Characterization of aptamer-small molecule interactions with backscattering interferometry

By

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CHAPTER 1: INTRODUCTION

Antibodies versus Aptamers:

At their foundation, all biological processes are composed of molecular interactions. These reactions occur between molecular species that, upon interaction, fulfill a function that affects the larger biological ecosystem. Molecular interactions cover a wide range of processes and can be as simple as the neutralization of sodium hydroxide by a buffering solution to the complex, multiple step interactions of proteins, DNA and biological complexes requiring multiple cofactors, and precise pH and ionic condition. Therefore, it is of utmost importance to biomedical researchers to be able to study these interactions in an accurate, quantitative fashion.

For biomedical applications, antibodies are the most common probe used in quantitative molecular interaction assays. Antibodies are used in diagnostics, therapeutics and treatments. However antibodies possess many qualities that make them difficult to work with. As therapeutics and even when they are humanized, they can elicit an antigenic response by the patient. Production of antibodies is nearly always done *in vivo*, which introduces batch-to-batch variability and a significant amount of cost, energy, and time required for development and production. Once made these proteins can be easily denatured, and therefore they require refrigeration and special handling to ensure their continued functionality. Even with proper handling, antibodies degrade over time which results in a limited shelf life. The use of antibodies for assays is ubiquitous, yet the presence of proteases in test solutions can significantly speed up degradation of the molecule. While there a several approaches used to modify antibodies so that they can be immobilized or made to fluoresce, these chemical modifications, such as the addition of conjugates, are time consuming, expensive and can cause the antibody to lose functionality..

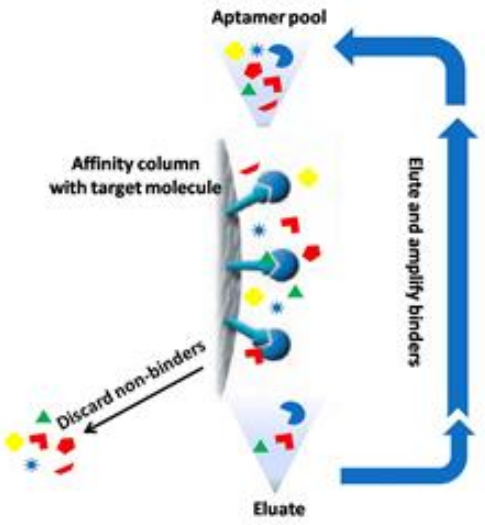

Figure 1: Simplified schematic of conventional, single-target DNA aptamer selection (SELEX).

An alternative to antibodies are aptamers [1]. Aptamers are small oligonucleotide or peptide molecules that can be selectively designed to fulfill a desired function in a desired environment, and are therefore represent viable alternative to antibodies for many applications. Oligonucleotide aptamers can be composed of ribonucleic acid (RNA) or single-strand deoxyribonucleic acid (ssDNA), and are usually between 15-60 bases long. The chain of nucleotides determines a folding pattern for the aptamer that usually results in loops and hairpins, and these structures yield an aptamer's ability to interact with a molecular target. Due to the stability of oligonucleotides, and their ability to maintain their structure and function over repeated denaturation/renaturation cycles, aptamers are extremely robust and can be much easier to work with than other macromolecules.

Aptamers are commonly identified by an *in vitro* method of selection referred to as Systematic Evolution of Ligands by EXponential enrichment or "SELEX". The process begins with a very large pool (~1015 unique sequences) of randomized polynucleotides which is generally narrowed to just a few aptamer binders per molecular target [2, 3]. **Figure 1** shows a simplified schematic for repeated rounds of SELEX of DNA aptamers. Once multiple (typically 10-15) rounds of SELEX are completed, the DNA sequences are identified by conventional cloning and sequencing. Aptamers have been developed as ligands to important peptides and proteins, rivaling antibodies in both affinity and specificity [4-6]. Aptamers have been also developed to bind small organic molecules and cellular toxins [7-10], viruses [11, 12], and even targets as small as heavy metal ions [13, 14].

Aptamers were first developed in 1990 [3, 15] and have become known for their specificity [16]. In fact, aptamers can be developed that are more selective than antibodies [17]. These molecules are developed and identified *in vitro* [16, 18] so they do not depend on the animals or cells, which is a significant advantage over antibodies. This development process also creates the advantage of being able to change the properties of the aptamer as needed [16]. Aptamers can also be synthesized quickly and precisely, eliminating batch-to-batch variations. They are also very stable, so shipments can be at ambient temperatures and they can be stored long-term without loss of activity [16]. Because of these advantages, aptamers are quickly gaining widespread acceptance and have been used in place of antibodies for numerous applications, including chromatography, flow cytometry, electrophoresis, and in several biosensors [16, 19, 20].

Measurement of Molecular Interactions Using Backscattering Interferometry

Backscattering Interferometry is particularly well suited to quick, easy, and reproducible measurement of the interactions between small molecules and aptamers, due to its label free, free solution, mix-and-read assay methodology. Recent theoretical advances have shown that the measured signal is produced by a change in the refractive index of the participating species in free solution upon binding as a result of changes in conformation and hydration [21]. Because the signal depends upon conformational change in the species, the interactions of aptamers and small molecules are detectable using this methodology unlike in other methods that require a mass change or a large calorimetric signal. The ability of BSI to measure aptamer interactions is important because of their growing applications. Antibody interactions currently play a large role in biological and biochemical research and the replacement of antibodies with aptamers in these fields is growing. Therefore a method to characterize the interactions between aptamers and target molecules quickly and efficiently is needed. BSI fits this need well, demonstrating the ability to accommodate free-solution, label-free binding assays using only a small amount of sample.

The optical train depicted in **Figure 2** is quite simple for a highly sensitive, small volume interferometer, consisting of a coherent source, an object (channel in a chip or capillary) and a transducer. Probing the object with an unfocused He-Ne beam at nearly 90^{o} ($\pm 7°$ to allow fringes to be viewed), results in a high-contrast interference fringe pattern (**Figure 2**) in the back-scattered direction. Depending on configuration, tracking the position of the fringes enables RI changes to be quantified in the range from 10^{-4} - 10^{-9} [22, 23], within picoliter – nanoliter probe volumes. A long effective path length results from multiple reflections at the fluid-channel interface and leads to the unprecedented sensitivity in constrained volumes [24]. The most common interferometer

configuration is based on a microfluidic chip containing a nearly semicircular isotropically etched channel that is 100μm deep and 210μm wide, and this was used for all experiments presented in this work. Based on empirical observations, we have found that fringe selection is best accomplished by filling the channel with the analysis solution (buffer, serum, etc.) and counting approximately 5 fringes from the centroid, then windowing or selecting 5-7 fringes in this region that exhibit a nearly single spatial. While the fringes closest to the centroid appear to exhibit a greater shift [25], a binding signal has yet to elude us in the region described above. With proper alignment, the fringe contrast ratio approaches 99% and this metric, combined with response to a change in RI (detection limits with glycerol solutions) serves to consistently produce the desired outcome. Good thermal stabilization and environmental isolation is also necessary and allows the device to produce a detection limit of $\Delta RI < 5 \times 10^{-7}$. Typically the sample/chip is probed with both planes of polarization as a result of coupling a linearly polarized laser into a non-polarization maintaining single mode fiber coupler.

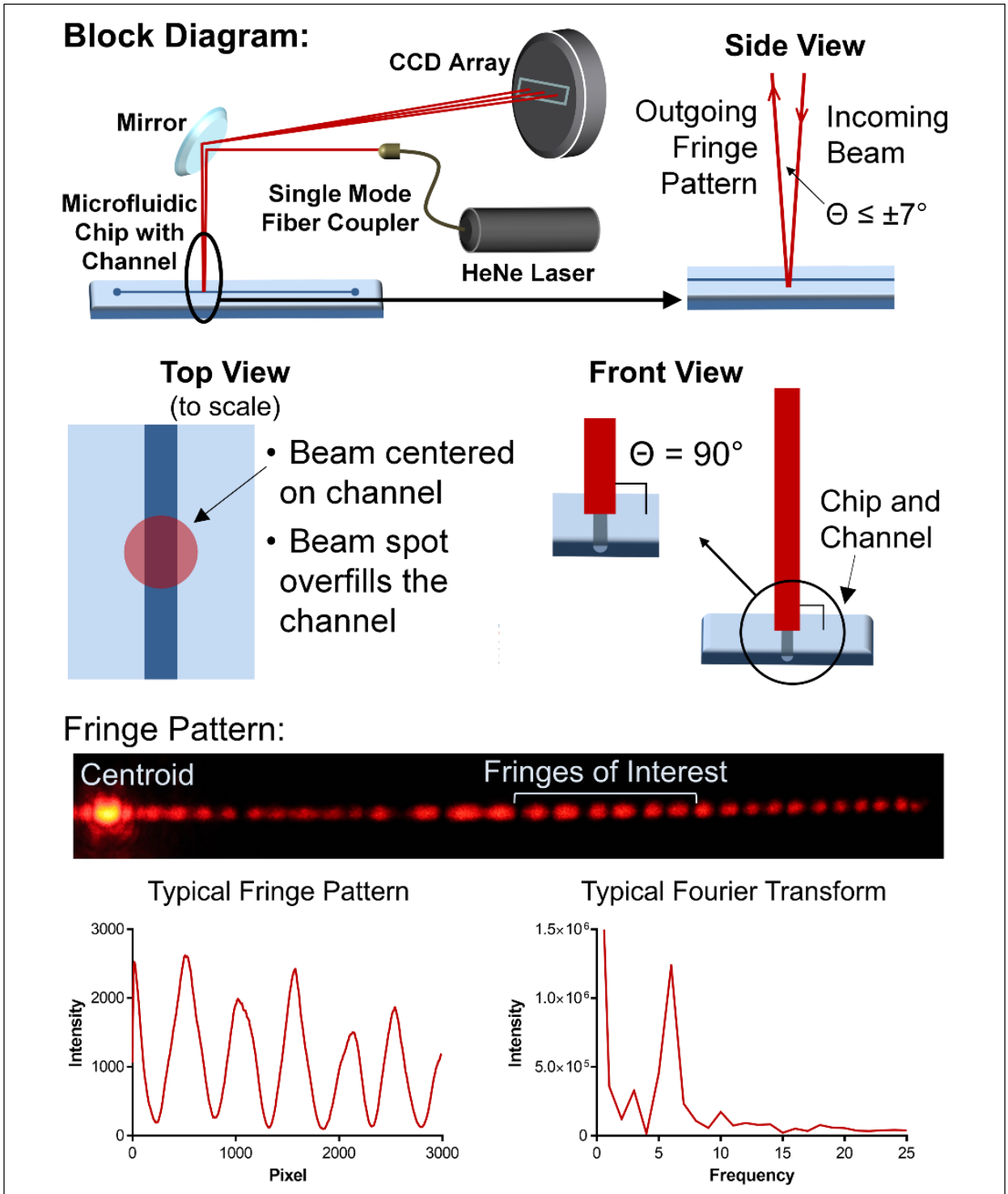

Figure 2: BSI Block diagram showing orientation of the beam relative to the chip, a photograph of the fringe pattern, the line profile of the region of interest for a good fringe pattern and the FFT spectrum for that region of interest (ROI).

CHAPTER 2: CHARACTERIZATION OF APTAMER SMALL MOLECULE INTERACTIONS WITH BACKSCATTERING INTERFEROMETRY – AS PUBLISHED IN *ANALYST,* NOVEMBER 21, 2014

Introduction:

Aptamers are small pieces of single-strand DNA or RNA that have been used in various applications. They can serve as sensors [26], therapeutics [27], and cellular process regulators [28], as well as drug targeting [10, 29-31]. The diversity of applications and the varied targets to which aptamers can bind (proteins, peptides, and small molecules) stem from the ability of aptamers to form complex three-dimensional shapes including both helices and single-stranded loops. Aptamers are then able to bind with targets through a variety of interactions including van der Waals forces, electrostatics, hydrogen bonding, and base stacking. Aptamers are selected *in vitro* and produced rapidly and inexpensively to bind with high affinity and specificity to a desired target molecule, as such are a promising alternative for applications that traditionally use antibodies. Until now, there has been no way to determine the binding affinity between aptamers and small molecules that is inexpensive, reliable, and does not require immobilization.

Currently, less than some 25% of existing aptamers have been generated for SM targets. Some of the earliest aptamers were selected against small organic dyes [2], which remained the focus of aptamer development during their infancy. However, methods were soon developed to easily select aptamers to larger biomolecules. Larger, more complex targets containing more functional groups and structural motifs yield a greater probability of finding sequences that can interact with the target via hydrogen bonds, electrostatic interactions, and hydrophobic interactions

[3]. As a result, the development focus switched to protein-binding aptamers and development of new aptamers for small molecules became less prevalent.

Recently, there has been a renewed interest in the design of aptamers that bind small molecules [32-34]. Identifying and characterizing new SM binding aptamers is important because they play key roles in a diverse range of biological processes, are involved in the environment, and are often difficult to quantify. Small molecules can be harmful toxins, beneficial drugs, such as antibiotics, nutrients, and serve as intercellular signal mediators or neurotransmitters. The vast majority of our most important therapeutics are small molecules [35], in part because they can often easily diffuse across cellular membranes [36]. Aptamers represent a unique class of chemical species because they can be selected to bind, manipulate, inhibit, control, or even detect small molecules in a complex matrix.

Currently there are six common methods of measuring aptamer-target binding affinity. These are surface plasmon resonance (SPR), biolayer interferometry (BLI), fluorescence anisotropy, isothermal calorimetry (ITC), microscale thermophoresis (MST) and backscattering interferometry (BSI). SPR measures the localized change in the refractive index (RI) near the surface of a substrate in order to detect binding [37, 38] and has been used in a multiplex format [39]. However, SPR is a heterogeneous method that requires complicated surface chemistry, tethering or immobilization of one of the species, typically the smaller of the two binding partners. Immobilization of one of the interacting species is known impact the binding, and the use of relatively expensive gold-plated slides or gold nanoparticles remains a limitation in SPR. Biolayer interferometry is another heterogeneous, label-free, optical analytical technique. In BLI the interference pattern of white light is produced from reflection of two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer. During the binding event

the reference surface region is compared to the sample region. BLI overcomes some of the limitations of SPR, yet it still requires surface immobilization. As with SPR, quantification of SM - aptamer binding is inherently difficult to measure with BLI, because it relies on a change in mass at the surface upon binding. Typically, the smaller of the interacting partners must be immobilized, so that the binding can be detected with reasonable finesse[40, 41]. Attempts to immobilize small molecules to the surface and detect the larger moiety are somewhat complicated and have practical limitations, since minimal changes in the structure can have a significant impact on the affinity [42]. ITC is a widely accepted method to measure rate and affinity of molecular interactions [43-45] that has recently demonstrated the ability to probe aptamer-target interactions [46], however this method suffers from low throughput, relatively large sample quantities, and poor detection limits. Fluorescence anisotropy (FA) has been used successfully for the characterization of aptamer-protein binding [47-49], however the rotational change upon binding between a large biomolecule and SM ligand can be small or not observable. Microscale thermophoresis (MST) is a relatively new free-solution method that can be applied to aptamer – SM interactions [50]. Like FA, MST still requires fluorescent labeling of the biopolymer and thereby increases the cost and complexity of the assay.

Backscattering interferometry (BSI) is a label-free, free-solution technology that can rapidly measure binding with little *a priori* knowledge of the system and allows for a broad range of binding affinities (pM-mM) to be measured [51, 52]. In the most common configuration of BSI, a microfluidic chip with a channel etched in glass acts as both the sample holder and the optics. BSI utilizes a red helium-neon (HeNe) laser (λ = 632.8 nm) to illuminate the chip in a simple optical train consisting of a source, object, and transducer. As the beam impinges on the channel, the beam reflects and refracts within the channel before exiting as a high contrast fringe

pattern. When a binding event occurs it results in a change in refractive index (RI) that produces a change in the spatial position of the fringes. This fringe shift is monitored using a CCD array in combination with Fourier analysis [22]. Because the BSI signal is a product of inherent properties of the sample (conformation, hydration and molecular dipole moment), there is no need for surface-immobilization or labeling to quantify a binding interaction. A more thorough explanation of BSI and the assay methodology is presented in our previous work. Specifically, these publications [52, 53] offer a detailed description of the instrument and would serve as a guide for in-house construction. Furthermore, the reader is encouraged to contact the Bornhop research group if questions arise during implementation at their site.

Our group has previously used BSI to measure aptamer-protein binding affinities in the low nanomolar range [54], showing also that it can quantify allostery in thrombin. Here, looking at a range of target molecules, we demonstrate the ability to quantitatively characterize the interaction between SM and aptamers with BSI. Ampicillin and tetracycline serve as common antibiotics, epirubicin as an anthracycline drug used for chemotherapy, and tenofovir an antiretroviral drug. We also studied BPA, which is a chemical toxin commonly used in plastics in the food and beverage industry. Finally, we studied the aptamer interaction with the hormone and neurotransmitter norepinephrine. We showed that BSI results compared well to published affinity values. In other cases, we did range-finding experiments to bracket the K_d, before building saturation isotherm plots with endpoint analysis. Our attempts to use MST or BLI for these analytes resulted in unreliable K_d values (data not shown). In all cases, BSI yielded high quality K_d values for the binding interactions, rapidly and with minimal sample manipulation.

Materials and Methods:

Previously unreported aptamers were selected by Base Pair Biotechnologies, Inc. (Pearland, TX). The sequence of the published BPA aptamer "12-5" was obtained from Jo *et al.* [55]. All binding affinity determinations by BSI were performed in an end-point format as previously described [51, 54, 56, 57], with the aptamer diluted in PBS buffer containing 150 mM NaCl and 1 mM $MgCl_2$. In short, the end-point (mix-and-read) assay consists of mixing a series of samples containing increasing concentrations of the SM target, from 0 nM to saturation, with a constant amount of aptamer, followed by a 2 hour incubation, and the subsequent BSI read. Samples are prepared in a manner such that the background solution composition is constrained throughout.

BSI provides a relative output using a series of blanks consisting of the same concentrations of target, in the absence of aptamer, as a reference allowing for correction for the analyte-induced bulk RI shift. A non-binding SM is also incubated with each aptamer, serving as a control and providing a measure for nonspecific-binding. Typically, 1 µL of sample is injected into the instrument and measured for 30 seconds, the channel then evacuated, rinsed if needed and the next sample introduced. The entire assay is run in triplicate, over a large ligand concentration range, allowing a saturation isotherm to be constructed and providing the most conservative outcome with respect to system drift. We calculate the difference between the binding sample and the blank and plot these values versus concentration. The data are fit to a single site saturation isotherm model using GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA). Using the coefficients of this nonlinear regression fit, K_d and B_{max} are calculated.

Results and Discussion:

Six aptamers were provided in a blinded fashion by Base Pair Biotechnologies to the Bornhop laboratory for binding affinity measurements. The binding affinity of various aptamers to their respective target was measured by BSI with no *a priori* knowledge of the binding system and only an estimate of the possible K_d. Three of the aptamers were studied previously by other methods (Table 1), tenofovir, epirubicin and bisphenol A (BPA); the other three have remained uncharacterized until this report. With a motivation to provide *true* K_d values [58, 59] we used concentrations of the aptamer at or significantly below the K_d value.

Figure 3A illustrates the saturation isotherm plot for the aptamer TFV_G2:125:256 binding to the antiretroviral drug (M.W. = 287 g/mol) tenofovir. The experiment yielded an R^2 value of 0.9885 for triplicate binding assay determinations. Our SM control experiment consisted of quantifying the TFV_G2:125:256 aptamer binding to 40 nM of ampicillin. At this concentration, the highest used for tenofovir, the aptamer showed a relatively modest binding signal suggesting decent specificity versus a commonly used SM drug. Table 1 illustrates the binding affinity of 9.0 ± 1.4 nM for tenofovir determined by BSI (a K_d of approximately 1 nM was determined by MST). While the affinity determinations were not designed to measure the limit of detection, we estimate these values to be 2.5/6.2nM for tenofovir. The limit of detection (LOD), is defined as 3 times the standard deviation of each individual phase reading, after signal settling, over 20 seconds, divided by the slope of the linear region of the saturation isotherm. In our case we calculate the limit of quantification (LOQ), as 3 times the pooled (average) standard deviation for replicate measurements divided by the slope. In our serum biomarker work we have found that the LOQ for an assay is typically 10 to 20-fold better than the K_d, thus a quantitative assay with an LOQ of 300 pM is well within reach for BSI using this aptamer – SM interaction.

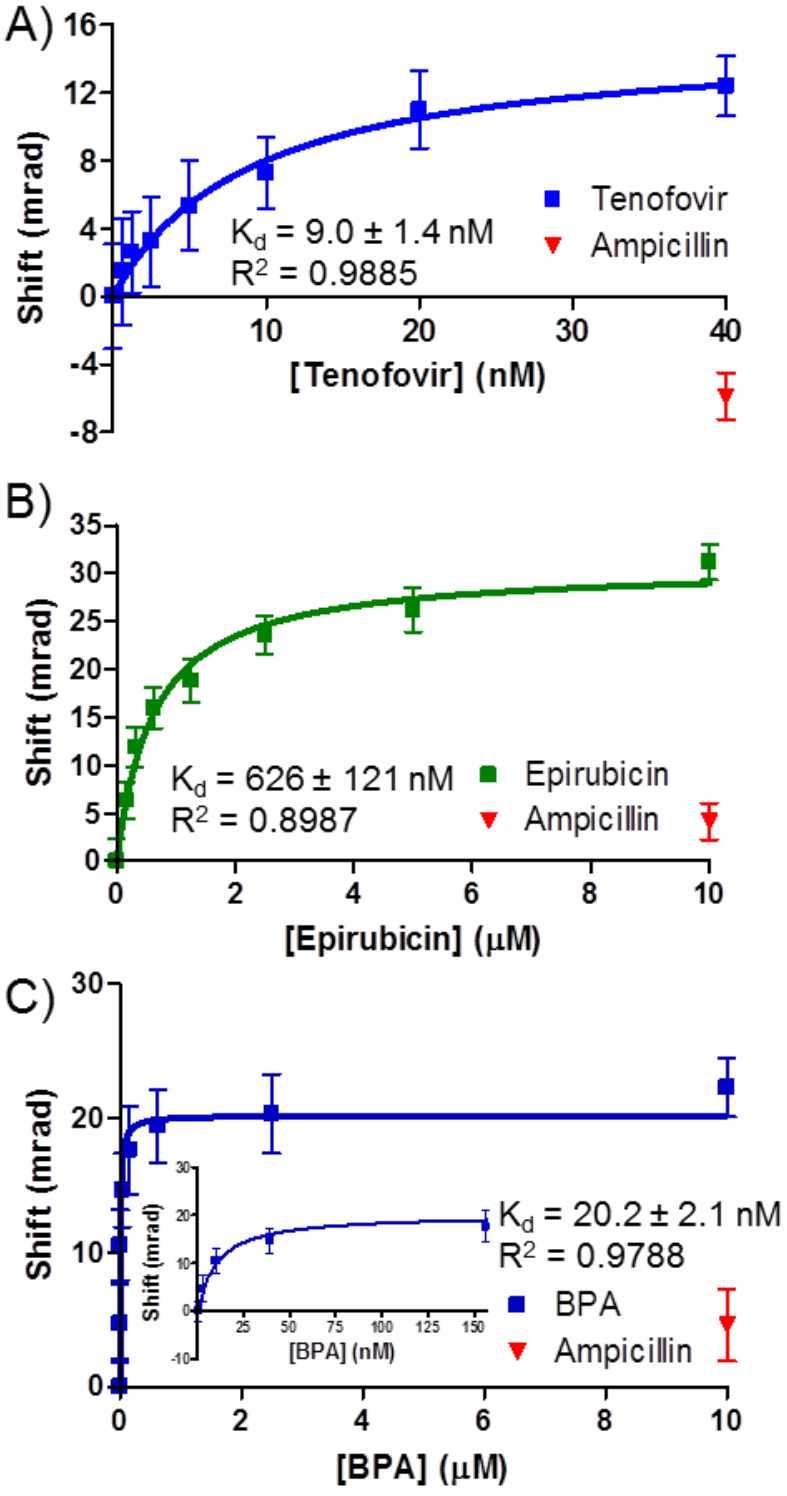

Figure 3: BSI K_d determinations for the three aptamer targets with a previously reported K_d. A) Tenofovir binding to TFV_G2:125:256, B) Epirubicin binding to Epirubicin286 aptamer and C) BPA binding to BPA aptamer.

The BSI determination of the K_d for Epirubicin286, the aptamer for the anthracycline drug used for chemotherapy epirubicin, is shown in **Figure 3B**. Our rapid, label-free and free-solution evaluation gave a binding affinity of 626 ± 121nM. Again our results compare well with the MST-measured value of 711+/- 30 nM for the binding of this 285 g/mole chemotherapy drug to the aptamer. In this case the control ampicillin had no quantifiable binding to the aptamer giving a BSI signal below the S/N threshold of 5 milli-radians (mrad) fringe shift. While the specificity of this aptamer, at least with respect to another common heterocyclic SM is quite high, the affinity would likely not be sufficient for a number of analytical applications. Nevertheless, the interaction produces a robust signal indicating that binding produces a significant change in conformation and hydration, suggesting that further refinement of the basic structure could produce an aptamer with higher affinity and robust S/N. These results are encouraging because label-free SM quantification has been challenging, yet ongoing studies are needed to determine if this level of performance will enable the construction of an assay for drug dosing and efficacy monitoring in cancer patients.

An inexpensive and robust assay for Bisphenol-A (BPA, M.W. = 228 g/mol), a once-common food-grade plastic resin now considered harmful to human health, is quite desirable. Current assays for BPA levels in human blood utilizing LC-tandem mass spectrometry have detection limits ranging from 0.43 nM [60] to 64 nM [61]. In an attempt to simplify the determination for BPA, aptamers with K_d values in the nanomolar range, directed against BPA have been recently described by Jo *et al*. [55]. These aptamers were also found to be highly specific for BPA, and did not bind to structurally related compounds Bisphenol B or 4,4'-Bisphenol [55]. Here we measured a binding affinity for the same clone, the "12-5" described by Jo *et al.*, allowing further benchmarking of our methodology. **Figure 3C** presents the BPA-

aptamer saturation isotherm produced in our BSI experiments, illustrating that we measured a binding affinity of 20.2 ± 2.1 nM. This value compares well to the10nM K_d reported (no technical replicates) by Jo *et al.* using equilibrium filter binding. In this case, 10 μM of the control molecule ampicillin showed no appreciable binding to the aptamer. As noted previously, affinity determinations are not designed to measure LOD/LOQ. Given our limited data, we estimate the LOD and LOQ to be 2.2/7.3 nM BPA. In our serum biomarker work we have found that the LOQ for an assay is typically 10 to 20-fold better than the K_D. Given our recent experience with serum and tissue [62]. the magnitude of the signal for this assay at low concentrations and that the LOQ for an assay is typically 10 to 20-fold better than the K_d, we are confident that a label-free BSI assay would be quantitative for BPA at the 100's of pM level.

As **Figure 3** and **Table 1** illustrates, collectively there is an excellent agreement between the two solution phase measurements, BSI and MST over a relatively wide range of K_d values.

The three other SM targets studied here represent species that are relatively difficult to measure, most typically by ELISA requiring multiple binding and wash steps [63-65]. In this case, the *mix-and-read* operation by BSI is particularly advantageous because it allowed rapid K_d range finding, on constrained sample quantities (microliter volumes), with no *a priori* information about the species.

Ampicillin is a widely used beta-lactam antibiotic (M.W. = 349 g/mol) and represents a potential contributor to over-use antibiotic resistance. Here our saturation isotherm (**Fig 4A**) yielded a K_d of 402 ± 99 nM for the aptamer "ampicillin284". For many applications, ampicillin is detected in the environment and biological samples by ELISA, capitalizing on enzymatic signal amplification, but needing a cold-train to insure protein functionality. While the typical detection limit using commercial ELISA kits is approximately 300 pM, no simple assay-analyzer

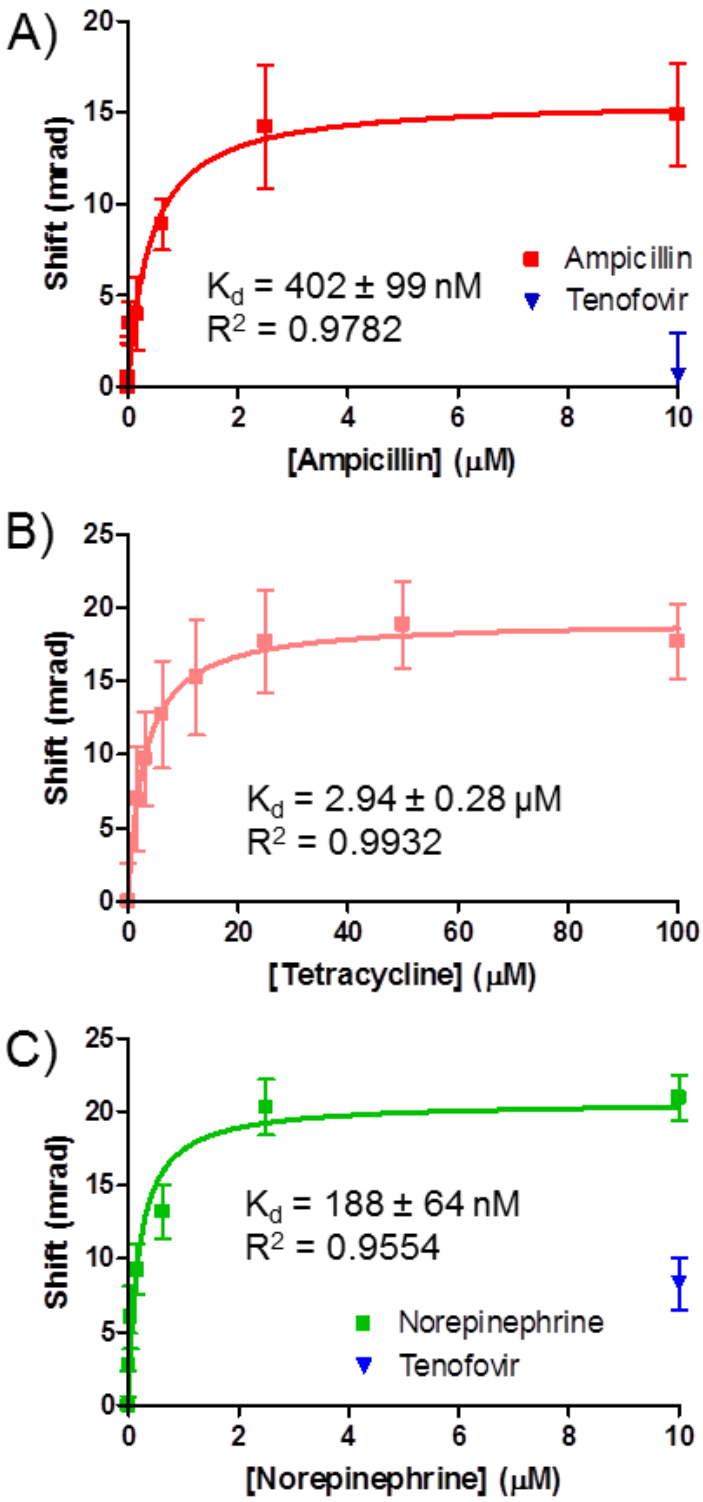

Figure 3: BSI K_d determinations for three aptamer targets with no previously reported values. A) Ampicillin binding to Ampicillin284, B) Tetracycline binds to Tetracyc_11C02, and C) Norepinephrine binds to Norepi_3932c_Prim.

combination exists that is capable of identifying a wide range of drugs at both low (e.g. <0.1 μg/ml) and high concentrations (>100 μg/ml) as desired by the research community. Since these assays were designed to measure affinity, the aptamer concentration was considerably lower than the K_d. For a detection limit/quantitation assay [62], we would employ an aptamer concentration in excess to allow the dynamic range and the lower limit of detection to be extended. Future investigations will be directed toward target quantification, but in this case we predict that an optimized BSI assay based on this target-probe interaction would have sensitivity in the 100s of pM range in complex milieu.

Tetracycline is a broad-spectrum polyketideantibiotic (M.W. = 444 g/mol) that continues to be a challenge to quantify [63] and given the rise in antibiotic resistance,[66] the potential to quantify this important drug with a simple method, in food or in the near-patient setting could be of great value. In an initial set of experiments we evaluated the affinity of our aptamer "tetracyc_11C02" with BSI. In free-solution, the saturation isotherm for tetracycline binding to this early generation aptamer yielded a K_d of 2.94 ± 0.28 μM. Admittedly, improvement on a K_d in the micromolar range to approximately1.4 μM would be required to facilitate a widely deployable assay for the food and serum. Yet, even at this level, our BSI would be competitive with the microbiological assay in milk and could be used to detect the drug in waste water.

Detection of norepinephrine is important because high plasma levels may be associated with reduced survival rates of otherwise healthy older individuals[67] as well patients with congestive heart failure [68]. Because norepinephrine is also secreted in urine, it may serve as a convenient biomarker as well for monitoring general neurotransmitter disruption and/or for monitoring treatments [69]. **Figure 4C** presents the binding assay for our norepinephrine aptamer "Norepi_3932c_Prim". Our aptamer for this SM hormone and neurotransmitter target (M.W. =

Table 1: Binding affinity of six aptamers to their respective SM targets.

Aptamer ID	Target	[Aptamer]	BSI K_d	Literature K_d	R^2
TFV_G2:125:256	Tenofovir	1 nM	9.0 ± 1.4 nM	10 nM	0.989
Epirubicin286	Epirubicin	50 nM	626 ±121 nM	711 nM	0.983
BPA Published	Bisphenol A	1 nM	20.2 ± 2.1 nM	10nM	0.979
Ampicillin284	Ampicillin	50 nM	402 ± 99 nM	569.8 nM*	0.978
Tetracyc_11C02	Tetracycline	50 nM	2.94 ± 0.28 μM	N/A	0.993
Norepi_3932c_Prim	Norepinephrine	50 nM	188 ±64 nM	N/A	0.955

*The value for the Ampicillin Literature K_d comes from an MST measurement.

169 g/mol), gave a binding affinity of 188 ± 64 nM. Given our recent biomarker results [62], at this affinity a BSI assay could be used for the detection of norepinephrine with high confidence and minimal optimizationin the range of 0.5-10 nM (0.85-1.7 ng/mL) in serum. By comparison, a commercially available ELISA for norepinephrine (Abnova, Taipei, Taiwan) reports a lower calibration range of 20 ng/ml in urine and 318 pg/ml in plasma (LOD = 118 nM in urine; 1.8 nM in plasma). Alternatively, a LC-mass-spec based approach achieved a LOD of 1.23 ng/ml in urine [70]. Thus, using BSI in conjunction with aptamers to small molecules has the potential to rapidly assay for these analytes without the multiple wash and enzymatic amplification steps of ELISA nor the complicated instrumentation required for LC-MS approaches. Mix-and-read, label-free operation also facilitates accelerated assay validation, important when attempting to translate a procedure to the clinical setting.

Conclusion:

It was shown here that BSI can be used to effectively measure the interaction between an array of small molecules and aptamers selected to bind to these targets. The results were quantitative, giving K_d values for the molecular interactions in a label-free, free-solution format. With the mix-and-read BSI assay, no immobilization of aptamers or small molecules is required, nor is any chemical modification such as labeling needed for either of the interaction species. Binding determinations took hours to complete, with no prior knowledge of the binding system. Where a K_d was previously known, K_d values determined by BSI agreed well with the literature. BSI also worked well for binding systems with unknown K_d values, and where we were unable to successfully obtain reliable values with other methods. These results were easily produced and gave reproducible K_d values within the expected range of affinity. Given our results, we predict that going forward BSI will enable the rapid characterization of high affinity aptamers, particularly those with pM to nM affinity values, helping to expedite their use in the main-stream.

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