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Free-Solution Interaction Assay of Carbonic Anhydrase to its Inhibitors Using Back-scattering Interferometry

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Abstract

Back-scattering interferometry (BSI) is a label-free, free-solution, small-volume technique used for characterizing binding interactions, which is also relevant to a growing number of biosensing applications including drug discovery. Here we use BSI to characterize the interaction of carbonic anhydrase (CAII) with five well known CAII inhibitors ($\pm$ sulpiride, sulfanilamide, benzene sulfonamide, dansylamide, and acetazolamide) in the presence of dimethyl sulfoxide (DMSO). Dissociation constants (K_D) calculated for each interaction were consistent with literature values previously obtained using surface plasmon resonance (SPR) and fluorescence-based competition assays. Results demonstrate the potential of BSI as a drug-screening tool which is fully-compatible with DMSO and does not require immobilization or labeling, therefore allowing binding interactions to be characterized in the native state. BSI has the potential for reducing labor costs, sample consumption and assay time while providing enhanced reliability over existing techniques.

Keywords

back-scattering interferometry; carbonic anhydrase; dissociation constant; drug screening; molecular interaction

1. Introduction

Studying biochemical interactions is essential to understanding cellular functions, assisting in diagnostic techniques, and discovering new disease treatments. The success of a drug discovery effort is especially dependent on efficient methods for characterizing the binding of a drug to its biological target. Ideally, molecular interaction assays should be relatively inexpensive and performed with both binding partners in their native state so that measurements will most accurately represent the *in vivo* interaction. Unfortunately, most widely-utilized techniques for drug discovery applications, such as surface plasmon resonance (SPR) and fluorescence-based assays, require labeling and/or immobilization of the biological target, adding both expense and possible disruption of the interaction [1-3]. These alterations to the interacting species could impede or block binding sites, leading to inaccurate affinity measurements or false negatives [1, 3]. Furthermore, in the case of

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fluorescence-based assays, the label itself can introduce high background or false positives from nonspecific binding of the fluorophore, and additional complications may arise if drug candidates exhibit autofluorescence [1]. Another important consideration for drug screening methods is that many drug candidates are hydrophobic and require additives such as DMSO to achieve solubility; molecular interaction assays for drug screening purposes should therefore readily accommodate solutions containing DMSO. Although it is possible to perform SPR assays using DMSO, extraordinary precautions and corrections are necessary to ensure no interference by this high refractive-index (RI) additive, which has introduced significant error in past SPR determinations even with attempted corrections [4]. Therefore, a label-free, free-solution technique which facilitates interaction measurements in the presence of DMSO would be highly desired for drug screening methods. Currently, isothermal titration calorimetry (ITC) is the most broadly-employed label-free, free-solution technique for measuring biochemical interactions. However, major drawbacks of this method include the need for large sample volumes, typically milliliters but at least 200 μ L when using low volume calorimeters, and limited sensitivity [5, 6]. ITC cannot be used to determine dissociation constants below the nanomolar range or above several hundred micromolar, nor can it measure binding events in which the reaction enthalpy is near zero [6]. These drawbacks make ITC a less desirable candidate for high-throughput screening applications in which sample quantities are limited and or binding affinities vary widely. Back-scattering interferometry (BSI) is a label-free, free-solution technique developed recently by the Bornhop lab which enables the interrogation of binding events using less than one microliter of sample at relevant concentrations and does not rely on reaction enthalpy or changes in mass [5]. BSI measures changes in refractive index (RI) using a simple optical train consisting of a He:Ne laser, mirror, microfluidic chip, and detector (CCD Camera). It is note worthy that BSI is essentially a detector of differences in molecular structures, with binding measurements relying on the comparison of signals between free and bound states. Modeling of the BSI phenomenon indicates that much of the signal derives from changes in conformation and solvation that occur when a molecule such as a protein interacts with a ligand. With a detection volume of approximately one nanoliter, BSI provides an environment for rapid, inexpensive, native-state characterization of binding interactions while constraining sample consumption. Furthermore, the sensitivity of BSI enables interactions to be studied at physiologically relevant concentrations over a wide dynamic range with binding affinities previously measured from low micromolar to tens of picomolar concentrations [5]. To illustrate the utility of BSI in drug screening applications, here we have used BSI to measure the binding affinities of well-characterized interactions between the carbonic anhydrase enzyme II (CAII) and five CAII inhibitors in the presence of DMSO.

CAII is a clinically relevant enzyme involved in several biochemical pathways [7-13]. Inhibitors of the enzyme are currently used in the treatment of glaucoma, cancer, and epilepsy, while recently showing potential for the treatment of obesity and other diseases [7-13]. As a consequence, the CAII system has been widely studied with SPR being used to characterize drug interactions with target biomolecules, including interactions of CAII with inhibitors [14]. As aforementioned, SPR requires immobilization of one binding partner, in this case CAII. Therefore, using SPR as a benchmark, we show that BSI can be used to

study free-solution, label-free molecular interactions of CAII with five of its inhibitors: $\pm$ sulpiride, sulfanilamide, benzene sulfonamide, dansylamide, and acetazolamide. These studies demonstrate the ability of BSI to perform binding studies for drug discovery applications which more closely represent *in vivo* binding characteristics than other current biosensing techniques.

2. Materials and Methods

2.1 Instrumentation

Analyses were performed using a research prototype BSI manufactured by Molecular Sensing, Inc (MSI), Montara, CA. This instrument had a basic optical train analogous to that described previously [5]. In summary, the beam from a HeNe laser was split into two beams with equal intensity, which was then directed onto two parallel channels, spaced 1 mm apart (Figure 1). The beams were off-set so that there was no overlap of the beam onto the other channel. The fringes were then directed onto a 2D CCD array camera using a mirror. The channels were isotropically etched in borosilicate glass, yielding a near semicircular cross section with dimensions 90 μ m by 40 μ m (Micronit, Netherlands). The position of the fringes during the assay was quantified at the millipixel level using a proprietary and patent-pending algorithm developed at MSI. $\pm$ Sulpiride, sulfanilamide, benzene sulfonamide, dansylamide, and acetazolamide were purchased from Fisher Scientific (Pittsburgh, PA, USA), carbonic anhydrase isozyme II (CAII) from bovine erythrocytes from Sigma-Aldrich (St. Louis, MO, USA), phosphate-buffered saline (PBS) tablets from Biomedicals (Solon, OH, USA), and dimethyl sulfoxide (DMSO) from Fisher Scientific (Pittsburgh, PA, USA).

2.2 Chip and Tube conditioning

The microfluidic chip was preconditioned with 100 mM of Potassium Hydroxide (KOH) for 30 minutes, rinsed with water, and then with 100 mM Hydrochloric Acid (HCl) for 30 minutes. The chip was then washed with water and left to equilibrate in 1M phosphate buffered saline (PBS). Siliconized Eppendorf tubes were washed and stirred in 50% Methanol:H₂O for one hour then washed with DI H₂O four times and left to dry in the oven at 80°C to remove biologically-active contaminants.

2.3 Sample Preparation

Small molecule solutions were initially prepared in 100% DMSO at a concentration of 1M, and then diluted to 100 mM in 100% DMSO. A stock solution of the small molecules of 1mM was prepared by 1:100 dilution of PBS buffer, resulting in a sample of 1% DMSO. Solutions were then diluted further to the desired stock concentrations using 1% DMSO PBS buffer. CAII was dissolved directly in 1% DMSO PBS buffer and then diluted using the same buffer to the desired concentration. A summary of the concentration range of each small molecule along with the fixed concentration of CAII used for each binding study is shown in Table 1. Assay solutions contained both CAII and the small molecule being studied, whereas the blank (i.e. calibration) solutions included only the small molecule at the same concentrations. Blank and assay solutions were made concurrently by taking two identical aliquots from each stock concentration and adding an equal amount of inhibitor (assay solutions) or 1% DMSO buffer (blank solutions). Samples and blanks were both

incubated at 4°C overnight to ensure complete binding and equilibration before taking measurements.

2.4 Analytical Procedure

End-point assays were performed similarly to those recently described by our group [15]. In short, the changes in refractive index arising from binding with CAII were measured for a series of inhibitor concentrations, allowing the determination of a dissociation constant (K_D) for the interaction. In contrast to real-time analyses which require on-chip mixing, end-point assays allow each assay concentration point (i.e. CAII and inhibitor) to be prepared in advance with a matching blank solution (i.e. inhibitor only), and solutions reach equilibration prior to analysis. The BSI measurements were then performed directly on these equilibrated samples without needing to remove the excess binding ligands.

For these analyses, all solutions were introduced into the BSI instrument using a P-10 pipette using a unique macro to micro interface. Prior to introduction of sample and blanks, the BSI instrument was calibrated using six concentrations of DMSO ranging from 1.0%-1.25% to ensure the sensitivity of the instrument was adequate to perform free-solution interaction assays in this sample medium. Blanks and samples were then introduced in order starting from the lowest to the highest concentration, and the signal was measured for 1 minute. This process was repeated three times collecting triplicate independent determinations of the assay. Following each assay, channels were cleaned using a regular acid/base cleaning followed by the equilibration of buffer in the channels.

3. Results

The signal obtained from BSI is measurement of the fringe shift the results from the change in refractive index (RI) that occurs during a binding event [5] and was quantified using a novel proprietary position sensing algorithm. Because the bulk RI can change as a function of analyte concentration, it is critical that the bulk contributions of the changing inhibitor concentration be subtracted from the overall BSI signal. As expected, the blank solutions (consisting of inhibitor only and no CAII) showed a small linear response due to the increasing concentration of the ligand. These bulk RI contributions were subtracted from the overall BSI signal for each concentration point, allowing the signal due solely to binding interactions to be calculated. This remaining BSI signal was plotted versus the concentration of the inhibitor ligand to create a saturation binding isotherm. The plots were then fit with a square hyperbolic function consistent with a single site binding model using Prism® (Graphpad, San Diego, CA) software and the binding affinities of CAII to each of the five inhibitors were calculated. As expected, all five small molecules demonstrated binding with CAII as shown by the dose-dependent increase in signal to yield a saturation isotherm (Figure 2). The binding curves of CAII with (A) $\pm$ Sulpiride (B) Sulfanilamide (C) Benzene Sulfonamide (D) Dansylamide (E) Acetazolamide are shown, with error bars representing three independent determinations of each concentration point in each assay and R^2 values calculated using the averages of these three determinations. The specific concentrations of CAII and each inhibitor are listed in Table I.

Table I also presents the comparison of the affinities obtained from BSI to those published previously using SPR [14]. While $\pm$ sulpiride gave a K_D values that was notably lower (i.e. higher affinity) than those found by SPR, the K_D values determined by BSI for the other inhibitors were remarkably close to the established affinities. In particular, benzene sulfonamide and dansylamide gave virtually the same K_D values as determined by SPR when taking into account the standard error of both determinations. While the K_D values determined using BSI were generally lower than those previously published, the measurements contain considerably less error. The differences in affinity may be attributed to the higher percent of DMSO used in the previous assay and/or and the fact that CAII was immobilized in the SPR experiments [14]. Binding assays performed using BSI were conducted in free solution; therefore, it is expected that the affinities of CAII for each of the small molecules may vary from those found using a tethered assay that is critically dependent on determining a change in mass. Furthermore, we think it is reasonable to believe that the binding activity of the non-immobilized CAII in the BSI assays is more similar to its activity *in vivo* since nonspecific binding, steric hindrance of binding sites, and mass transport problems have been identified as potential problems in using SPR [16]. Comparison with a fluorescent competition assay (Table I) also showed notably similar affinity values [17], further supporting the values determined using BSI. The acetazolamide and sulfanilamide had calculated K_D values that were nearly identical for the BSI and the competition assay methodologies [17].

4. Discussion

It has been shown here that BSI can be used to quantify protein-small molecule interactions in the presence of DMSO, in free-solution, with relative ease (mere pipetting for sample introduction), speed (approximately 1 hr for an 8-concentration point, $n=3$ determination following equilibration of samples) and small sample quantities. The ability to use small volumes (approximately 1 μ L per measurement), at physiologically relevant concentrations allows for the assay to be performed using only a few nanograms of enzyme. For example, the entire binding assay between the CAII and $\pm$ sulpiride, which required the highest concentrations of both protein and inhibitor, used only 12 picomoles of enzyme and less than 3 nanomoles of inhibitor. Taken together with the capability to perform assays label-free and in free-solution, this advantage represents a reduction in cost versus method that requires a fluorescent labeling. Additionally, the ability to work in DMSO solutions can not be understated and demonstrates that BSI can be considered as a practical screening tool for a variety of drugs and targets. As mentioned previously, drug candidates are typically insoluble in water and are often prepared and analyzed in the presence of DMSO (commonly up to 5% DMSO), and minute deviations in DMSO concentration can cause large RI shifts [4]. When using SPR, the nature of immobilizing one binding partner and measuring the change in refractive index at the surface when ligand is introduced requires tedious accommodations to relieve error from using solutions containing DMSO. Therefore, SPR must not only overcome this high RI background invading the surface, but also requires near-perfect precision when constructing calibrations to subtract these bulk RI effects of DMSO. In contrast, BSI's sensitivity does not suffer due the presence of DMSO. BSI has an extremely large dynamic range with respect to affinity, and enables the complete

cancellation of bulk effects since calibration solutions of ligand (i.e. inhibitors) are drawn from the same stock solutions as those used in the free-solution interaction. Creating a separate DMSO calibration to subtract the bulk effects of DMSO alone is unnecessary with BSI, eliminating preparation error that could be present in other methodologies. BSI not only allows binding partners to remain in their native state without potentially-perturbing labels or immobilizations, but also more easily accommodates assays which require DMSO, complex matrices [15] or other high-RI additives. Finally, BSI is not a mass-sensitive sensor making it compatible with a wide range of solutes and targets including small molecule-protein interactions, protein-DNA or small molecule-DNA interactions and large protein complex-inhibitor interactions.

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Abbreviations

BSI	Back-scattering Interferometry
CAII	Carbonic Anhydrase Enzyme II
CCD	Charge-coupled Device
DMSO	Dimethyl Sulfoxide
MSI	Molecular Sensing, Inc.
ITC	Isothermal Titration Calorimetry
K_D	Dissociation Constant
PBS	Phosphate Buffered Saline
RI	Refractive Index
SPR	Surface Plasmon Resonance

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Figure 1.
Block diagram of the optical train of BSI.

Figure 2.

Binding curves of CAII with (A) sulpiride (B) sulfanilamide (C) benzene sulfonamide (D) dansylamide (E) acetazolamide are shown with K_D values as determined with BSI analyses. Blank solutions have been subtracted from each concentration point to eliminate bulk RI contributions.

Table 1

indicates ranges of small molecule concentration with the corresponding concentration used for CAII. Error bars represent the standard deviations of three independent trials. All R^2 values were determined using the average of three trials.

Inhibitor	[CAII] (nM)	[Inhibitor] (μ M)	K_D (μ M) BSI ^a	K_D (μ M) SPR ^b [14]	K_D (μ M) Competition Assay ^a [17]
$\pm$ Sulpiride	500	3.9-250	5.90 ± 1.24	48 ± 14	N/A
Sulfanilamide	50	0.39-25	1.76 ± 0.22	3.1 ± 1.1	1.0 ± 0.03
Benzene Sulfonamide	10	0.16-5.0	0.57 ± 0.09	0.80 ± 0.28	0.19 ± 0.01
Dansylamide	0.5	0.039- 2.5	0.45 ± 0.10	0.44 ± 0.12	1.0 ± 0.1
Acetazolamide	0.5	0.0039- 0.25	0.011 ± 0.001	$0.031 \pm .011$	0.011 ± 0.002

^a 1% DMSO

^b 3% DMSO