Quantitative assessment of biomolecular interaction parameters is often carried out in the core research facilities of large multi-disciplinary research centers in academia and industry. However, factors such as the use of different equipment models, standards of maintenance, and variability in the quality of starting materials mean that data from different sources may be difficult to standardize. In addition, the inherent sophistication of the technologies involved, together with the care required in experimental planning and data analysis, all contribute to a complex picture from which it is difficult to derive data that can be relied upon as gold standards for specific interactions. In order to estimate the quality and consistency of data generated from a disparate group of users of three of the most commonly used biomolecular interaction technologies, namely analytical ultracentrifugation (AUC), isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR) (Figure 1), the Association of Biomolecular Resource Facilities (ABRF) invited them to analyze a selected interaction using one of these three methods. Myszka et al. reported the comparative results of this field trial in full in the Journal of Biomolecular Techniques (2003). Results from the Biacore users who participated in the trial are now summarized in this Application Note.
The experience of the Biacore users in the study varied widely and they carried out their analysis on equipment ranging from the semi-manual system, Biacore® X, to Biacore® S51, specifically designed for small molecule analysis in drug discovery.

The interaction between carbonic anhydrase II (CA II) and a specific inhibitor, 4-carboxybenzenesulfonamide (CBS) was selected as a model system principally because crystallographic studies indicate 1:1 stoichiometry, implying relatively simple kinetics that would likely generate the high-quality data demanded by global analysis of the results. Reagents and experimental guidelines were dispatched to all participants in the trial (AUC, ITC, and SPR users). Each participant received lyophilized CA II and a stock solution of CBS in PBS buffer. A detailed protocol was supplied to those who volunteered to use SPR. It covered instrument preparation, immobilization strategies, and running conditions. SPR participants were asked to measure the kinetics and affinity of complex formation. Selected users were also requested to carry out a thermodynamic analysis of the interaction. 28 Biacore-using groups participated in the study, some of them repeating the analysis. In total, 59 Biacore data sets were returned to ABRF.

Summary of instructions to SPR users

Instrumentation and reagents
The experimental protocol was designed for use with Biacore® 3000 and Biacore® 2000 systems, although other systems including Biacore X, Biacore® 1000 and Biacore S51 were used with appropriate instrument-specific modifications to the protocol. Sensor Chip CM5, Biacore’s versatile and high-binding capacity sensor chip with a carboxymethylated dextran matrix, was used. Recommended reagents for the amine coupling procedure were from Biacore AB, Uppsala, Sweden.

Instrument preparation
Each newly docked Sensor Chip CM5 was prepared as follows:
- an automated Desorb routine using 0.5% v/v SDS followed by 50 mM glycine, pH 9.5
- a precondition routine using two 12-second injections of 50 mM NaOH, 0.1% v/v HCl, 0.1% v/v SDS and 0.085% v/v H₃PO₄
- normalization of the detector with the appropriate concentration of glycerol after three primes with freshly degassed PBS running buffer
- a blank run using buffer-only samples at a flow rate of 100 µl/minute

Ligand immobilization
The ligand, CA II (molecular weight of 30,000 Da), was immobilized on the surface of the sensor chip using an EDC/NHS-mediated amine coupling procedure. A freshly prepared solution of 50 mM NHS and 0.2 M EDC was injected for 7 minutes to activate the flow cell. CA II was reconstituted in 125 µg/ml in 10 mM Na acetate, pH 5.0 and injected for 10 minutes at a flow rate of 10 µl/minute. Excess activated ester groups on the surface were deactivated using a 7-minute injection of 1 M ethanolamine-HCl, pH 8.5. Unmodified flow cells were reserved as control and reference surfaces.
Interaction analysis
The analysis temperature was set to 25°C. CBS was freshly diluted in PBS running buffer in three-fold serial increments spanning 82 nM to 20 µM and injected over the immobilized CA II at 100 µl/minute. CBS and CAII were allowed to interact for one minute and their dissociation was monitored for three minutes. The samples were randomized, along with samples containing buffer only. Ten additional start-up buffer (blank) cycles were run and excluded from the analysis.

Data processing
Each volunteer was instructed on how to process their raw data. This included setting the baseline to zero, cropping the data so that the staggered start times could be analyzed globally, referencing responses across flow cells by subtracting data generated across an unmodified flow cell and double referencing within flow cells by subtracting an average buffer (blank) response from data collected within each flow cell.

Data analysis
The binding responses were analyzed globally by fitting data to a simple 1:1 interaction model using BIAevaluation or CLAMPTM software (Myszka and Morton, 1998). The association rate constant (k_a) and dissociation rate constant (k_d) were calculated for each data set and the equilibrium dissociation constant (K_D) was derived from their ratio, k_d/k_a.

van’t Hoff analysis
A van’t Hoff thermodynamic analysis of the interaction was carried out by repeating the CBS binding experiment described above at 5, 15, 25 and 35°C. The K_D values provided by the ratios of the kinetic rate constants determined at each temperature were used to obtain a van’t Hoff enthalpy change for the binding interaction. A plot of 1/T vs K_D yielded a slope from which the ΔH_van’t Hoff was extracted, according to the linear form of the integrated van’t Hoff equation. i.e., ln K_D = ΔH_van’t Hoff /RT - ΔS/R, where R = 1.987 cal/(mol K) and T is in units of Kelvin.

Results
Quality control test
To estimate the basic condition of the equipment used by each trial participant, a series of running buffer injections over unmodified flow cells was monitored for steadiness of the baseline response. The volunteers were asked to inject running buffer 30 times and to overlay the responses. Most participants reported flat baselines although baseline drift was occasionally a feature of early buffer injection. However, this phenomenon diminished with repeated runs, emphasizing the importance of fully conditioning the sensor chip surface before running samples for analysis.

Ligand immobilization
The maximum response due to CBS binding (R_max) was expected to be 150 times lower that the immobilization level of CA II, reflecting the differing molecular
weights of the interactants; approximately 200 Da and 30000 Da, respectively. To compensate for the relatively small response predicted for CBS binding to a much larger immobilized target, the protocol was designed to yield a dense CAII surface with a high CBS-binding capacity. The average immobilization level from the 59 responses was 6600 ± 2300 RU.

**Binding profiles**

Samples containing CBS at concentrations spanning 82 nM to 20 µM in running buffer were injected over immobilized CA II and ligand-free surfaces for one minute. No regeneration was necessary between injections because the dissociation was complete after a two minute-dissociation phase. Unmodified flow cells served as reference and control surfaces. Binding responses showed a CBS concentration-dependent increase in response and appeared to approach saturation as seen from the plateau in the sensorgram traces obtained for the high-concentration samples. The superimposed traces resulting from triplicate injections (Figure 2) demonstrate that the assay was highly reproducible.

**Data analysis and evaluation**

Response data were analyzed by simulating a set of curves based on a simple bimolecular reaction model to derive $k_a$ and $k_d$ values. In most cases, an excellent fit was obtained, with simulated responses varying by less that 1 RU from all data points collected for the interaction. All 59 data sets received from participants were processed and analyzed by the authors in the same way to unify data transformation steps that are often assessed subjectively. All data were amenable to kinetic interpretation. Several participants who reported high immobilization levels of CA II (>8000 RU) also reported a small degree of baseline drift during the binding experiments, possibly reflecting non-covalent trapping of a small
population of the enzyme molecules during the immobilization step and their gradual leaching from the chip surface during the assay. It is also notable that the maximal responses observed fell below the predicted $R_{\text{max}}$ (for example, 6600 RU of immobilized CA II should bind approximately 45 RU CBS if all the protein is active), suggesting that not all the CA II remained active after immobilization.

The most common Biacore systems used in the trial, Biacore 2000 (25 data sets) and Biacore 3000 (27 data sets) were shown to be well-suited to small molecule analysis with both systems providing reproducible results, although Biacore 3000 generated data with a somewhat higher signal to noise ratio. Biacore S51, however, produced the highest signal to noise ratio and the best reproducibility of all systems tested.

It is likely that the optimal performance of this system, which is specifically designed for small molecule analysis, is due to its unique flow cell system that ensures simultaneous delivery of analyte to two discrete spots from a single injection of analyte. The mean values for the rate constants and the corresponding binding affinity for all (59) returned data sets yielded kinetic and affinity parameter values of $k_a = 4.0 \pm 0.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $k_d = 0.036 \pm 0.007 \text{ s}^{-1}$, and $K_D = 0.90 \pm 0.22 \mu\text{M}$. Figure 3 shows representative sensorgrams for three of the Biacore systems used. Despite the range of equipment used and the difference in experience of the users, the overall variation in the parameter values reported across the panel of Biacore users was only around 20%.

Figure partly reproduced, with permission, from Myszka et al., J Biomolecular Techniques 14: 247-69 (2003).
van’t Hoff analysis

Six data sets were returned for the binding of CBS to immobilized CA II at 5, 15, 25 and 35°C. The affinity of the interaction weakened six-fold (from 0.28 µM to 1.83 µM) as the temperature increased over the 30°C range explored. The reaction was deduced to be exothermically driven with a mean enthalpy change of $\Delta H_{\text{van't Hoff}} = -10.6 \pm 1.4$ kcal/mol. Within experimental error the enthalpy determined by Biacore was identical to that measured directly by ITC (-10.4 ± 2.5 kcal/mol).

Discussion

One of the main motivations for initiating this study was to compare the results of an identical interaction using surface-based SPR technology and solution-based ITC. Intuitively, some researchers have stated a preference for ITC over surface-based technologies due to a commonly held perception that the process of immobilization may influence the conformation and hence binding characteristics of macromolecules. However, this study showed that, in addition to confirming the quality of data reported by independent Biacore users, the thermodynamic parameters ($K_D = 0.90 \pm 0.22$ µM and $\Delta H_{\text{van't Hoff}} = -10.6 \pm 1.4$ kcal/mol) for the interaction were indistinguishable from those reported by ITC users ($K_D = 1.00 \pm 0.22$ µM and $\Delta H_{\text{ITC}} = -10.4 \pm 2.5$ kcal/mol). Figure 4 shows a summary of affinities measured using the surface- and solution-based methods. These data confirm that binding behavior is not affected by being restricted in the surface analysis compared to the free solution approach.

Although Biacore-based assays are surface-based, it is worth considering the chemistry at the chip surface. Unlike other surface-based assays, e.g. ELISA, in which large molecules are linked to a planar surface, the surface of Sensor Chip CM5 comprises a carpet of long and flexible dextran molecules with carboxymethyl groups to which ligands can be covalently attached using one of several ligation chemistries.

Figure 4. Comparative affinity determinations for the CBS:CAII binding interaction using Biacore ($n = 59$) and ITC ($n = 14$).
The flexibility and length of these dextran chains create a scaffold that allows ligated molecules to retain much of their entropic freedom. There are no reports in the literature to date that indicate that immobilization to Biacore’s CM5 sensor chips seriously compromises the binding activity of a ligand except in cases where the amino acids within the binding site itself participate in the immobilization process.

This study demonstrated that Biacore-derived data collected from a large pool of volunteers were remarkably consistent despite the variety of Biacore systems used and the range in user experience. Further, comparisons of affinity and thermodynamic data with those returned by ITC users for the same interaction showed that both technologies generated similar parameter values. In addition to requiring less material than ITC, the constantly improving sensitivity and signal to noise specifications of the latest Biacore systems are reducing the limitations imposed by the inherently small signals that are generated when low molecular weight analytes bind to large ligands. These technological advances are now enabling systems like Biacore 3000 and Biacore S51 to accurately characterize the kinetics and affinities of small molecule interactions.

References