Label-free determination of kinetic constants for small molecule binding to proteins using FortéBio's Octet Red Multi-Channel Platform

Charles Wartchow, Danfeng Yao, Pu Li, Bettina Heidecker, Krista Witte, Sae Choo, Jing Wei, Don Perillat, Huddee Ho, Lian-She Zhao, Tim Ellis, Greg Carricato, Nancy Stainton, Scott Lockard, Hong Tan, and Robert Zuk, FortéBio, Inc. Menlo Park, CA

Abstract

The determination and evaluation of the affinity of small molecule binding to a therapeutic target is a significant component of drug discovery and lead optimization. We report a label-free method for determining kinetic constants for the binding of small molecule inhibitors (<500 Daltons) to carbonic anhydrase, including rate constants and affinity constants. These parameters are derived from data obtained using FortéBio's Octet RED System, a label-free, 8-channel system that is compatible with 96-well microplates. The system is based on BioLayer Interferometry, a technique that generates an interference pattern by monitoring visible light reflected from two surfaces in a fiber-optic biosensor. When binding events occur at the tip of a fiber optic biosensor, the interference pattern shifts to higher wavelength, and these changes are monitored in real time.

In a typical experiment, biotinylated protein targets are immobilized onto a high-binding capacity streptavidin surface, and the association and dissociation of small molecules with molecular weights ranging from ~150-350 Daltons is monitored in parallel. Kinetic constants for the binding of compounds to carbonic anhydrase with micromolar and nanomolar affinities agree closely with published values. In addition, evaluations conducted on proteins obtained from major pharmaceutical partners show agreement of the results between Octet RED and other SPR-based methods.

Measuring Protein - Small Molecule Interactions using BioLayer Interferometry (BLI) Technology

Octet provides real-time monitoring for protein: small molecule interactions and binding events using BioLayer Interferometry (BLI) technology. Any change in the number of molecules bound to the biosensor tip changes the optical layer thickness. Changes in optical thickness cause a shift in the interference pattern ($\Delta\lambda$)that can be measured in real time (Figure 1).

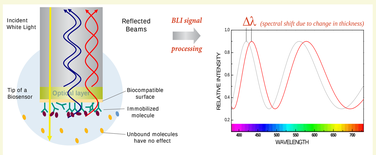


FIGURE 1: Octet BioLayer Interferometry (BLI) Technology Principle

Molecular binding events at the sensor surface increase the thickness of the biological layer, which results in a shift of the interference pattern to higher wavelength. Dissociation reduces the thickness and shifts interference pattern to lower wavelength.

Methods

- **Protein biotinylation:** Proteins were biotinylated at a 1:1 molar coupling ratio of Biotin-LCLC-NHS (Pierce) in PBS at ~30 µM protein. Unreacted biotin was removed by dialysis with a Pierce 10kD MWCO dialysis cassette.
- **Preparation of Super Streptavidin Biosensors:** Biotinylated protein was attached to streptavidin-coated biosensors by incubating the protein conjugate at 30-100 µg/mL PBS with biosensors for ~30-60 minutes on a benchtop at room temperature.
- **Sample preparation:** Compounds were dissolved in DMSO at a final concentration of 1-20 mM and diluted in PBS followed by serial dilution in PBS with DMSO at the concentrations indicated. Samples including PBS/DMSO for baselines and dissociations were placed in a 96-well sample plate for analysis.

Biomolecular Interaction Analysis on the Octet RED System

Biosensors were placed in buffer matching the composition of the assay prior to analysis and equilibrated for ~10-15 minutes followed by cycles of baseline, association, and dissociation ranging from 30-60 seconds, 60-180 seconds, and 120-360 seconds, respectively, at 1000 RPM and 30 C. References include the streptavidin surface blocked with biocytin (with and without compound), and biotin-protein surface that is monitored in assay buffer throughout the run. Reference subtractions were performed in FortéBio software, and kinetic analysis was performed with the Scrubber application (BioLogic Software, Campbell, Australia) or Octet software. Acetazolamide results were obtained using a mass transport term, and other results were relatively unaffected by this term.

Assay setup and workflow on the Octet RED System for unknown small molecule compounds

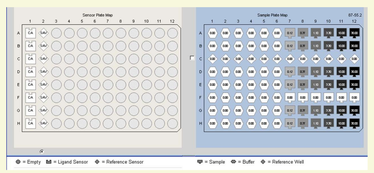


FIGURE 2: Sensor set-up (left) and plate map (right).

SMALL MOLECULE ASSAY WORKFLOW AND ANALYSIS

1. Target Immobilization

- Load biotinylated target to saturation
- Equilibrate sensors for 15-20 minutes

2. Optimal Analysis Range Determination

- Obtain compound binding data at 10, 1, 0.1 µM
- Determine optimal concentration range

3. Data Analysis

- Subtract System noise and drift
- Examine non-specific binding to the reference sensor
- Sensorgrams : Identify artifacts due to secondary binding, non-system drift, and high signals
- Examine results for concentration-dependent responses
- Determine preliminary kinetic constants: K_D

4. Kinetic Constant Determination

- Obtain binding data for a 3X dilution series of the compound against the immobilized target
- Substract noise and drift
- Determine kinetic constants by global fitting or steady-state analysis

References:
1 Papalia et al, Analytical Biochem. 359 (2006), 94-105
2 Myszka, Analytical Biochem. 329 (2004), 316-323

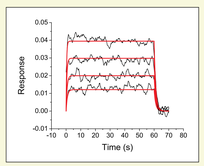
Evaluation of Carbonic Anhydrase binding to small molecules

Label-free analysis of the association and dissociation of a small molecule with the target protein of interest results in the determination of kinetic constants including the association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant (K_D). To assess the utility of the Octet Red platform for the determination of kinetic constants, we examined the binding of inhibitors of carbonic anhydrase, a well-established model system. These inhibitors were selected to demonstrate the robustness of the Octet RED to measure low molecular weight compounds with respect to accuracy, reproducibility, and DMSO compatability.

ACCURACY: CARBONIC ANHYDRASE : INHIBITOR PANEL <350 Daltons

Accuracy against SPR was determined on the Octet RED system with sulpiride, furosemide, benzenesulfonamide, and acetazolamide, which are high, medium, and low affinity inhibitors of carbonic anhydrase.

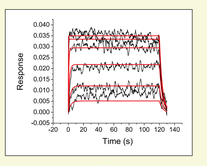
LOW AFFINITY: Sulpiride (341 Daltons)



$k_{on} = 3900 \pm 200\ M^{-1}s^{-1}$, $k_{off} = 0.93 \pm 0.04\ s^{-1}$, $K_D = 239 \pm 4\ \mu M$

FIGURE 3: Analysis of a weak binding interaction: sulpiride:carbonic anhydrase binding data at 500-63 µM furosemide (2X dilution series) in PBS with 1% DMSO.

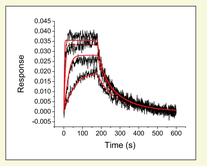
LOW MOLECULAR WEIGHT Benzenesulfonamide (165 Daltons)



$k_{on} = 89{,}000 \pm 3{,}000\ M^{-1}s^{-1}$, $k_{off} = 0.174 \pm 1.006\ s^{-1}$, $K_D = 1.96 \pm 0.02\ \mu M$

FIGURE 4: Analysis of furosemide:carbonic anhydrase binding data at 30-0.12 µM furosemide (3x dilution series, N=5) in PBS with 0.5% DMSO.

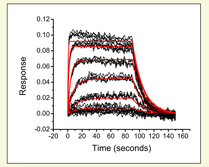
HIGH AFFINITY: Azetazolamine (222 Daltons)



$k_{on} = 8.2E5 + 0.1E5\ M^{-1}s^{-1}$, $k_{off} = 0.0325 + 0.0004\ s^{-1}$, $K_D = 39.4 + 0.2\ nM$, $k_T = 10{,}000$

FIGURE 5: Analysis of a high affinity interaction: acetazolamide:carbonic anhydrase binding data at 1-0.012µM acetazolamide (3X dilution series) in PBS with 0.5% DMSO. For this analysis, a mass transport term (k_T) was applied in Scrubber.

MEDIUM AFFINITY: Furosemide (330 Daltons)



$k_{on} = 58{,}200 + 400\ M^{-1}s^{-1}$, $k_{off} = 0.0674 + 0.005\ s^{-1}$, $K_D = 1.157 + 0.005\ \mu M$

FIGURE 6: Analysis of furosemide:carbonic anhydrase binding data at 30-0.12 µM furosemide (3x dilution series, N=5) in PBS with 0.5% DMSO.

Reproducibility

To demonstrate the reproducibility of results obtained from the Octet RED System, 2 different inhibitors binding to carbonic anhydrase were assayed. Kinetic constants for multiple analyses agree within <30% for benzenesulfonamide and within <17% for furosemide.

Benzenesulfonamide (157 Daltons)

Analysis	Rmax (Δnm)	Rmax Error	k_{off}(1/s)	k_{on}(1/Ms)	k_{on} Error	K_D (M)	Chi²	R²
1	0.036	0.0001	2.63E-01	1.11E+05	2.02E+03	2.37E-06	0.021	0.960
2	0.040	0.0001	3.04E-01	8.41E+04	1.74E+03	3.62E-06	0.027	0.962
3	0.030	0.0001	2.49E-01	2.08E+05	8.70E+03	1.20E-06	0.089	0.691
4	0.035	0.0001	3.29E-01	1.34E+05	2.76E+03	2.45E-06	0.021	0.956
5	0.032	0.0001	3.31E-01	1.96E+05	3.73E+03	1.69E-06	0.017	0.959
6	0.037	0.0001	3.42E-01	1.26E+05	2.85E+03	2.71E-06	0.028	0.955
Average	0.035	0.0001	0.30	143183	3633	2.34E-06	0.034	0.914
SD	0.003	0.0001	0.04	48779	2579	0.8E-06	0.027	0.109

Furosemide (330 Daltons) in 0.5% DMSO

Analysis	Rmax (Δnm)	Rmax Error	k_{off}(1/s)	k_{on}(1/Ms)	k_{on} Error	K_D (M)	Chi²	R²
1	0.0975	0.0001	7.83E-02	6.49E+04	4.57E+02	1.21E-06	0.053	0.99
2	0.1017	0.0001	7.88E-02	5.75E+04	3.84E+02	1.37E-06	0.050	0.99
3	0.0951	0.0002	8.52E-02	6.76E+04	5.66E+02	1.26E-06	0.067	0.99
4	0.0976	0.0002	7.97E-02	6.20E+04	4.69E+02	1.28E-06	0.059	0.99
5	0.0931	0.0001	8.32E-02	8.72E+04	6.97E+02	9.54E-07	0.063	0.99
Average	0.097	0.0002	0.081	67836	515	1.22E-06	0.058	0.989
SD	0.003	0.0002	0.003	11453	121	1.57E-07	0.007	0.002

Blind Study of Protein:Small Molecule Interactions using BLI on the Octet RED System and Surface Plasmon Resonance

In a blind study, ten compounds (162-330 Daltons) were analyzed with the Octet RED, and the results were compared to results from Biacore. Accuracy between the two methods is generally within 2-3X. The comparative values between results obtained from the Octet RED System and Biacore is listed in the table below.

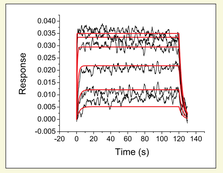
Compound	Size (Daltons)	ForteBio K_D (µM)	Biacore K_D (µM)
1	258	0.6	0.4
2	165	0.1	0.5
3	168	1.2	3.3
4	269	12	4.6
5	269	1.3	0.4
6	221	1.7	1.9
7	296	0.6	0.3
8	295	1.5	1.3
9	324	No binding	No binding
10	300	No binding	No binding

Comparison of Octet RED results to SPR

Compound	ForteBio	Biacore[1,2]
Acetazolamide (222 Daltons)	K_D = 0.039 µM k_{on} = 8.2E5 $M^{-1}s^{-1}$ k_{off} = 0.033 s^{-1}	K_D = 0.039, 0.019 µM k_{on} = 3.0E6, 2.9E6 $M^{-1}s^{-1}$ k_{off} = 0.079, 0.056 s^{-1}
Benzenesulfonamide (157 Daltons)	K_D = 2.4 µM k_{on} = 1.1E5 $M^{-1}s^{-1}$ k_{off} = 0.26 s^{-1}	K_D = 0.8, 0.85 µM k_{on} = 1.7E5, 1.7E5 $M^{-1}s^{-1}$ k_{off} = 0.12, 0.14 s^{-1}
Furosemide (330 Daltons)	K_D = 1.2 µM k_{on} = 6.4E4 $M^{-1}s^{-1}$ k_{off} = 0.078 s^{-1}	K_D = 1.0, 0.51 µM k_{on} = 6.3E4, 9.7E4 $M^{-1}s^{-1}$ k_{off} = 0.061, 0.05 s^{-1}
Sulpiride (341 Daltons)	K_D = 239 µM k_{on} = 3.9E3 $M^{-1}s^{-1}$ k_{off} = 0.93 s^{-1}	K_D = 48, 186 µM k_{on} = 8.0E3, 3.4E3 $M^{-1}s^{-1}$ k_{off} = 0.38, 0.64 s^{-1}

TABLE 2: Comparison of results obtained from the Octet RED System in comparison to surface plasmon resonance.

DMSO COMPATABILITY: Furosemide in 5% DMSO



$k_{on} = 3.1E5\ M^{-1}s^{-1}$, $k_{off} = 0.085\ s^{-1}$, $K_D = 2.7\ \mu M$

FIGURE 7: Analysis of furosemide : carbonic anhydrase binding data at 30-1.1 µM furosemide (3X dilution series) in PBS with 5% DMSO.

Summary

The ForteBio Octet Red platform is a precise and accurate platform for the characterization of small molecule binding kinetics to protein targets

- **Accuracy:** Results for K_D with the carbonic anhydrase model system and a collaborator's system generally agree within 2-3 fold in comparison to published literature. and previously obtained results, respectively.
- **Reproducibility:** Kinetic constants agree within <30% for benzenesulfonamide (157 Daltons) and within <17% for furosemide (330 Daltons).
- **DMSO compatability:** All determinations were tested with DMSO ranging from 0.5-5% v/v.
- **Low Molecular Weight Molecules:** Analysis was achieved for benzenesulfonamide at 157 Daltons.

ForteBio, Inc. 1360 Willow Road, Suite 205 Menlo Park, CA 94025 **t**: 1-888-OCTET-QK or 650-322-1360 **web:** www.fortebio.com