been observed in previous studies of Chinese hamster P-gp binding to the non-hydrolyzable ATP analogue ATP-γ-S, which revealed that when one NBD occludes nucleotide with relatively tight affinity (K_D: 4 µM), the other NBD also remains associated with the nucleotide, albeit more weakly (K_D: 740 µM). This group's work represents a case study for the use of the BLI platform for characterizing low affinity binders.

Reproducibility	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
Replicate 1	8.64E+05	4.58E-05	5.30E-11
Replicate 2	8.60E+05	4.81E-05	5.59E-11
Replicate 3	8.60E+05	4.68E-05	5.44E-11
Replicate 4	8.57E+05	4.20E-05	4.90E-11
Mean	8.60E+05	4.57E-05	5.31E-11
Standard Deviation	2.87E+03	2.62E-06	2.96E-12
%CV	0.33%	5.73%	5.57%

Table 2: Repeatability assessment of the binding of a receptor to human IgG on the Octet RED96e system. Samples were prepared in bulk to minimize pipetting errors and loaded onto different columns of a 96-well microtiter plate. Each replicate was analyzed from an independent column of the sample plate.

Octet platform repeatability assessment: human IgG binding to a receptor molecule

Just as important as accuracy is the reproducibility of an assay. Assuming that the analyzed reagents are of high integrity, *i.e.* stable, assay reproducibility is critical to the validity of the method used. In this example, we show that for affinity characterization of stable binding pairs, ForteBio's high precision Streptavidin biosensors (SAX2) can be used to generate assays that are highly reproducible. The biosensors are well suited for any downstream applications, including QC where reagent characterization such as kinetics assessment is required, and where biotinylated ligands are in use.

Conclusion

Many analytical laboratories are today equipped with different biophysical tools for characterization of biomolecular interactions. Of the three methods discussed, SPR and ITC are mainly used for binding characterization. The Octet system on the other hand is a workhorse that can be used for both binding characterization and screening of different types of biologics due to its better ease of use configurations and low maintenance attributes. For antibody:antigen or protein:protein interactions where molecules tend to be large in size, data in the literature suggests that the three techniques provide comparable affinity constant data when similar model pairs are investigated. Instrument configuration differences, however, may require that some assay optimization be performed in specific situations. In these situations, the Octet system's ease of use and plate-based sampling format is an added advantage as they enable comparatively faster assay development. In addition, the Octet platform in combination with disposable off-the shelf biosensors allows for greater versatility with use over a diverse range of sample types that may not be possible in either ITC or SPR techniques.

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