



Determining kinetics and affinities of protein interactions using a parallel real-time label-free biosensor, the Octet

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ABSTRACT

ForteBio's Octet optical biosensor harnesses biolayer interferometry to detect and quantify molecular interactions using disposable fiber-optic biosensors that address samples from an open shaking microplate without any microfluidics. We recruited a monoclonal antibody against a panel of peptides to compare the Octet directly with Biacore's well-established 3000 platform and Bio-Rad's recently launched ProteOn XPR36 array system, which use surface plasmon resonance (SPR) to detect the binding of one analyte over four surfaces and six analytes over six surfaces, respectively. A sink method was used to prevent analyte from rebinding the ligand-coated Octet tips and enabled us to extract accurate kinetic rate constants, as judged by their close agreement with those determined by SPR. Although the Octet is not sensitive enough to detect the binding of small molecules directly, it can access their affinities indirectly via solution competition experiments. We conducted similar experiments on the SPR instruments to validate these measurements. The Octet is emerging as a versatile complement to other more sophisticated biosensors, and the ProteOn provides high-quality data near the sensitivity of Biacore but in a more multiplexed format. Our results provide a benchmark for assessing the performance of the above-mentioned sensors.

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Cutting-edge biosensors are revolutionizing our understanding of biomolecular interactions and are established as routine biophysical tools in research laboratories worldwide. Until recently, Biacore AB (now part of GE Healthcare) had been the only significant vendor of commercial biosensors due in part to its patent surrounding the use of a hydrogel matrix for monitoring interaction analysis by exploiting surface plasmon resonance (SPR)¹ and its expanding repertoire of customized platforms. Recently, however, other companies have emerged with complementary technologies that differ in detection, sample delivery, and/or throughput [1]. One such platform is the Octet, commercialized by ForteBio (Menlo Park, CA, USA). This simple dip-and-read assay harnesses biolayer interferometry incorporated onto inexpensive disposable optical fiber biosensors to measure a parallel set of eight interactions from an open shaking plate (Fig. 1). Thus, the sensors move to the samples, rendering unnecessary the microfluidics that traditionally deliver samples to a stationary Biacore sensor chip. By handling samples on single-use tips without microfluidics, the Octet offers a unique

platform with significant advantages over other biosensors that pertain to the way in which the assay can be configured. For example, ligands can be coupled onto tips offline in large batches and do not need to be regenerated. If samples are sufficiently stable under experimental conditions, they can be reused within the assay or recovered for use in other assays, and this is particularly appealing when they are precious. Furthermore, clogging is not an issue on the Octet, and virtually no maintenance is required on the unit.

We report on how the Octet can be used to quantify the kinetics and affinities of protein interactions and validate these measurements by comparing them directly with those collected on SPR-based biosensors, namely the recently released parallel ProteOn XPR36 array and the widely used serial flow Biacore 3000 platform, which we consider our standard. We address three main themes. First, we establish that the direct binding of small molecules such as peptides to ligands on the tip is beyond the sensitivity of the Octet, limiting its use to the study of large analytes (generally > 50 kDa in molecular mass depending on the specifics of the interaction). Second, we demonstrate that the Octet can return accurate kinetic rate constants for large analytes through the use of a sink method that abolishes any rebinding of the analyte to ligand-coated tips, a strategy that has been commonly employed for years in other well-based systems such as the IAsys technology [2]. Third, we highlight how solution competition experiments on all three systems confirm the affinities measured by direct binding.

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¹ Abbreviations used: SPR, surface plasmon resonance; CGRP, calcitonin gene-related peptide; rCGRP α , rat CGRP- α ; hCGRP α , human CGRP- α ; Mes, 2-(N-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HBS-EP, Hepes-buffered saline EDTA polysorbate P20; EDC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide; NHS, N-hydroxysuccinimide; BSA, bovine serum albumin.

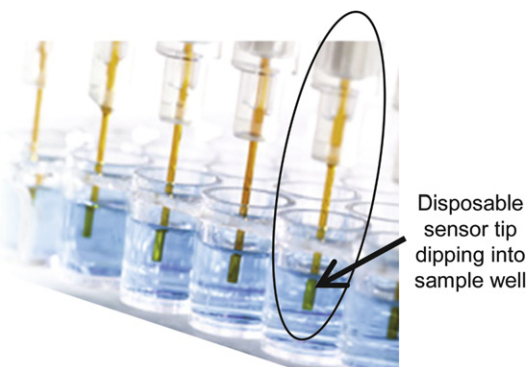


Fig. 1. The Octet's dip-and-read well-based format. An isolated view of an individual sensor is circled. Samples are handled without any microfluidics because a row of up to eight sensors moves to the samples held in an open shaking microplate. The actual plate used in the instrument is black. Reproduced with permission from ForteBio Inc., Menlo Park, CA, USA.

For the purpose of illustrating these applications, we adopt a model system, namely the interactions of a murine monoclonal antibody called "4901" with its antigen, the calcitonin gene-related peptide (CGRP). This interaction was chosen because the reagents are commercially available, they regenerate well, and their affinities fall within a measurable range. Furthermore, CGRP is implicated in migraine and other types of pain, and interfering with its biological activity is of therapeutic interest, rendering this model system relevant from a drug discovery perspective [3]. Raised against a C-terminal epitope of rat CGRP- α (rCGRP α), antibody 4901 also binds other isoforms [4], and we include full-length and truncated forms of human CGRP- α (hCGRP α) in this study. The four peptides studied here varied in molecular mass from 609 to 3806 Da and bound 4901 with a 1000-fold range in affinity. Our results demonstrate that the Octet and ProteOn can return kinetic rate constants and binding affinities as accurately as the well-established Biacore 3000. The main limitation of the Octet is its sensitivity, meaning that only certain assay orientations are possible. The results of our study can be used as a benchmark to test the performance of any or all of the above-mentioned instruments.

Materials and methods

Materials

Octet QK equipped with amine reactive (AR) and streptavidin (SBC or FA) biosensor tips and coupling buffer (100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.0) were purchased from ForteBio. Biacore 3000 equipped with CM5 and streptavidin sensor chips, HEPES-buffered saline ethylenediaminetetraacetic acid (EDTA) polysorbate P20 (HBS-EP) buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) polysorbate P20), and coupling reagents (10 mM sodium acetate (pH 5.0), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and 1 M ethanolamine-HCl (pH 8.5)) were purchased from Biacore (Uppsala, Sweden, now part of GE Healthcare, Piscataway, NJ, USA). ProteOn XPR36, GLM and neutravidin (NLC) sensor chips, and coupling reagents (sulfo-NHS and EDC) were purchased from Bio-Rad (Hercules, CA, USA). Monoclonal anti-CGRP antibody 4901 (cat. no. C7113) produced in mice and polyclonal anti-rat CGRP (cat. no. C8198) produced in rabbit were purchased from Sigma Chemical (St. Louis, MO, USA). rCGRP α (1–37 and 19–37) and hCGRP α (1–37, 1–19, 26–37, and 32–37) were custom-ordered from Global Peptide (Fort Collins, CO, USA) amidated at the C terminal. Full-length (1–37) peptides were also purchased modified at their N terminal with LC-biotin. The following immuno-

pure-grade reagents were purchased from Pierce (Rockford, IL, USA): streptavidin (cat. no. 21122), Gentle IgG Elution Buffer (cat. no. 1851520), and Fab Preparation Kit (cat. no. 44885). The Fab kit was used as directed to digest full-length 4901 antibody into Fab fragments using papain resin, and the product was confirmed to be more than 90% pure by standard SDS-PAGE under reducing conditions.

Standard experimental conditions

All interaction analyses were conducted at 25 °C in HBS-EP running buffer unless stated otherwise. Temperature control on the Octet was accomplished by holding the instrument in a 22 °C temperature-controlled room so that it could be heated to 25 °C. Sensor tips were prewet for 5 min in buffer immediately prior to use, and the microplates used in the Octet were filled with 200 μ l of sample or buffer per well and agitated at 1000 rpm.

Direct binding of CGRP to amine-coupled 4901 on tip

Amine reactive AR tips were prewet in 0.1 M MES buffer (pH 5.0), which served as the background buffer for the immobilization. This involved establishing a stable baseline (5 min), activating the sensors with a freshly mixed solution of 0.2 M EDC + 0.05 M NHS (5 min), coupling 100 μ g/ml 4901 (15 min), and then blocking excess reactive esters with ethanolamine (5 min). Final immobilization levels were 2.5 ± 0.1 nm (full-length IgG) and 2.0 ± 0.1 nm (Fab fragment) within a row of eight tips. A new baseline was established in HBS-EP + 1 mg/ml bovine serum albumin (BSA) (5 min) that provided the running buffer for all subsequent binding steps. rCGRP α was prepared as a serial dilution (0, 1.2, 3.7, 11, 33, and 100 nM) and allowed to bind the 4901-saturated tips for 15 min. This was followed by the binding of polyclonal anti-rCGRP α (15 min) at a single concentration that corresponded to a sevenfold dilution of the commercial stock. The full-length 4901 IgG and the 4901 Fab were compared in the same experiment by coupling each onto its own row of tips.

Optimizing sink conditions on the Octet

Streptavidin-coated FA tips were saturated with 20 μ g/ml N-biotinylated full-length CGRPs (5 min). Typical capture levels were 0.42 ± 0.06 nm within a row of eight tips, with the standard deviation being within instrument noise. Then 100 or 300 nM 4901 Fab was bound to rCGRP α or hCGRP α for 15 min and allowed to dissociate for 1 h into buffer spiked with a threefold concentration gradient of a competing peptide (0, 1, 3, 9, 27, 81, and 243 μ M rCGRP α 19–37) or an irrelevant peptide (100 μ M hCGRP α 1–19). Dissociation buffer was used only once to ensure its potency. Blank binding cycles containing no Fab were used to correct for baseline drift.

One-shot kinetics by Octet

FA tips were saturated with 20 μ g/ml N-biotinylated full-length CGRPs (5 min). The 4901 Fab was prepared as a five-membered threefold serial dilution with a highest concentration of 100 or 300 nM to study the interactions with rCGRP α or hCGRP α on the tip. A duplicate of the middle Fab concentration and two buffer blanks completed a row of eight samples, which were allowed to associate for 15 min. Dissociation was measured for 1 h into buffer spiked with 100 μ M rCGRP α 19–37 to ensure optimal sink conditions as described above. The analysis was sometimes repeated on the same set of tips by reusing them if the binding signal returned to baseline at the end of the dissociation phase (hCGRP α on tip) or regenerating them if it did not (rCGRP α on tip). When

used, regeneration was accomplished using a 2:1 (v/v) cocktail of Gentle IgG Elution Buffer/4 M NaCl (3 min). Replicate measurements were collected both within a single experiment by reusing all samples and tips and in independent experiments on fresh samples and tips.

Solution affinity by Octet

FA tips were saturated with N-biotinylated rCGRP α . Solution affinity was determined in two steps. First, a twofold concentration gradient of antibody 4901 typically starting at 5-nM (IgG) or 10-nM (Fab) binding sites provided a standard curve. Then a constant concentration of antibody binding sites equivalent to the highest one used in the standard curve was mixed with titrating concentrations of CGRP α at typical highest concentrations of 0.2 μ M rat, 4 μ M human, 4 μ M human 26–37, and 30 μ M human 32–37 to provide the inhibition curves. The standard curve and inhibition curves were generated by allowing the samples to bind the rCGRP α tips for 30 min. All four peptides were run in the same assay, typically as a fivefold series, allowing a fresh row of tips for each peptide tested. The standard curve was measured at the beginning and end of the assay to confirm that it was reproducible and valid over the time taken to run all rows of samples.

One-shot kinetics by ProteOn

To address CGRP kinetics, 4901 IgG was amine-coupled as a gradient onto a GLM chip, leaving one channel unmodified to provide an additional reference surface. This was achieved by varying the concentration of the activation reagents used in each channel. Five activation solutions were prepared using a threefold serial dilution of a stock mixture containing 0.2 M EDC + 0.05 M sulfo-NHS and injected for 30 s. Then 4901 IgG at 30 μ g/ml in sodium acetate (pH 4.5) was coupled for 1 min at 25 μ l/min. Excess reactive esters were blocked for 5 min with ethanolamine. This created uniform strips of 4901 IgG spanning final immobilized levels of 1700 to 11,000 RU with less than 2% variation within each strip. The chip was swiveled to the “analyte” direction to address “one-shot” kinetics of an array of CGRPs, each of which was prepared as a five-membered serial dilution (fivefold for the full-length CGRPs and fourfold for the truncated CGRPs) starting at 0.1 μ M rCGRP α , 0.3 μ M hCGRP α , 0.4 μ M hCGRP α 26–37, or 10 μ M hCGRP α 32–37. A single injection delivered a full concentration series of each peptide, using buffer to complete a row of six samples and provide an in-line blank for double-referencing the response data. Association and dissociation phases were typically measured for 200 s and 10 min, respectively, except rCGRP α , which was allowed to dissociate for 1 h. Immobilized 4901 was regenerated with 0.8 mM phosphoric acid for 30 s after each binding cycle, and hCGRP α 26–37 and 32–37 were analyzed in duplicate injections within the same experiment to confirm cycle-to-cycle reproducibility.

To address Fab kinetics, a gradient of CGRP was captured on an NLC chip, leaving one channel unmodified to provide a negative control surface. Thus, 0.003 to 3 μ g/ml N-biotinylated full-length CGRPs was injected for 1 min at 30 μ l/min in the “ligand” direction, resulting in capture levels spanning 27 to 550 RU rCGRP α and 4 to 63 RU hCGRP α on different chips, with less than 3% variation along each strip. The chip was then swiveled to the “analyte” direction to deliver a full concentration gradient of 4901 Fab in a single injection (0, 1.2, 3.7, 11, 33, and 100 nM Fab over rCGRP α and 0, 4.5, 14, 41, 122, and 366 nM 4901 Fab over hCGRP α). The in-line buffer blank was used for double-referencing the data. Association was measured for 3 min (rCGRP α) or 1 and 4 min (hCGRP α) at 100 μ l/min, and dissociation was allowed for up to 40 min. The hCGRP α -coated chip was regenerated with 0.8 mM phosphoric acid to

enable replicate injections to be performed within a single experiment to confirm reproducibility.

Solution affinity by ProteOn

A gradient of N-biotinylated rCGRP α was captured on an NLC chip to probe for free antibody. Standard curves were prepared using a twofold serial dilution of full-length 4901 IgG or 4901 Fab starting at 1- or 2-nM binding sites, respectively. Inhibition curves were prepared by titrating CGRP α as a fivefold series typically starting at 0.087 μ M rat, 0.63 μ M human, 1.9 μ M human 26–37, and 13 μ M human 32–37 into 1 nM 4901 IgG or 2 nM 4901 Fab (in binding sites). Samples were injected for 10 min at 30 μ l/min, and surfaces were regenerated with 0.8 mM phosphoric acid. The standard curve was always analyzed in duplicate to confirm that it was reproducible.

Single-cycle CGRP kinetics by Biacore

Full-length 4901 IgG was immobilized onto a CM5 chip at three different levels using standard amine coupling chemistry addressing flow cells individually. Briefly, this involved activating each flow cell with a freshly mixed solution of 0.2 M EDC in 0.05 M NHS for 7 min and coupling 30 μ g/ml 4901 IgG in 10 mM sodium acetate (pH 5.0) for various contact times until the desired level was reached (1600, 3200, and 7600 RU). Finally, excess reactive esters were blocked using a 7-min injection of ethanolamine. One flow cell was left unmodified to provide a reference surface. CGRPs were prepared as five-membered threefold concentration series starting at 100 nM rCGRP α , 100 nM hCGRP α , 1 μ M hCGRP α 26–37, and 20 μ M hCGRP α 32–37. Each CGRP was titrated from low to high concentration in a single-cycle mode for 3 min at 50 μ l/min, allowing a 20-min dissociation phase. Surfaces were regenerated with a cocktail of 1:1:1 (v/v/v) Gentle IgG Elution Buffer/4 M NaCl/100 mM phosphoric acid.

Multicycle Fab kinetics by Biacore

Streptavidin was amine-coupled onto CM5 sensor chips to saturating levels ($\sim$ 3000 RU) using a standard protocol. Briefly, this involved activating all flow cells with a freshly mixed solution of 0.2 M EDC in 0.05 M NHS (7 min), coupling 30 μ g/ml streptavidin in 10 mM sodium acetate (pH 5.0) (7 min), and blocking excess reactive esters with ethanolamine (7 min). N-biotinylated CGRPs were diluted to approximately 0.01 μ g/ml and captured at different levels on individual flow cells using 30-s pulses at 10 μ l/min until the desired level was captured ($<$ 10 RU). One streptavidin-coated flow cell per chip was left unmodified to provide a reference channel. The 4901 Fab was prepared as a threefold serial dilution using a highest concentration of 366 nM, and samples were injected for 1 min at 100 μ l/min in a classical multicycle mode. Dissociation was followed for 1 h. Buffer injections provided blanks for double-referencing the data. Regeneration was accomplished with 0.8 mM phosphoric acid. At least one concentration of the Fab series was injected in duplicate to confirm the assay's reproducibility.

Solution affinity by Biacore

A high level (800 RU) of N-biotinylated rCGRP α was captured on a streptavidin chip to probe for free antibody. Standard curves were prepared using a twofold serial dilution of full-length 4901 IgG or 4901 Fab starting at 0.4- or 0.5-nM binding sites, respectively. Inhibition curves were prepared by premixing 0.4 nM 4901 IgG or 0.5 nM 4901 Fab with threefold serially diluted CGRPs using highest concentrations of 657 nM rCGRP α , 792 nM hCGRP α , 7.8 μ M hCGRP α 26–37, and 54.5 μ M hCGRP α 32–37. Samples were

injected for 5 min at 20 $\mu\text{l}/\text{min}$, and surfaces were regenerated with the Pierce Elution buffer-salt-acid cocktail as above.

Data processing and analysis

Shift data from the Octet were exported as text files for processing and analysis in BiaEvaluation (version 4.1, Biacore) or Scrubber (version 2.0, BioLogic Software, Campbell, Australia). These programs were also used to analyze the Biacore data. ProteOn data were analyzed in ProteOn Manager (version 2.0). To deduce a direct binding affinity via the kinetic rate constants ($K_D = k_{\text{off}}/k_{\text{on}}$, where K_D = equilibrium dissociation constant, k_{on} = association rate constant, and k_{off} = dissociation rate constant), the buffer-subtracted Octet data or double-referenced SPR data (using either interspot or blank channel referencing on the ProteOn) were fit globally to a simple 1:1 Langmuir model. A special model was used to analyze single-cycle kinetics on Biacore [5]. SPR data that were collected across multiple-capacity surfaces simultaneously were constrained to the fit if the binding capacities fell within an acceptable range based on whether including them did not significantly deviate the kinetic values from those obtained on the optimal capacity surface.

The affinity for the direct binding of hCGRP α 32–37 over immobilized antibody by SPR was also determined via a steady-state approach. Thus, the equilibrium response values obtained during the association phase from duplicate CGRP injections tested simultaneously over multiple capacity surfaces (four on ProteOn and three on Biacore) were normalized by the maximum binding response measured on each surface and expressed as “percentage bound” values, and these mean values were plotted as a function of injected CGRP. This curve was fit to a single-site isotherm in Kaleidagraph (version 3.5).

To deduce an affinity from solution competition experiments, the relationship between the antibody binding site concentration and the blank-subtracted (Octet) or double-referenced (SPR data) binding responses from a standard curve was used to infer the “free 4901 concentration” in mixtures containing the competing peptides. These converted values were then fit to a solution affinity model using the BiaEvaluation software, allowing the “initial concentration of antibody binding sites” to be a global parameter for all mixtures derived from a common antibody sample. All concentrations were in terms of binding sites.

Results

Exploring the sensitivity of the Octet

The first assay orientation that we explored was binding rCGRP α to antibody 4901 amine-coupled onto the tips, but the direct binding of a 4-kDa molecule was below the detection limit of the Octet. To confirm that the peptide was indeed bound despite being at an undetectably low level that was within instrument

noise, we formed a sandwich complex with a polyclonal anti-rCGRP α antibody in a subsequent binding step (Fig. 2). The dose-dependent binding responses observed when we probed with a fixed concentration of the polyclonal reflected the concentration gradient of rCGRP α that was bound to 4901 on the tip. The full-length 4901 IgG and Fab fragment behaved similarly in this regard.

Small molecule detection by SPR biosensors

In contrast, the direct binding of CGRPs to amine-coupled 4901 IgG was easily detectable on ProteOn (Fig. 3) and Biacore (Fig. 4) platforms. The kinetic rate constants determined for an array of peptides, ranging in molecular mass from 609 to 3806 Da, were so similar on these two platforms that they were considered to be identical (Table 1). The rCGRP α had a 10-fold tighter affinity than the hCGRP α (K_D values of 0.6 and 5 nM, respectively), consistent with the known binding specificity of antibody 4901. Truncating the full-length hCGRP α to 26–37 and 32–37 weakened the wild-type affinity 2-fold and a further 20-fold (K_D values of 10 and ~ 200 nM, respectively), with the off-rate being mainly responsible for the loss in affinity. This pattern of activity is consistent with the known epitope of 4901 that reportedly resides within the 10 most C-terminal residues [4]. The analysis of hCGRP α by Biacore (Fig. 4B) was hindered by rebinding, as seen by the poor fit of the measured data to the simulated off-rate. This artifact was less noticeable on the ProteOn (Fig. 3B) for data collected on similar capacity surfaces as the Biacore; however, the noise was larger on the ProteOn (± 4 RU) than on the Biacore (± 1 RU), and this may have masked any potential deviation.

Use of a sink to achieve reliable kinetic data

Another assay orientation that we explored was the direct binding of the 50-kDa 4901 Fab fragment to CGRP-saturated tips. Binding responses of this large analyte were easily discernible above instrument noise (± 0.1 nm), with maximum signals reaching 3.0 nm. An appealing feature of the Octet's parallel format means that a full concentration gradient of the analyte can be monitored in a single binding cycle. This one-shot kinetic mode has been described previously in the context of another parallel biosensor, namely Bio-Rad's ProteOn XPR36 array system [6]. Similarly, but without any microfluidics, the Octet can address up to eight samples simultaneously, and they can be re-used to provide replicate measurements within the same experiment or retrieved for use in other experiments. However, in the absence of flow, the analyte may tend to rebinding the ligand-coated tips and, thereby, introduce an artifact into the observed dissociation phase that hinders a kinetic analysis. The black lines in Fig. 5 show that the dissociation phase data collected in this one-shot mode over rCGRP α (Fig. 5A) appeared to be mildly biphasic, whereas hCGRP α (Fig. 5B) appeared to be markedly biphasic. However, any rebinding observed on the

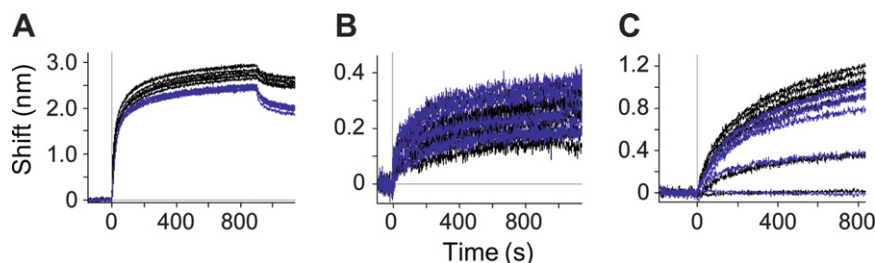


Fig. 2. Sandwich assay to detect rCGRP α binding to 4901-saturated Octet tips: Amine coupling of 4901 (A), binding of rCGRP α (0, 1.3, 3.7, 11, 33, and 100 nM) (B), and sandwich detection using a polyclonal rCGRP α at a fixed concentration (C). Black and blue curves distinguish the intact antibody from the Fab fragment.

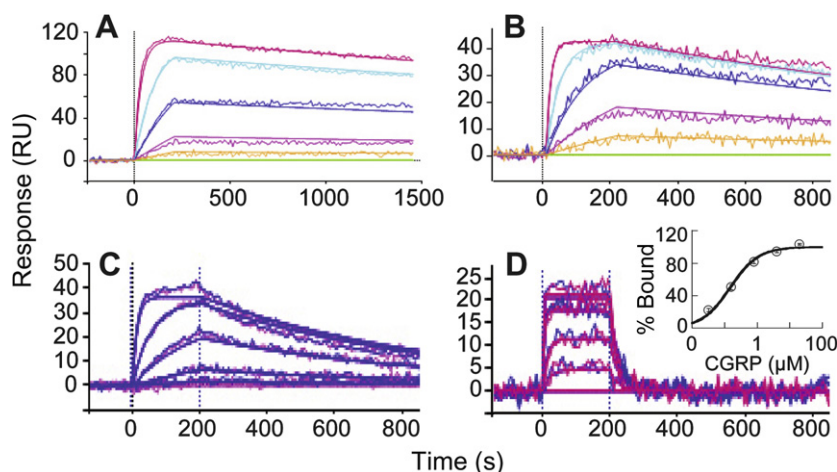


Fig. 3. One-shot kinetic analysis of rCGRP α (A), hCGRP α (B), hCGRP α 26–37 (C), and hCGRP α 32–37 (D) binding amine-coupled 4901 IgG on ProteOn. Each peptide was injected as a five-membered threefold series starting at 0.1, 0.3, 0.4, and 10 μ M, respectively, and analyzed in duplicate binding cycles (except full-length CGRPs, each of which was analyzed in a single injection) over five differing capacity reaction surfaces, one of which is shown per peptide. Noisy and smooth lines distinguish measured data from the global fits (Table 1). The inset shows a steady-state analysis where the error bars are the standard deviations of the mean responses that were normalized by the level of antibody on the chip and expressed as “percentage bound” values.

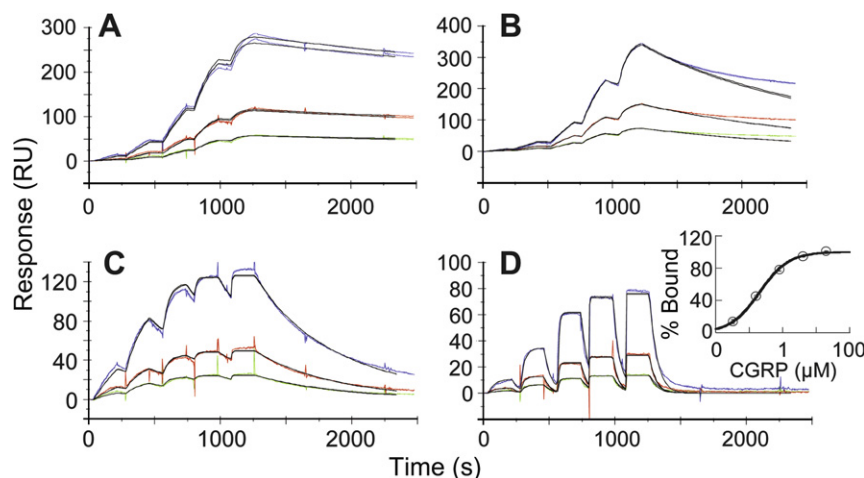


Fig. 4. Single-cycle kinetic analysis of rCGRP α (A), hCGRP α (B), hCGRP α 26–37 (C), and hCGRP α 32–37 (D) binding amine-coupled 4901 IgG on Biacore. Each peptide was injected as a five-membered threefold series with highest concentrations of 0.1, 0.1, 1, and 20 μ M, respectively, and analyzed in duplicate binding cycles over three differing capacity reaction surfaces. Green, red, and blue represent the measured data over low-, medium-, and high-capacity surfaces, respectively, and black lines show the overlaid global fits (Table 1). The inset represents a steady-state analysis where the error bars are the standard deviations of the mean responses that were normalized by the level of antibody on the chip and expressed as “percentage bound” values.

Table 1

Direct binding kinetics for an array of CGRPs binding 4901 IgG immobilized on Biacore and ProteOn biosensors

Figure	Biosensor	CGRP α	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
3A	ProteOn	Rat	$2.093 (0.001) \times 10^5$	$1.316 (0.004) \times 10^{-4}$	$0.565 (0.002)^a$
3B	ProteOn	Human	$1.156 (0.001) \times 10^5$	$6.71 (0.02) \times 10^{-4}$	$5.78 (0.02)^a$
3C	ProteOn	Human 26–37	$1.755 (0.003) \times 10^5$	$1.430 (0.002) \times 10^{-3}$	$8.13 (0.02)$
3D	ProteOn	Human 32–37	$1.926 (0.008) \times 10^5$	$3.00 (0.01) \times 10^{-2}$	$155.1 (0.9), 150 (20)^b$
4A	Biacore	Rat	$2.017 (0.002) \times 10^5$	$1.146 (0.003) \times 10^{-4}$	$0.569 (0.002)$
4B	Biacore	Human	$1.762 (0.003) \times 10^5$	$7.04 (0.03) \times 10^{-4}$	$4.00 (0.02)$
4C	Biacore	Human 26–37	$1.761 (0.004) \times 10^5$	$1.685 (0.003) \times 10^{-3}$	$9.55 (0.02)$
4D	Biacore	Human 32–37	$9.32 (0.03) \times 10^4$	$1.882 (0.005) \times 10^{-2}$	$201.9 (0.8), 200 (10)^b$

Note. Each value represents the global affinity (and standard error of the fit) for duplicate binding cycles, unless stated otherwise, collected simultaneously over four (ProteOn) or three (Biacore) differing capacity reaction surfaces.

^a Single injection.

^b Steady-state analysis.

Octet was abolished by taking advantage of the platform’s well-based format, which allowed dissociation into any buffer of choice. The red curves in Fig. 5 demonstrate that when we spiked the dissociation buffer with a competing antigen, namely

the tight-binding rCGRP α , the dissociation phase data cleaned up significantly well to justify a kinetic analysis.

Under optimal sink conditions, which were empirically determined for each interaction (see Materials and Methods), the mean

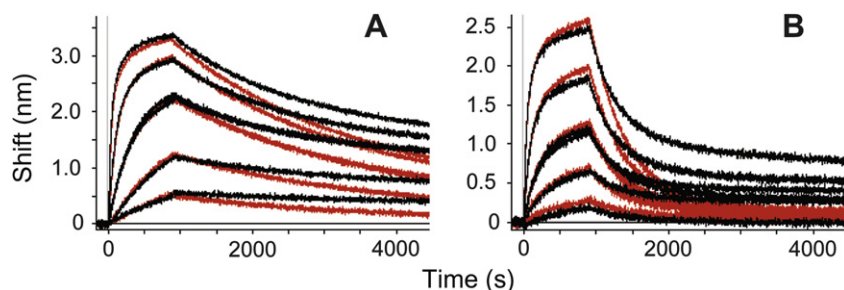


Fig. 5. Primary Octet data for one-shot 4901 Fab kinetics over rCGRP α (A) and hCGRP α (B) on tips in the presence of no sink (black) or optimal sink (red). The highest concentrations of 4901 Fab used in the five-membered threefold dilution series were 100 and 300 nM, respectively.

affinities of 4901 Fab binding CGRP-saturated tips were determined to be $K_D = 2.0 \pm 0.4$ nM (rCGRP α) and $K_D = 36 \pm 1$ nM (hCGRP α), where the standard deviation is for two and three independent Octet experiments, respectively, one of which is shown for each in Fig. 6A and B. The Octet data were highly reproducible both with respect to tip-to-tip performance, as judged by duplicating the middle concentration of the Fab gradient binding rCGRP α -saturated tips (Fig. 6A), and from cycle to cycle, as demonstrated by the overlapping binding responses obtained by repeatedly analyzing the same samples four times on a single row of hCGRP α -saturated tips (Fig. 6B).

Furthermore, the Octet data collected under optimal sink conditions returned kinetic rate constants that agreed within twofold of those generated in similar experiments on SPR biosensors. Fig. 6 directly compares the 4901 Fab kinetic data obtained on three platforms, and Table 2 summarizes the global kinetic analyses of these data. The ProteOn and Biacore values were essentially identical to one another and provided independent measurements on parallel and serial flow biosensors, respectively, as well as on different types of chips (neutravidin and streptavidin, respectively). In agreement with the Octet data, the analysis of SPR data for 4901 Fab binding hCGRP α surfaces was hindered by rebinding, meaning

that only the initial portion of the dissociation phase conformed to a simple model. However, there was sufficient decay ($> 5\%$) in the truncated dissociation phase data to justify a global fit. The fact that we observed rebinding on Biacore and ProteOn systems confirmed that this issue was not unique to the Octet. An advantage of the Octet was that, of the three biosensors used, it was uniquely configured to abolish this artifact through the use of a sink method. Because the Octet was able to reveal a full and clean dissociation profile for the hCGRP α interaction, the off-rate determined on this platform was perhaps more accurate than those determined on SPR platforms. The fact that the on-rates agreed closely with those determined on the SPR sensors (Table 2) suggested that any rebinding that may have occurred during the association phase was no more prominent on the Octet than on the SPR sensors.

Solution affinity determination

An advantage of determining a solution affinity is that it offers a method that is unbiased by assay orientation or the immobilization process; the ligand-coated tip merely probes the free concentration of analyte that is not complexed to the solution competitor of interest. Furthermore, the reactants can be studied in their na-

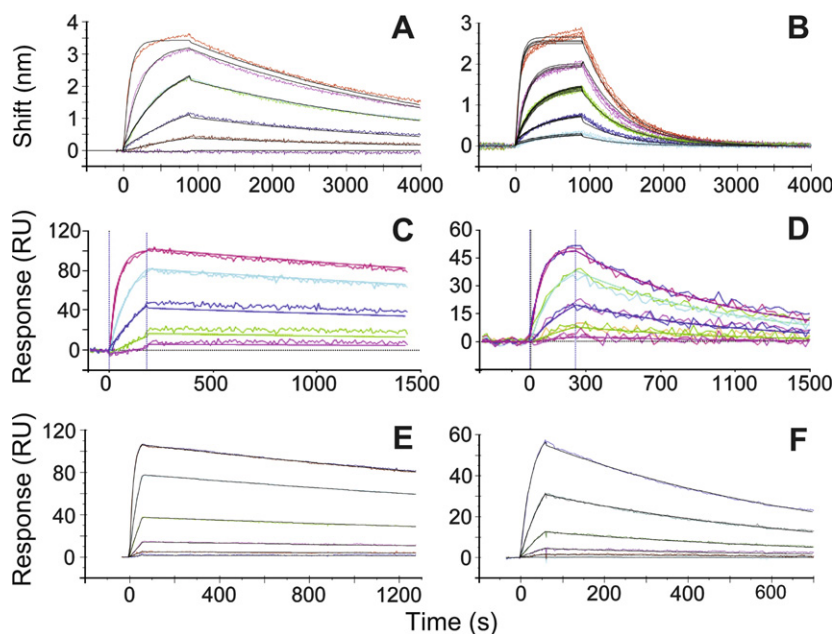


Fig. 6. Direct comparison of binding responses for 4901 Fab interacting with rCGRP α (A,C,E) and hCGRP α (B,D,F) measured on Octet (A,B), ProteOn (C,D), and Biacore 3000 (E,F) biosensors. A representative data set is shown for each interaction. The 4901 Fab was injected as a threefold serial dilution starting at 100 nM (A,C), 122 nM (E), 300 nM (B), and 366 nM (D,F). The level of CGRP coated on the sensor varied according to the platform, as did the timing of the binding steps. All dissociation steps were performed in buffer except the Octet data (A,B), which were collected under optimal sink conditions (see Materials and Methods). Colored and black lines in panels A, B, E, and F distinguish the measured data from the overlaid fits, whereas noisy and smooth lines in panels C and D distinguish the measured data from the simulated fits (Table 2).

Table 2

Global kinetic analysis of 4901 Fab binding CGRPs on different sensors

Fig. 6	Biosensor	CGRP α	k_{on} ($\text{M}^{-1} \text{s}^{-1}$)	k_{off} (s^{-1})	K_D (nM)
A	Octet	Rat	$1.5 (0.3) \times 10^5$	$2.85 (0.09) \times 10^{-4}$	$2.0 (0.4)^a$
B	Octet	Human	$5.1 (0.4) \times 10^4$	$1.8 (0.2) \times 10^{-3}$	$36 (1)^a$
C	ProteOn	Rat	$2.729 (0.002) \times 10^5$	$1.667 (0.004) \times 10^{-4}$	$0.612 (0.002)^b$
D	ProteOn	Human	$5.571 (0.006) \times 10^4$	$8.998 (0.009) \times 10^{-4}$	$16.2 (0.02)^b$
E	Biacore	Rat	$5.31 (0.03) \times 10^5$	$2.29 (0.02) \times 10^{-4}$	$0.432 (0.006)^c$
F	Biacore	Human	$8.9 (0.3) \times 10^4$	$1.54 (0.03) \times 10^{-3}$	$17.3 (0.4)^a$

Note. Each error in parentheses was obtained in different ways, as specified in the footnotes, depending on the platform and the data analysis software used. Octet data were obtained under optimal sink conditions.

^a Mean (and standard deviation) for two or three independent experiments.

^b Global fit (and standard error for the fit) for one (rat) or two (human) injections over five differing capacity surfaces.

^c Mean (and standard deviation) for independent fits from two differing capacity surfaces analyzed in a single experiment.

tive form in solution. This is similar to a KinExA setup [7] but offers a label-free real-time version and is simpler to run. However, the scope of this assay is restricted in two respects. First, the assay must be oriented so as to compete out binding of a large analyte to generate a clear binding signal. Second, only fairly weak interactions can be studied because the probe must be able to detect the analyte at a low enough concentration to be at or below the K_D being measured in solution. Tight binders will just titrate out the competing molecule in a stoichiometric manner, thereby instead giving an active concentration measurement where either the analyte or competing molecule can be used as a standard to determine the concentration of the other binding partner.

An appealing feature of our model system was that the tight affinity of rCGRP α for antibody 4901 could be used to probe the affinities of the weaker isoform hCGRP α and its fragments 26–37 and 32–37 that do not contain the full epitope. Fig. 7 shows the primary data collected for the inhibition of the intact antibody using the smallest fragment tested, namely hCGRP α 32–37 (in red), superimposed with a standard curve (in black). Each data set was collected on a fresh row of Octet tips. A typical inhibition analysis that included a set of four peptides is shown in the overlay plot in Fig. 8A for the full-length antibody and in Fig. 8B for the Fab fragment. These results were corroborated by similar analyses on ProteOn (Figs. 8C and D) and Biacore (Figs. 8E and F) platforms. In most cases, the affinities determined by the Octet agreed closely with those determined on SPR sensors, with the Biacore and ProteOn platforms giving very similar results as one another. Two discrepancies are noteworthy. First, the higher sen-

sitivity of the Biacore meant that solution competition could be performed in a background of a lower antibody concentration than was used on the other sensors, enabling the tight affinity of the rCGRP α /4901 interaction to be resolved more precisely (K_D of ~ 0.5 nM), agreeing well with our direct binding measurements. Second, all peptides tested bound the intact antibody and the Fab fragment similarly except the full-length hCGRP α , which discriminated between them. The SPR sensors resolved a threefold tighter affinity for the full-length 4901 IgG than the Fab fragment in binding hCGRP α , consistent with our direct binding measurements. The Octet was unable to discern this difference, likely due to our working at a relatively high antibody binding site concentration to overcome its worse sensitivity than the SPR platforms.

Discussion

Tables 1, 2, and 3 summarize the affinities determined for a panel of four CGRPs binding antibody 4901 in various assay orientations on three different biosensor platforms. The main conclusion from this study is that the orientation in which an interaction is studied can affect the results as much as, or more than, the biosensor used. For example, the affinity determined for the 4901/rCGRP α interaction was very similar in all three assay orientations tested by SPR (K_D values of ~ 0.6 nM), confirming that the immobilization methods used in exploring different assay strategies was not altering the activity of the reagents. In contrast, when hCGRP α was flowed over amine-coupled 4901 IgG, the affinity appeared to be threefold tighter than when the 4901 Fab fragment was flowed over N-biotinylated CGRP on the chip by SPR (K_D values of 5 and 17 nM, respectively). These affinities were corroborated by solution competition measurements that revealed that hCGRP α discriminates between the full-length 4901 IgG and the Fab fragment, and this was responsible for the apparent discrepancy in binding affinities determined in the direct binding methods. In a separate experiment, we confirmed that the N-biotinylated and native forms of hCGRP α bound identically to one another when flowed over 4901 IgG amine-coupled to the chip (data not shown), confirming that the activity of the native peptide was unaltered on biotinylation.

Regardless of the assay orientation used, the Octet yielded affinities that were typically within twofold of those determined by Biacore in the same assay orientation, and this held true across a nearly 1000-fold range of affinities within the set of four peptides tested here. The main limitation of the Octet is its poorer sensitivity compared with SPR platforms, meaning that the direct binding of small molecules is currently beyond the detection limit of the Octet, which in turn dictates how the assay must be oriented. Some assay orientations were not explored, including the binding of Fab to truncated peptides on the sensor, because we worried that tethering small molecules

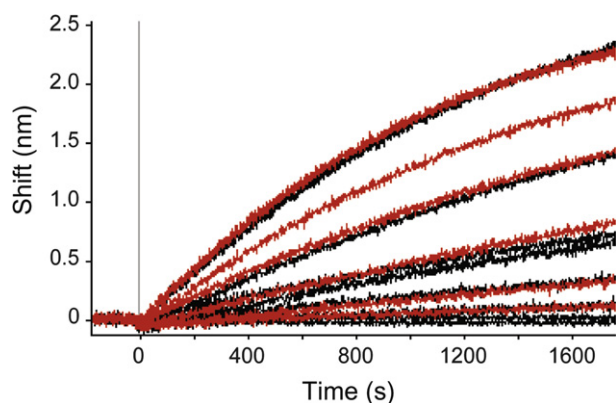


Fig. 7. Typical solution competition analysis on the Octet. The overlay plot depicts the primary data from a standard curve (black) and an inhibition curve (red) on separate rows of rCGRP α -saturated tips. The standard curve was constructed using 0 (in duplicate), 0.23-, 0.46-, 0.91 (in duplicate), 1.82-, and 3.65-nM binding sites of the intact 4901 IgG. The inhibition curve was constructed by titrating hCGRP α 32–37 (0 in duplicate, 135, 405, 1216, 3649, 10,950, and 32,840 nM) into 3.65-nM antibody binding sites. Samples were allowed to bind the tips for 30 min.

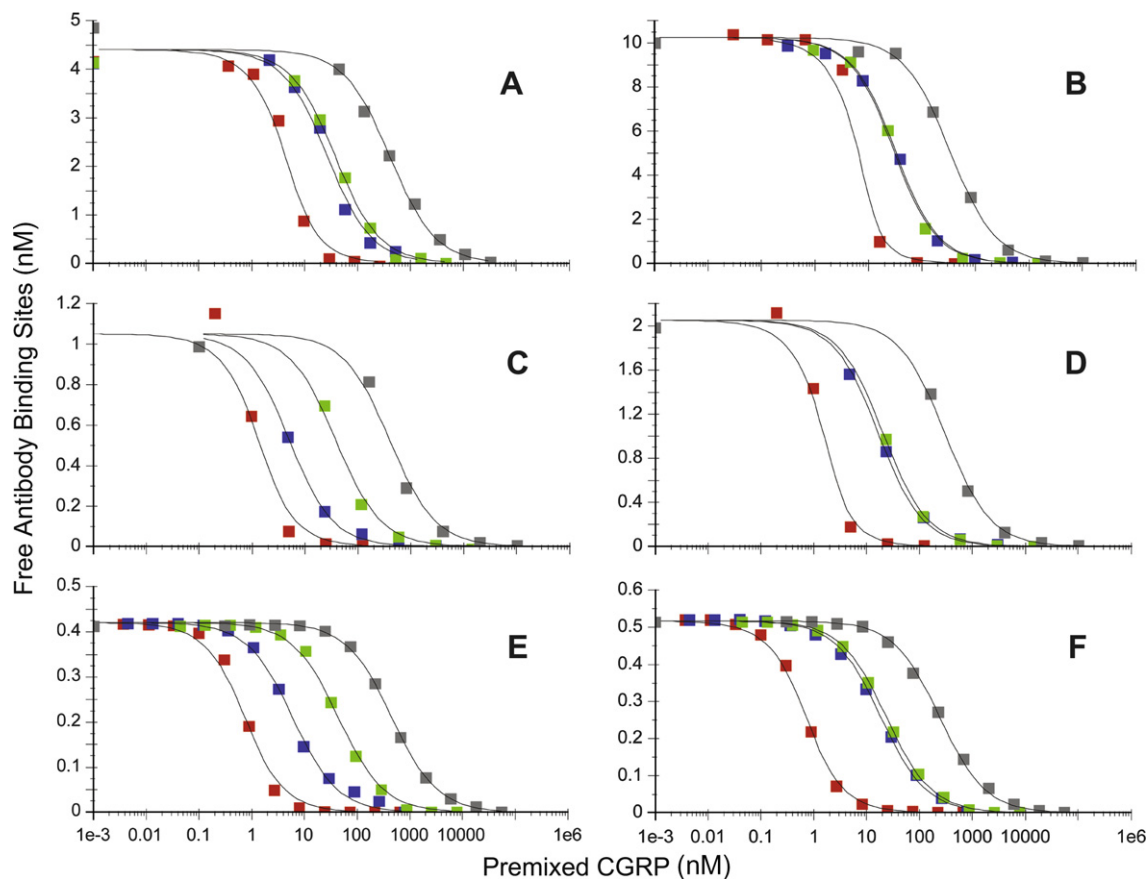


Fig. 8. Performance comparison of three biosensors—Octet (A,B), ProteOn (C,D), and Biacore (E,F)—in determining the solution affinities of antibody 4901 for an array of peptides. Panels A, C, and E show reactions with the full-length IgG, and panels B, D, and F show reactions with the Fab fragment. Colors distinguish the CGRP α peptides as follows: red (rat), blue (human), green (26–37 human), and gray (32–37 human). The black lines are the simulated K_D fits in which all four curves within a panel are constrained to a global initial antibody binding site concentration.

Table 3
Solution affinities of antibody 4901/CGRP interactions determined on three biosensor platforms

CGRP α	Octet		ProteOn		Biacore	
	IgG	Fab	IgG	Fab	IgG	Fab
Rat	3 (1)	1.5 (0.3)	0.9 (0.1)	0.3 (0.3)	0.54 (0.04)	0.45 (0.02)
Human	28 (5)	22 (7)	7 (4)	20.3 (0.3)	5.8 (0.4)	18.2 (0.7)
Human 26–37	38 (4)	27 (3)	34 (5)	23.6 (0.8)	44 (3)	23.1 (0.8)
Human 32–37	500 (100)	280 (70)	370 (40)	224 (2)	460 (30)	239 (9)
Initial antibody concentration (nM) ^a	4.41 (0.07)	7.8 (0.2)	1.05 (0.03)	1.94 (0.05)		
	5.24 (0.05)	10.2 (0.1)	1.19 (0.04)	2.03 (0.05)	0.421 (0.002)	0.517 (0.001)

Note. Values shown are K_D (in nM). Each value is the mean (and standard deviation) from two independent experiments, except for the Biacore data where the value is from a single experiment, so the standard error is for the fit.

^a Globally determined number of initial antibody binding sites (and standard error for the fit) for each experiment that incorporated all four peptides.

may compromise their binding activity. However, the measurement of affinities of small molecules weaker than approximately 1 nM was possible on all platforms via solution competition, which also provided a measurement that was unbiased by assay orientation and enabled the study of native reagents.

The use of a sink method enabled us to generate high-quality Octet kinetic data that were essentially free of any rebinding artifact. Rebinding can be caused by mass transport effects on the SPR machines and either mass transport or the lack of flow on the Octet. We confirmed that a specific competitor was needed to elicit a sink effect because neither an irrelevant peptide (100 μ M hCGRP α 1–19) nor a carrier molecule (10 mg/ml BSA) caused any sink effect when spiked into the dissociation buffer. An unusually high concentration of competing antigen was needed to achieve optimal

sink conditions because the dissociated 4901 Fab may be distributed unevenly within the well in the absence of flow such that the free, solution-based rCGRP α close to the tip is perhaps depleted more quickly than expected. For some systems, any potential competitor may be too precious to use at such high concentrations, although it can be reused as a sink even if it is no longer pure enough to be used in other applications. The SBC and FA sensors behaved alike, confirming that rebinding was not unique to a particular sensor. We also investigated whether immersing the sensors deeper into the wells or moving them farther from the center of the well had any effect, but none of these new positions improved data quality relative to the default setting. The fact that we diagnosed rebinding in the analysis of 4901/hCGRP α interaction by SPR confirmed that the issue was not unique to the Octet.

The different ways in which the three biosensors addressed samples introduced diversity in the ways in which data could be replicated. The one-shot kinetic mode that is available for the parallel biosensors (Octet and ProteOn) is appealing because a full titration series of analyte can be monitored in a single binding cycle. The Octet further enabled samples to be reused in the same analysis or to be recovered for use in other assays, and this is particularly useful when working with precious samples. One-shot kinetics on ProteOn and single-cycle kinetics on Biacore enabled a global and simultaneous analysis of data collected over multiple surface capacities that increased the number of curves that were constrained in the fit and made for a robust analysis. The standard error for such an experiment is for the fit, which reflects the consistency in kinetics obtained across different surface capacities and from cycle to cycle when analyte injections were repeated within a single experiment. We recognize that the confidence in experimentally determined parameter values demands the use of replicate independent measurements, which we performed for some interactions. We have distinguished between these types of errors in the tables.

An advantage of the Octet's dip-and-read format from an open plate means that there is virtually no limit to the length of association and dissociation phases that can be measured other than evaporation-related concerns. To reduce the rate of evaporation on the Octet and to standardize data collection across the study, we performed all binding analyses at 25 °C. Although the Biacore and ProteOn are capable of monitoring interactions over a wide range of temperatures (4–40 °C), the Octet can only heat above ambient temperature. We therefore placed the Octet in a 22 °C temperature-controlled room and set the assay temperature to 25 °C. The only significant difference in the way data were collected is in the timing of the binding steps, and this should be taken into account when visually comparing the sensorgrams obtained on the different platforms. We took advantage of the longer association times possible on the Octet relative to the SPR systems, which are subject to the limits imposed on volume by

microfluidics. There are, however, a few literature examples of how association phase data have been collected for extended periods of time on SPR biosensors through the use of recirculating analyte through the flow cell of Biacore's FlexChip [8], redirecting analyte through the buffer syringe, or installing a peristaltic pump on a standard Biacore 3000 instrument [9].

The Octet lacks the sensitivity required to investigate some assay orientations. However, its ability to measure interactions on disposable tips that do not need to be regenerated, to address samples without any microfluidics, and to not necessarily consume precious samples makes it a versatile complement to Biacore. In addition, the ProteOn's ability to generate high-quality data with sensitivity similar to that of a Biacore 3000, but in a higher throughput manner, opens up exciting opportunities for this array system in influencing drug discovery.

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