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Kinetic Curvature: Assessment of Small Molecule Kinetics and Affinity Using OneStep[®] Injections in SPR Screening

Stuart Knowing, PhD, Senior Scientist

Sartorius Corporation, 47661 Fremont Blvd, Fremont, CA 94538 USA

Correspondence

Email: octet@sartorius.com

Introduction

Fragment-based drug design (FBDD) has become a popular platform for the identification of lead candidates in drug discovery programs alongside structure-based drug design, and high throughput screening. In 2021 over 70% of the drugs approved by the FDA were non-protein based and therefore the ability to measure the kinetics & affinity across a range of molecular sizes is critical during initial selection.

The detection and characterization of these binding events is facilitated by sensitive biophysical technologies capable of detecting low affinity interactions of low molecular weight compounds. Surface plasmon resonance (SPR) is a core technology in many pharma and biotechnology settings for this purpose as SPR has the required sensitivity and throughput to provide complete fragment screens on libraries of several thousand compounds in just a few weeks per target.

Traditional initial SPR screening of weak affinity compounds using a single fixed concentration injection can present numerous challenges, which include low molecular weights, weak affinities (μM – mM), and solubility limitations that make it impractical to test for binding using sample concentrations above the K_D . When combined this can frequently result in the need to make decisions based on inappropriate (small, square-shaped) sensorgrams.

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Due to these restrictions, there is a need for techniques that can resolve these limitations and increase hit confidence, shorten timelines, and allow screening groups to provide richer datasets for more comprehensive analysis.

OneStep® Injections, which are unique to the Octet® SF3, use a continuous analyte titration method and can dramatically improve SPR-based screening by providing higher content information that allows for confident, rapid characterization of hits. OneStep® injections allow users to obtain accurate kinetic rate constants (k_a , k_d), affinity (K_D) and ligand efficiency (LE) data directly from the primary screen, and as such combine the first three steps (clean screen, primary yes/no screen, kinetics and affinity hit confirmation) in the traditional SPR workflow into a single assay.

Here, using a well-characterized small molecule interaction system, we demonstrate that standard multi-cycle kinetics (MCK) do not provide sufficient data from a single concentration injection, and that reliable kinetics and affinity can be obtained from significantly less data than is commonly used. In addition, the number of measurements necessary for accurate kinetics and affinity determination can be reduced to a single measurement by using OneStep® injections, thus increasing sample throughput and saving sample material.

This removes the barrier for SPR as a tool for large screens and allows rapid progression for additional assay development of potential therapeutics.

Methods

Instrument and Reagents

All assays were performed using an Octet® SF3 SPR system. Phosphate buffered saline with 0.05% Tween 20 (PBS-T), pH 7.4 was used as running buffer throughout. Unless indicated, all assays were performed at 25 °C.

PBS was purchased from Gibco. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Thermo Fisher Scientific. All other reagents were purchased from Sigma Aldrich and prepared in-house.

Kinetics and Affinity Determination

Carbonic Anhydrase II

Bovine carbonic anhydrase II (CAII) was immobilized on an Octet® SPR PCH Sensor Chip using standard amine coupling chemistry. Briefly, a 50:50 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 0.1 M N-Hydroxysulfosuccinimide (NHS) was injected across flow cells 1, 2 and 3 using a flow rate of 10 μ L/min for 7 minutes. CAII (100 μ g/mL in sodium acetate pH 5.0) was then injected across flow cells 1 and 3 using a flow rate of 10 μ L/min for 7 minutes. The surfaces were then deactivated by injecting 1 M ethanamine HCl pH 8.5 across flow cells 1, 2 and 3 using a flow rate of 10 μ L/min for 7 minutes.

Furosemide (330 Da) was dissolved directly in the PBS-T running buffer and analyzed using both a three-fold (10 μ M–0.123 μ M) and ten-fold dilution series (100 μ M–0.01 μ M). Acetazolamide (222 Da) was dissolved directly in the PBS-T running buffer and analyzed using a single concentration (1 μ M).

Samples were placed into Octet® SPR 0.9 mL vials and placed into a mixed format sample rack. 3% sucrose was prepared using PBS-T as the bulk reference standard for OneStep® injections. The sample rack was sealed using a resealable septa and placed in the sample tray set to 20 °C.

The Octet® SF3 system was primed 5 times into PBS-T running buffer and the sensor chip hydrated and conditioned using 10 injections of PBS-T. A standard assay format was used for fixed concentration injections and multi-cycle kinetics with a common association time of 60 seconds and a dissociation time of 140 seconds at 100 μ L/min. Association parameters for OneStep® are fixed based upon the volume of the injection loop used, which was set to 50% and the same dissociation parameters used as for multi-cycle kinetics. A buffer blank injection was performed for each analyte concentration to generate accurate double referenced data.

All multi-cycle kinetics data was globally fitted to a simple 1:1 interaction model for kinetics and affinity. Data assessing single fixed concentration using fixed concentration injections and OneStep® injections were locally fitted to a simple 1:1 interaction model for kinetics and affinity determination.

Results and Discussion

The objective was to demonstrate that standard fixed concentration injections (FCI) used in traditional SPR screens, which provide a flow of uniform analyte concentration across the sensor chip surface, are often outside the limits that produce sufficient curvature for kinetic analysis as a single concentration. In contrast OneStep[®] injections provide adequate kinetic curvature over a much wider range from a single concentration injection and can generate kinetic and affinity constants that compare favorably to those derived from standard multi-cycle kinetic assays. In addition, as the affinity of the drug-therapeutic is often unknown, the flexibility in determining the initial analyte concentration choice is considered.

Determining Reliable Kinetic and Affinity Parameters From a Minimal Data Set

Previous attempts at determining the minimum data set required for multi-cycle kinetics have shown that in a confidently measurable region, significant curvature is required to determine accurate kinetics.³ Even then, a prior screening process is required to determine the necessary concentrations.

The well-characterized small molecule interaction between furosemide and carbonic anhydrase II has literature values for the interaction that are traditionally in the range of 500–1000 nM.^{2,4,5}

Initial analysis of the kinetics and affinity determined by fixed concentration injections ranging from 0.01–100 μM are shown in Table 1 and the associated sensorgram shown in Figure 1A–C.

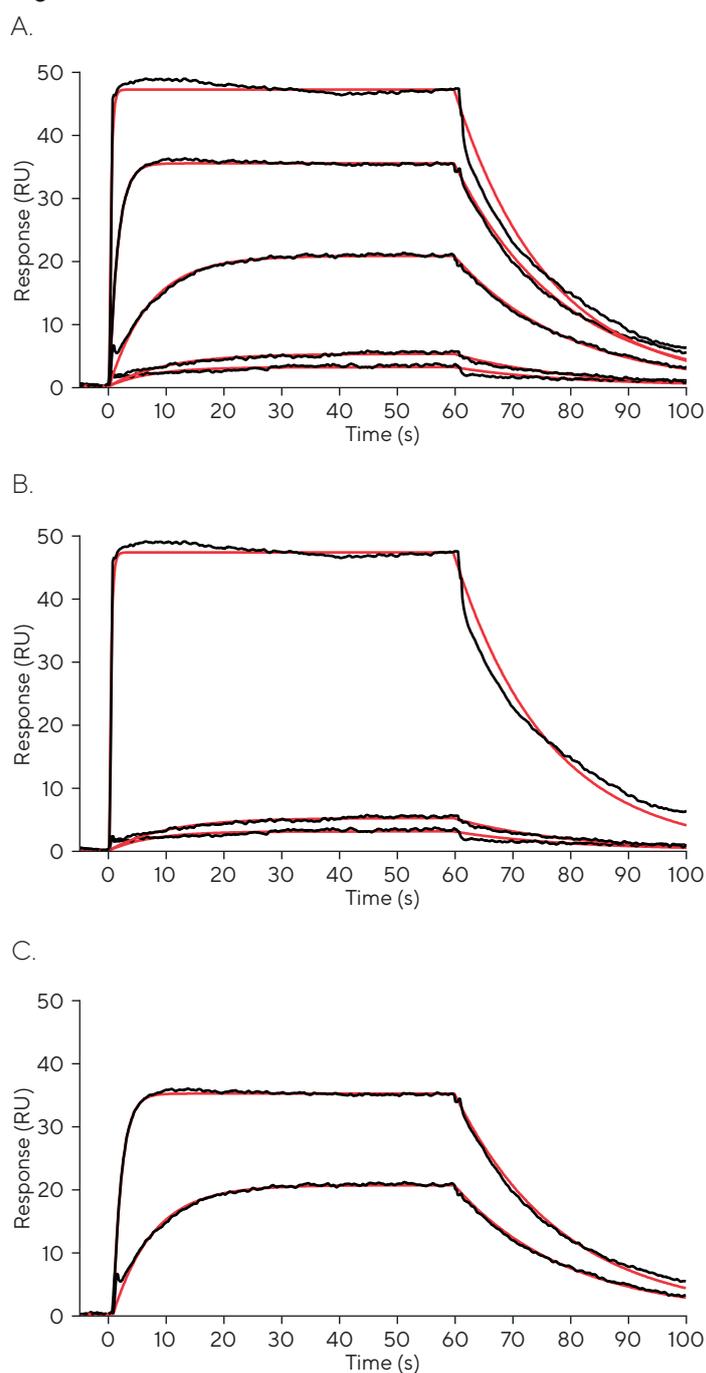
As shown in Figure 1A and highlighted in Figure 1B, the highest fixed concentration injection of 100 μM exhibits an association phase that is too rapid and only a few data points occur within the kinetic phase. At the lowest fixed concentration injections of 0.01 μM and 0.1 μM the binding responses can be viewed as linear and therefore also lack detailed information about the reaction kinetics. This lack of usable kinetic information is reflected in the determined affinity values of 5.8 nM, 105 nM, and 2050 nM for 0.01, 0.1 and 100 μM fixed concentration injections, respectively.

Figure 1C shows the fixed concentration injections of 1 and 10 μM both of which show sufficient curvature and reach equilibrium, which allows for accurate kinetic rate constants to be determined.³ Affinity values of 552 nM and 851 nM for 1 μM and 10 μM are broadly in line with published values.

Table 1

	Concentration (μM)	k_a ($\text{M}^{-1}\text{s}^{-1}$)	k_d (s^{-1})	K_D (nM)
Furosemide	100	2.99×10^4	0.0614	2050
	10	6.22×10^4	0.0529	851
	1	9.21×10^4	0.0509	552
	0.1	4.93×10^5	0.0518	105
	0.01	8.60×10^6	0.0498	5.8

Figure 1

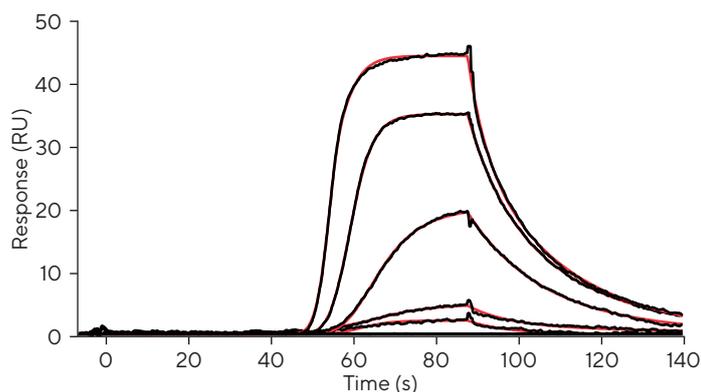


A single injection of each Furosemide concentration was then injected across the same surface using OneStep® injections. It is important to note that multiple OneStep® injections are not required in normal screening and the rationale of performing multiple injections here was to determine the tolerance levels between fixed concentration injections and OneStep® injections. Kinetics parameters for local fits of each concentration are shown in Table 2, corresponding sensorgrams in Figure 2.

Table 2

	Concentration (μM)	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (nM)
Furosemide	100	5.36×10^4	0.0448	835
	10	8.49×10^4	0.0529	623
	1	8.14×10^4	0.0539	660
	0.1	1.20×10^5	0.0518	430
	0.01	1.00×10^6	0.0100	10

Figure 2



Unlike fixed concentration injections, OneStep® injections show curvature throughout the concentration series, allowing reliable kinetic rate constants to be determined over a much wider range of concentrations. This is reflected by an improvement in the determination of kinetic and affinity parameters for values outside of the range of fixed concentration injections, 0.1 μM and 100 μM. Unlike the fixed concentration injection for 100 μM, the OneStep® injection shows significant curvature and as such allows a reliable kinetics and affinity value to be determined, which is in agreement with published values (835 nM). This improvement can also be seen in the 0.1 μM OneStep® injection, with an affinity value of 430 nM compared to the 105 nM from the fixed concentration injection. Therefore, a single OneStep® injection is sufficient to determine accurate association kinetics compared to standard fixed concentration injection.

Kinetic Analysis of Furosemide and Acetazolamide Binding Carbonic Anhydrase II

Unlocking the potential of SPR screening involves generating accurate data quickly that allows potential candidates to progress quickly through downstream stages. As shown for furosemide binding to carbonic anhydrase II, multiple analyte concentrations are able to produce accurate kinetics and affinities using OneStep® injections. A single concentration injection that accurately resolves association and dissociation kinetics is highly desirable in terms of saving precious sample, time and importantly, how many samples can be assessed in a single run.

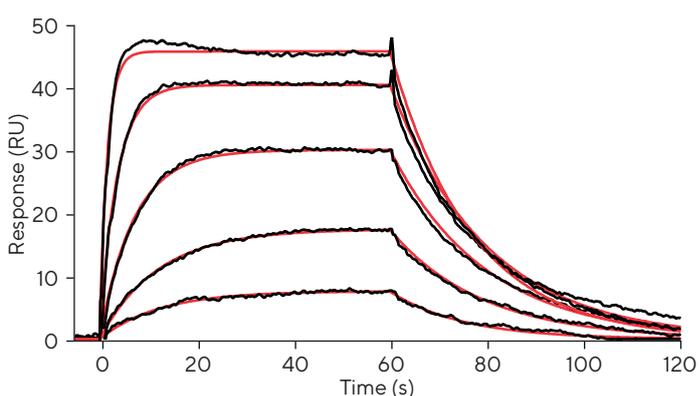
Here, carbonic anhydrase II was immobilized as described in the methods section binding to the small molecule furosemide assessed using standard multi-cycle kinetics and a single top concentration OneStep® injection.

Initial analysis of the Furosemide Carbonic Anhydrase II kinetics determined by multi-cycle kinetics (10 μM–0.123 μM) is shown in Table 3 and the associated sensorgram shown in Figure 3.

Table 3

	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (nM)
Furosemide	7.96×10^4	0.0535	671

Figure 3

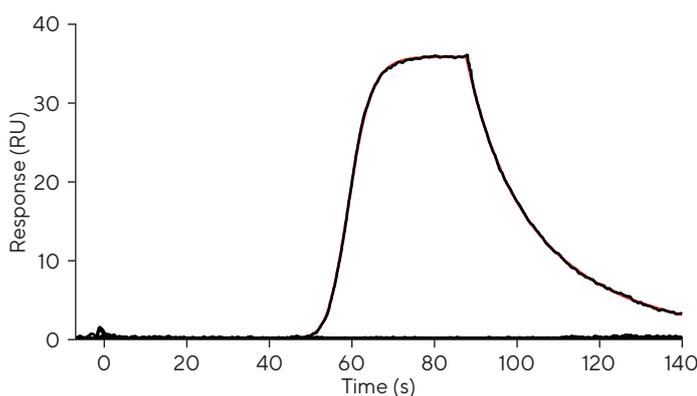


A single injection of 10 μM was then injected across the same surface using a OneStep[®] injection (Table 4 and Figure 4).

Table 4

	k_a ($\text{M}^{-1}\text{s}^{-1}$)	k_d (s^{-1})	K_D (nM)
Furosemide	8.49×10^4	0.0529	623

Figure 4



As shown, both multi-cycle kinetics and OneStep[®] injections performed on the Octet[®] SF3 system show kinetics and affinity values that are in excellent agreement with literature values.^{2,4,5}

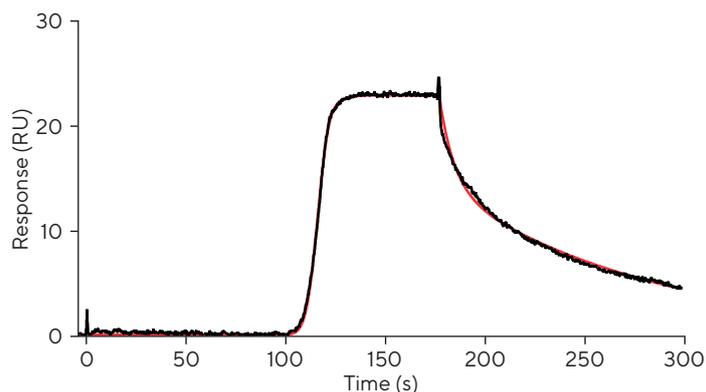
As a proof of principle, a single injection of acetazolamide (1 μM) was then injected across the same surface using a OneStep[®] injection (Table 5 and Figure 5) and its kinetics and affinity value found to be in excellent agreement with those in literature.^{2,4,5}

Table 5

	k_a ($\text{M}^{-1}\text{s}^{-1}$)	k_d (s^{-1})	K_D (nM)
Acetazolamide	6.43×10^5	9.69×10^{-3}	15.1

Previous attempts at determining the minimum data set required for multi-cycle kinetics have shown that in a confidently measurable region significant curvature is required³ to determine accurate kinetics, and even then, a screening process is required beforehand to determine the necessary concentrations. As shown here for furosemide and acetazolamide binding to carbonic anhydrase II and exemplified in Quinn,⁶ OneStep[®] injections provide significant curvature during the single injection thanks

Figure 5



to the analyte gradient formation, which allows accurate association kinetics to be rapidly determined. A single top concentration matching that used for multi-cycle kinetics is sufficient to derive accurate kinetics and affinity but importantly as shown for furosemide binding to carbonic anhydrase II, even if the affinity of the interaction is unknown, a wide range of analyte concentrations can be used to determine precise association and dissociation kinetics. As shown, these concentrations are typically linear in standard SPR injections and therefore, yield no usable kinetic information.

In addition to a significant sample saving of ~50% due to the lack of necessity for serial dilutions, OneStep[®] injections offer significant time savings. The total time taken for the furosemide multi-cycle kinetics assay (including buffer blanks) was approximately 24 min and 5 min for the OneStep[®] injection (including buffer blank). This represents a significant time saving of almost 20 min for a single analyte, which would only be compounded at a higher number of samples.

OneStep[®] injections offer additional benefits when assessing binding interactions thanks to the necessity of only a single injection. Surface regeneration plays an integral part in assessing interactions and unlike standard multi-cycle kinetics, where several concentrations require an equal number of regenerations, OneStep[®] injections only require a single regeneration step. Over the course of a high throughput screen, this reduction in the number of regeneration cycles is critical to achieving stable baselines, regardless of whether the ligand is directly immobilized to the sensor chip surface or a capture molecule is being used.

As shown above, OneStep[®] injections compared favorably to those derived from standard multi-cycle kinetic assays at a single top concentration injection but also allows flexibility in initial assay design through initial analyte concentration.

Conclusions

High-throughput assays that require minimal development times are key to identifying molecules from compound libraries. As shown here, standard fixed concentration injections used in fragment and small molecule SPR screens, which provide a flow of uniform analyte concentration across the sensor chip surface, are often outside the limits that produce sufficient curvature for kinetic analysis and produce no reliable kinetic information. Therefore, after an initial screen at a single concentration, multiple concentration injections (multi-cycle kinetic) are required to generate accurate kinetics and affinity data. In contrast, OneStep® injections produce adequate kinetic curvature over a much wider range from a single concentration injection and can generate kinetic and affinity constants that compare favorably to those derived from standard multi-cycle kinetic assays. OneStep® injections significantly reduce time costs and produce a complete kinetic profile from a single experiment. In addition, as the affinity of the drug-therapeutic is often unknown, OneStep® injections allow flexibility in determining the initial analyte concentration to assess.

The detection and characterization of fragment binding events is dependent upon ultrasensitive technologies that are well suited to detect interactions of weak affinity and low molecular weight compounds. Here, we show using a well-characterized small molecule interaction system that OneStep® injections can generate accurate kinetics and affinity using much less sample and data than is commonly

used today, which has the extraordinary advantage of increasing sample throughput and data content, while decreasing sample preparation time and reducing human error by eliminating the preparation of multiple sample dilutions.¹ Thanks to the use of fewer reagents and the use of a single sensor chip, assay costs are significantly reduced too.

The Octet® SF3 and OneStep® injections remove the guess work from high throughput screens and allows a full kinetic panel to be determined in a fraction of the time compared to standard techniques that require initial screening and then potentially extensive assay development. Full kinetic information early in drug discovery allows rapid progress of potential therapeutics through the development pipeline and forms a solid foundation for any additional assay development. Thanks to full automation and flexible sample format, up to 768 unique analytes can be assessed in a single run, allowing you to screen libraries in days rather than weeks.

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Germany

Sartorius Lab Instruments
GmbH & Co. KG
Otto-Brenner-Strasse 20
37079 Goettingen
Phone +49 551 308 0

USA

Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
Phone +1 888 OCTET 75
Or +1 650 322 1360

 For additional information,
visit www.sartorius.com