

Characterization of Small Molecule to Protein Binding

Summary

Label-free technologies are mostly used for detection of biomolecular interactions between proteins or other macromolecules. Characterization of small molecule to protein binding has often been a challenge because label-free signal depends on molecular weight of the analyte and analyte-to-target molecular weight ratio. In this study we show that Nanopore Optical Interferometry (NPOI) can be used for accurate measurement of an enzyme interaction with inhibitors as small as 95 Da. We provide statistical analysis of data fidelity and correlate our results with published data obtained by Surface Plasmon Resonance (SPR). In addition, we show that the same enzyme binding surface can be used multiple times in automated runs for screening and characterization of multiple analytes.

Introduction

Technologies for detection of small molecule interaction with target protein have been at the core of new drug discovery process. Procedures for primary high throughput screening (HTS) typically rely on measurement of changes in fluorescence or luminescence in high-density microwell plates. Primary screening campaigns are done with the use of advanced liquid handling equipment and often completed quickly. The next stage, lead selection and in-depth characterization, often becomes a bottleneck due to lengthy and labor-intensive assay development. Label free technology offers an attractive alternative to traditional bioassays. It eliminates the need for preparation and characterization of labeled biomolecules. Also it automatically eliminates common artifacts caused by optical properties of small molecules or their interference with

secondary reagents in fluorescence and luminescence-based assays.

The Silicon Kinetics SKi Pro system uses Nanopore Optical Interferometry (NPOI) for label-free measurement of biomolecular interactions. Nanoporous silicon biosensors provide extensive hydrophilic liquid-like surfaces for immobilization of target proteins. Advanced multi-channel optics ensure highest sensitivity at low background noise. SKi Pro flow cell systems deliver analyte to sample and reference channels with two parallel continuous flow pumps. That ensures constant analyte concentration and further reduces systems noise with accurate referencing.

In order to evaluate the SKi Pro flow cell instrument for small molecule to protein binding we characterized interactions of an enzyme Carbonic Anhydrase II (CAII) with a number of its sulfonamide inhibitors. This protein - small molecule system was used extensively for assessment of SPR-based instruments and benchmarking of different label free platforms.[\[1\]](#),[\[2\]](#),[\[3\]](#) The results clearly show that SKi Pro instruments can be used for accurate characterization of small molecule binding to a target protein in a drug discovery process.

Method

Carboxyl coupling reagents (P/N SUPPLY-0309) and sensor chips (SKI-SENSOR-FC-8PACK-C) were from Silicon Kinetics. Carbonic Anhydrase II from bovine erythrocytes and sulfonamide inhibitors were purchased at Sigma-Aldrich, St. Louis, MO. All experiments were done on a SKi Pro X10 flow cell instrument. CAII was immobilized onto sample channels of carboxyl SKi Sensor flow cartridges using standard amine coupling procedure. The enzyme was dissolved in water immediately prior to immobilization.

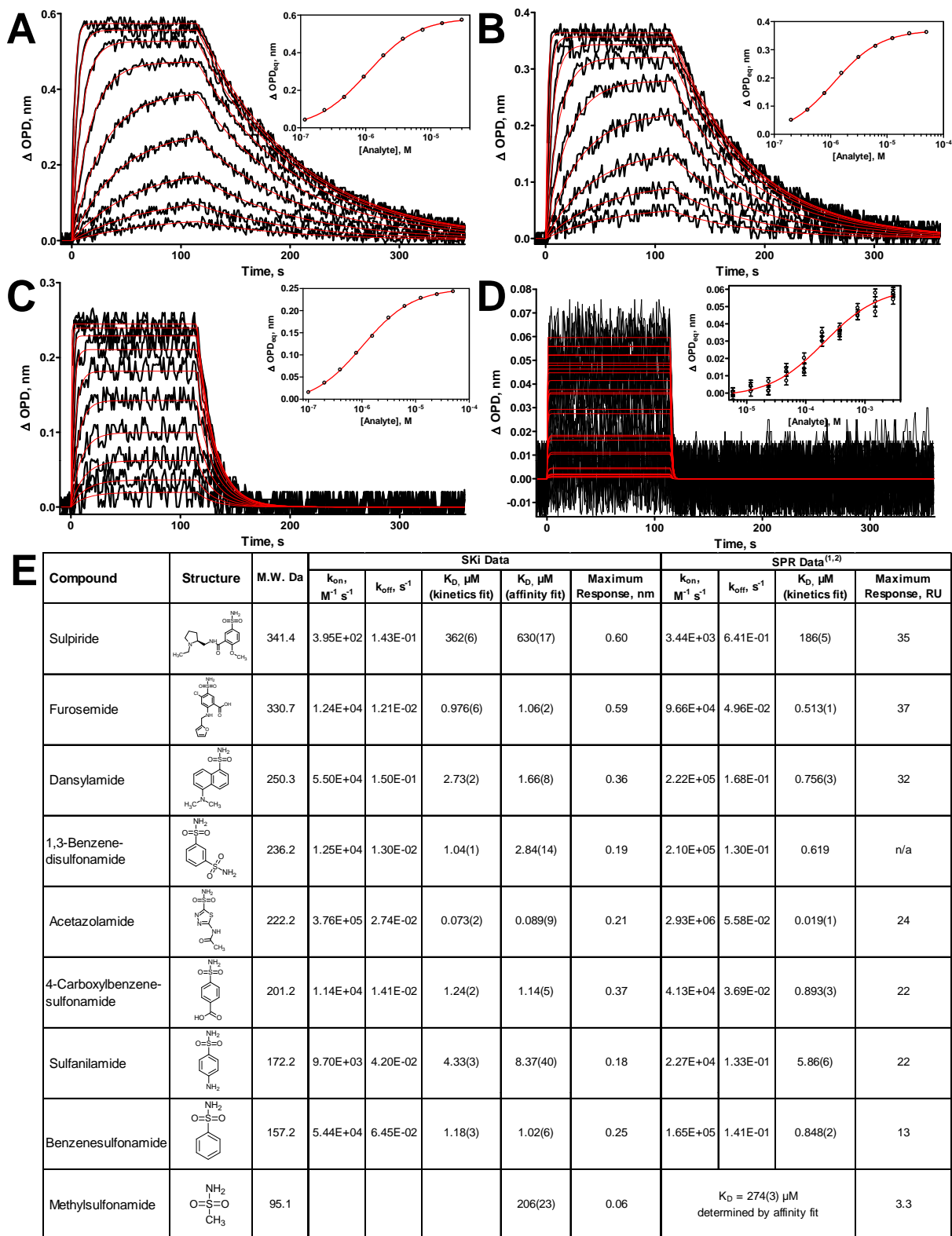


Figure 1: A-D: Representative binding curves for small molecule inhibitor binding to CAII. Maximum concentrations in 2-fold dilution series were 30 μM for Furosemide (A), 50 μM for 4-Carboxybenzenesulfonamide (B), 50 μM for Benzenesulfonamide (C) and 3 mM for Methanesulfonamide (D). Equilibrium affinity fits are shown in inserts. E: Summary of results for small molecule to CAII binding. Data on the right represents published results obtained by SPR

Surface was activated with a 7 minute injection of EDC/sulfo-NHS mixture at 200/50 mM concentration. Protein was diluted to 200 $\mu\text{g/ml}$ into 20 mM Acetate buffer pH 5.0 and injected for 10 minutes. Active sites were quenched with 1 M Ethanolamine buffer pH 8.0 and washed with phosphate-buffered saline pH 7.2 (PBS). Binding surface was stabilized with 3-5 blank injections of PBS.

PBS was used as a running and sample dilution buffer in all experiments. The tested sulfonamide CAII inhibitors were Furosemide, Acetazolamide, Benzenesulfonamide, 1,3-Benzenedisulfonamide, 4-Carboxybenzenesulfonamide, Dansylamide, Sulfanilamide, Sulpiride and Methylsulfonamide. All inhibitors were first prepared as 100 mM stock solutions in Dimethyl Sulfoxide (DMSO) and further diluted into PBS immediately prior to the experiments. Each analyte binding was analyzed in automated runs of 10 two-fold dilution series plus 2 "zero concentration" injections. No regeneration steps were necessary since all bound compounds dissociated quickly to baseline.

Data was analyzed with SKi Report software. Referenced and blanked binding-dissociation curves were fit globally with two-state binding kinetics model. Sigmoidal dose-response fit was used for affinity analysis. For statistical analysis of data fidelity two populations were selected. One included all data points during last 10 seconds before inhibitor injection and represented background. The second included all data points during last 10 seconds of inhibitor injection and represented signal.

Results

Direct immobilization of CAII onto carboxyl sensors typically resulted in 60-70 nM binding surfaces (data not shown). While even larger binding surfaces can be achieved with higher protein concentration, we chose to do binding experiments with lower immobilization levels that ensure better integrity of the target protein and are more universally applicable to other protein targets.

Detection of inhibitor binding

Figure 1 shows typical binding curves for 4 inhibitors with molecular weights in the range from 331 to 95 Da and data summary for all tested inhibitors. These results demonstrate that small molecule to protein binding is accurately detected on SKi Pro instrument. Max-

imum response varies from 0.6 nM for 331 kDa compound to 0.06 nM for the smallest 95 kDa inhibitor and generally consistent with compound to protein molecular weight ratio. Since binding reaches equilibrium for most concentrations, these data suitable for both kinetics and affinity fit. In the case of the smallest inhibitor, Methylsulfonamide, we show the results of 3 separate multi-concentration runs. While binding signals here are low, 0.06 nm and less, they are reproducible and allow to measure dissociation constant based on equilibrium binding values.

Data quality

The short-term noise is 0.02-0.03 nm which results in signal to noise ratio of more than 6 for small molecules larger than 150 kDa. Statistical analysis of data fidelity shows that Z-factor values for inhibitors larger than 150 Da are more than 0.4 for inhibitor concentrations corresponding to 0.3-0.5 of respective compound K_D and higher (Figure 2).

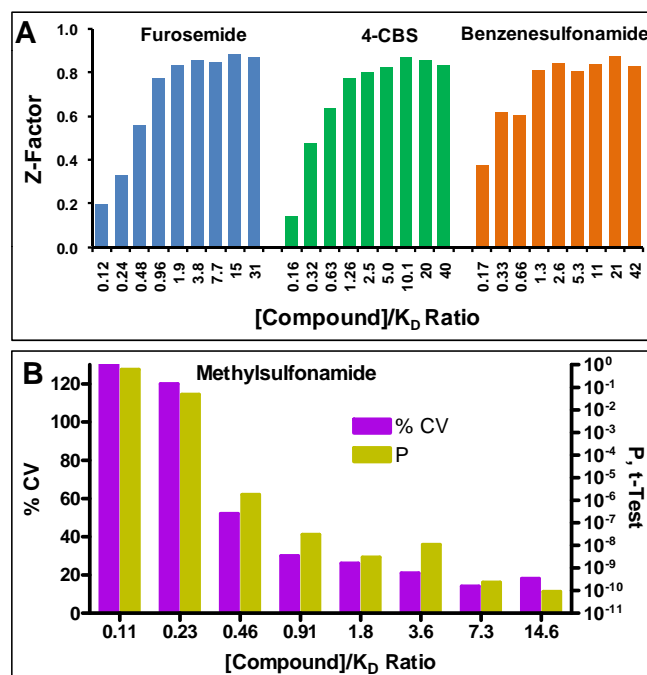


Figure 2: Statistical analysis of detection of small molecule to CAII binding. All data points during last 10 seconds of injection (*Signal*) were compared to all data points during last 10 seconds before injection (*Background*). **A:** Z-factor values for Furosemide, 4-Carboxybenzenesulfonamide and Benzenesulfonamide. **B:** Coefficient of variation and t-Test probability for Methylsulfonamide.

Z-factor values for the smallest and lowest affinity inhibitor, Methylsulfonamide, were less than 0.4 at

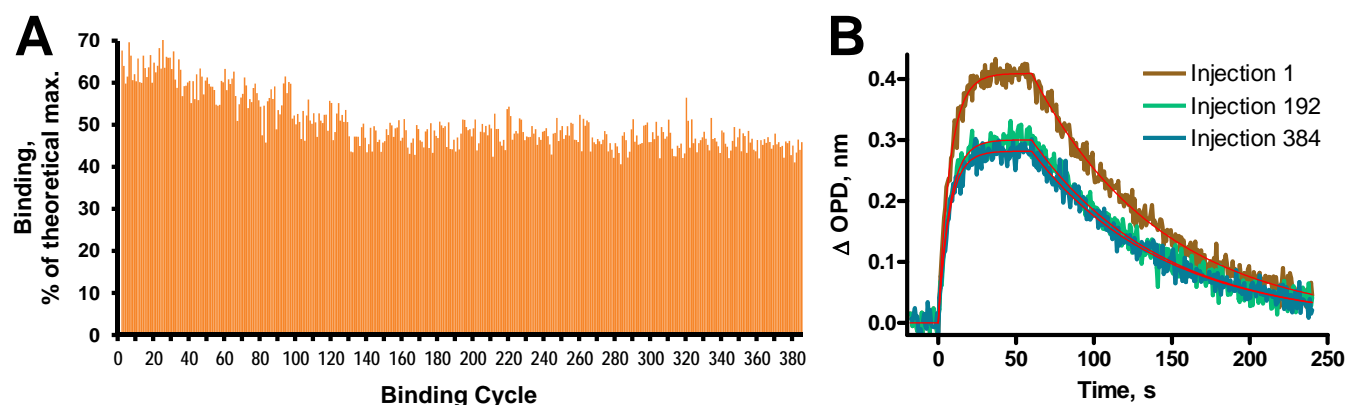


Figure 3: Repetitive binding of Furosemide to CAII surface. CAII was immobilized onto a carboxyl SKi Sensor at 68 nm. Furosemide at 10 μ M was injected for 384 cycles with 60 second binding and 180 second dissociation times. **A:** Maximum binding signals were calculated as percentage of theoretical maximum binding of 0.64 nm. **B:** Binding-dissociation curves for injections 1, 192 and 384

all concentrations. Coefficient of variation (Figure 2 B) changes from very high values at low concentrations to close to 50% at about half K_D to 15–25% at higher concentrations. The probability associated with non-paired t-Test is only high at inhibitor concentrations of about quarter K_D and less while differences between signal and background populations are statistically significant at higher concentrations. The results of consecutive runs (Figure 1 D) show good repeatability and low inter-run variation. Taken together, these results indicate that measurements of Methylsulfonamide interaction with CAII, while not conforming to stricter criteria of $Z' < 0.4$, can still be reliably used for detection of binding and estimation of K_D .

Stability of binding surface

Robust characterization of inhibitor to enzyme binding involves multiple automated runs over the same binding surface. We performed a 384-run study for Furosemide-CAII interaction as an example of such experiments. The results (Figure 3) show that binding signals decreased from initial 60–70% of theoretical maximum within first 120 runs and then stabilized at 45–50%. That amounts to less than one-third decrease in signal compared to initial runs and still affords reliable detection of binding with typical Z-factor values over 0.8.

Discussion

In this study we show that Nanopore Optical Interferometry (NPOI) can be used for accurate measurement of small molecule to protein binding. We measure interactions of CAII with its sulfonamide inhibitors, the same protein – small molecule system that was used extensively for assessment of SPR-based instruments and benchmarking of different label free platforms. Our results are similar to the published data obtained by SPR (Figures 1 and 4).

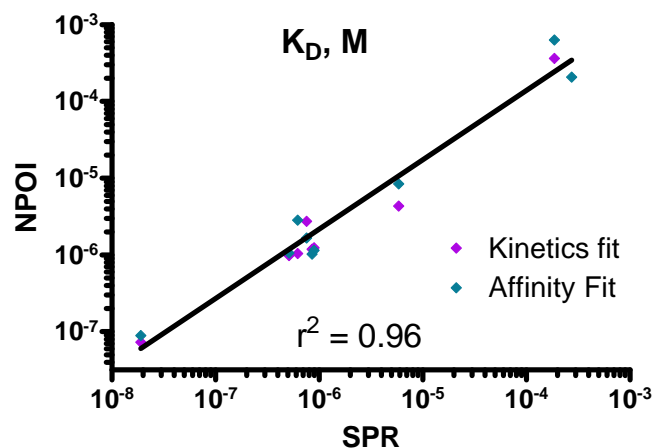


Figure 4: Comparison of NPOI and SPR data

Although systemic differences in k_{on} and k_{off} numbers can be seen, the overall correlation of the results obtained with two different technologies is remarkably high. NPOI benchmarking against SPR is further discussed in Application Note 7.[4]

Statistical analysis of data fidelity shows that

binding signals can be measured precisely for small molecules of 150 Da and larger at concentrations as low as 0.3–0.5 of their respective K_D values. In the case of 95 Da low affinity inhibitor, the measurements still provided highly significant and repeatable results, similar to the data obtained by SPR.[1]

Multi-injection study of the immobilized enzyme stability has shown that its inhibitor binding capacity decreased gradually by 25–30% over first 120 binding cycles and then stabilized for the remainder of the 384-injection run. These numbers are expected to depend strongly on the nature of immobilized target and on reversibility of small molecule binding. In our experiment, the quality of data obtained after multiple binding-dissociation cycles remained sufficiently high for accurate measurements.

References

- [1] D. G. Myszka. Analysis of small-molecule interactions using biacore s51 technology. *Analytical Biochemistry*, 329:316.
- [2] G. A. Papalia et al. Comparative analysis of 10 small molecules binding to carbonic anhydrase ii by different investigators using biacore technology. *Analytical Biochemistry*, 359:94.
- [3] M. J. Cannon et al. Comparative analyses of a small molecule/enzyme interaction by multiple users of biacore technology. *Analytical Biochemistry*, 330:98.
- [4] Application Note 7. *Benchmarking Nanopore Optical Interferometry against SPR*. www.siliconkinetics.com.

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