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Universal Sensing by Transduction of Antibody Binding using Backscattering Interferometry

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Antibodies are the most widely used agents for biomolecular recognition because of their combination of high affinity, high specificity, broad range of compatible analytes, and commercial availability. For analytical purposes, the antibody binding event must be converted to a detectable signal, for which several methods are commonly employed. ELISA-type techniques rely on the binding event to immobilize either the antibody or its antigen, allowing for a colorimetric, fluorescent, or radioactive signal to be generated by direct attachment to the antibody or by a secondary antibody-antibody or antibody-antigen interaction.[1] Despite their convenience and utility, ELISAs do not always accurately reflect a true affinity measurement because excess binding and non-binding species must be removed in washing steps, and because the binding events take place on a surface rather than in solution.[2] The former limitation has the effect of biasing existing antibody-antigen interactions towards higher observed apparent affinities, or completely removing lower-affinity interactions from the system of interest. The latter can affect binding by limiting the conformational flexibility of one or more of the binding partners, blocking or otherwise changing the accessibility of binding sites, and increasing the occurrence of non-specific interactions that get folded into the overall signal. Though ameliorated through the use of blocking reagents and other procedures, such factors add to the time and effort required to

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obtain quantitative binding information.[3] ELISA methods can also require substantial amounts of expensive reagents, particularly labeled primary or secondary antibodies. Perhaps as a result of these factors, specific affinity (K_d) values are rarely provided for commercial antibodies, even though antibody-antigen affinities can vary widely.[4]

While less convenient and more expensive, label-free techniques such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) have also been used to offset some of the limitations of ELISA.[2a, 5] However, such measurements often have a limited range of affinities over which they are applicable. SPR, being a surface-bound mass-sensitive technique, is also impractical for the use of immobilized antibodies to detect small analytes.

To solve these problems we turned to measurement of refractive index (RI). It was previously established that molecular interactions can be detected by monitoring RI changes at a surface, and that the signal can be sensitive to changes in conformation and hydration.[6] We have recently demonstrated backscattering interferometry (BSI) to be remarkably sensitive to the binding of analytes by dissolved or or adsorbed molecular agents,[7] causing changes in molecular structure, dipole moment, and polarizability, all of which can affect RI.[8] We describe here the mating of this technique with the broad and specific molecular recognition properties of antibodies, to give a general method for quantifying the presence of almost any type of molecule.

The BSI measurement is made in a microfluidic chip engineered to provide sufficiently long effective path length in a small volume. Incident coherent light is allowed to interfere with scattered light to produce fringes that can be captured on a standard CCD camera (Figure 1). Fourier analysis of these interferometric fringes can be correlated in real time to changes in refractive index that result from receptor-ligand interactions occurring in solution.[7a] No labels are required, and all binding partners can be observed in physiologically-relevant fluids.

Five small molecules were chosen for antibody detection (Figure 2), including three amines of biological or metabolic relevance (serotonin, histamine, and dopamine), one modified amino acid (3-nitrotyrosine), and one molecule of interest in explosives detection (2,4,6-trinitrophenol, TNP). In addition, we also tested antibody binding to human holo-transferrin, a protein of interest in metabolic disease and cancer cell targeting. Each of these molecules is recognized by a commercially-available (Abcam and BD Biosciences) antibody, either monoclonal (the first four compounds) or polyclonal (trinitrophenol and transferrin). In each case, a compound of similar structure that does not bind to the antibody, or binds with much lower affinity, was chosen as a negative control. In two cases, this involved the addition of a carboxylic acid group (tryptophan *vs.* serotonin, and histidine *vs.* histamine). More subtle changes distinguish 3-methoxytyramine from dopamine (methylation of one of the two phenolic oxygens) and tyrosine from 3-nitrotyrosine. Lastly, phenol was used as the negative control for TNP and the denatured protein served as the control for functional transferrin.

Individual samples of each antibody were incubated at room temperature separately with a range of concentrations of antigen; in each case identical samples were prepared with the negative control in place of the analyte. BSI measurements were performed on these samples after equilibration and without removal of excess binding ligands. In each case, a dose-dependent change in refractive index, manifested as a shift in phase of the interference fringe pattern, was observed for the positive analyte relative to the control. Plots of these values against the concentration of analyte gave sigmoidal curves that fit well to a simple single-site binding model resulting in an equilibrium binding constant (Figure 3), expressed as an adsorption isotherm.[9] Little or no BSI signal was observed for even the highest concentrations of the negative control compounds, showing that the BSI signal reports on specific antibody-antigen interactions.

The binding constants derived from these data are listed in Table 1. Precise values are not reported or provided by the suppliers, so detailed comparisons to literature values cannot be made. In general, the values obtained by BSI are in the low nanomolar range expected for optimized antibodies, with the exception of the dopamine case, which is reported by the manufacturer to be a relatively weak binder (approx. 100 μM). Particularly interesting are the polyclonal cases of trinitrophenol and transferrin, for which K_D values similar to those of the monoclonal examples were observed. This suggests that BSI reports on the affinities of the most tightly-binding members of the polyclonal library.

It has been suggested that the production of highly-specific antibodies to such species as trinitrophenol (picric acid), in combination with a sensitive biosensing apparatus such as SPR, could be used as a detection platform for explosives.[10] However, SPR detection requires modification of the species to be detected in order to increase its mass and obtain sufficient signal.[11] Optical methods, relying on infared or Raman spectroscopy, are useful for detection and classification of explosive substances, but require microgram quantities of samples. Elegant systems employing signal- amplifying fluorescence polymers and other materials provide extremely high sensitivity, but are specific to one molecule or class of molecules.[12] As a potential alternative, we show here that an easily-generated polyclonal anti-TNP antibody allows BSI to selectively detect TNP in a straightforward, “plug-and-play” fashion, without the use of enhancing molecules of any kind. Using this unoptimized setup, the pure compound was easily detected at a concentration of 200 parts per trillion, similar to SPR, AFM, and other techniques.[13]

To test the tolerance of the BSI method to uncontrolled contaminants, we spiked two soil samples from public areas near our laboratory (garden soil and beach sand) with 100 nM (23 ng/mL) TNP by mixing 1 gram of soil with 2 mL of an aqueous solution of TNP. Samples were dried overnight under vacuum and reconstituted the next day in 2 mL of 1× PBS buffer, pH 7.4. Each soil sample was then resuspended, filtered once through a 100 nm pore-diameter filter, and the resulting solution analyzed by BSI using a ~12 nM solution of the anti-TNP antibody. Each TNP-spiked soil sample was compared to an untreated soil sample processed in the same way (Figure 4). While the baseline BSI signal in each case was different, the comparative analyses (signal from analyte-containing sample minus signal from analyte-free sample) revealed the presence of TNP in the soil extracts in statistically significant fashion without chromatographic purification or enrichment.

Backscattering interferometry has been shown here to provide direct, convenient, and quantitative transduction of antibody binding into an optical signal, for both small- and large-molecule analytes. Since BSI is a highly sensitive refractive index detector, and since RI varies greatly with temperature, one might suspect that BSI reports on the change in enthalpy of a binding interaction. However, in such a case, the BSI signal would be transient, since the temperature of the mixture would return to the starting value shortly after the binding event takes place on the temperature-controlled sample stage. Instead, the signals persist indefinitely; the measurements here were taken as long as 8 hours after mixing.

BSI instrumentation and sample handling are simple, and very little sample is required, with approximately one microgram of each antibody used to generate complete binding curves. No blocking agents are needed, and excess analytes do not have to be separated from the reporter (antibody). Since the BSI measurements are done in solution, we believe that they provide information more representative of true antibody-antigen affinities than surface-based techniques. The washing steps common to surface measurements are not used, allowing for less perturbation of weaker interactions, and avoiding the displacement of equilibria that sometimes occurs in ELISA, equilibrium dialysis, filtration, or other assays. Its compatibility with both monoclonal and polyclonal antibodies allows BSI to be used for the detection of a limitless variety of molecules, taking advantage of the abilities of the immune system or *in vitro* evolution techniques to generate potent and selective binding agents at will.

Acknowledgments

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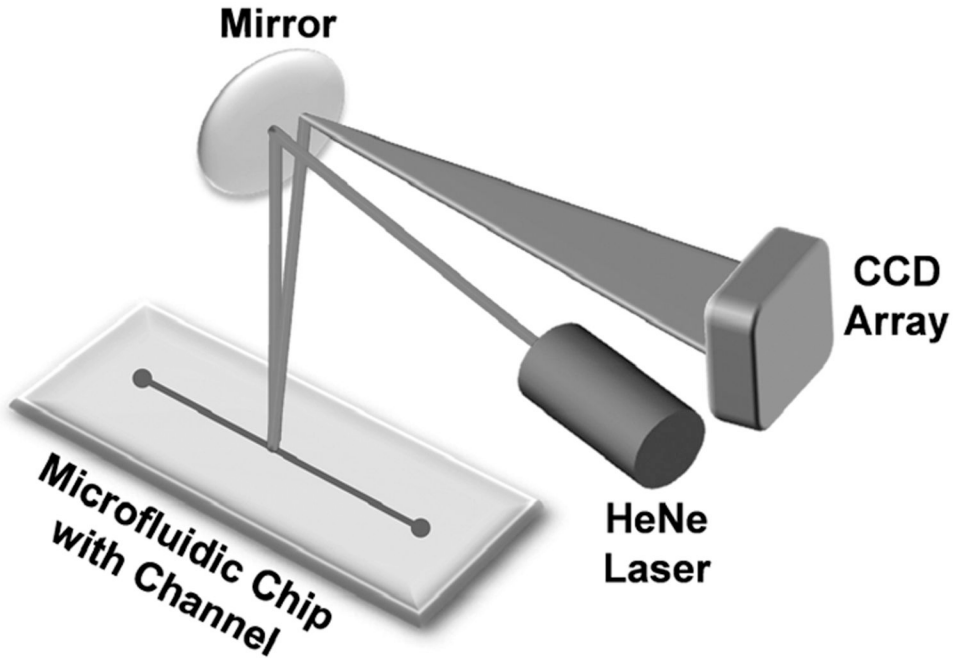

Figure 1.

Schematic representation of the BSI apparatus. Pre-equilibrated solutions of antibodies and ligands in buffer are pipetted into the channels of the microfluidic chip. Channels are interrogated with a 633 nm HeNe laser, and the resulting interference fringes are captured using a CCD camera.

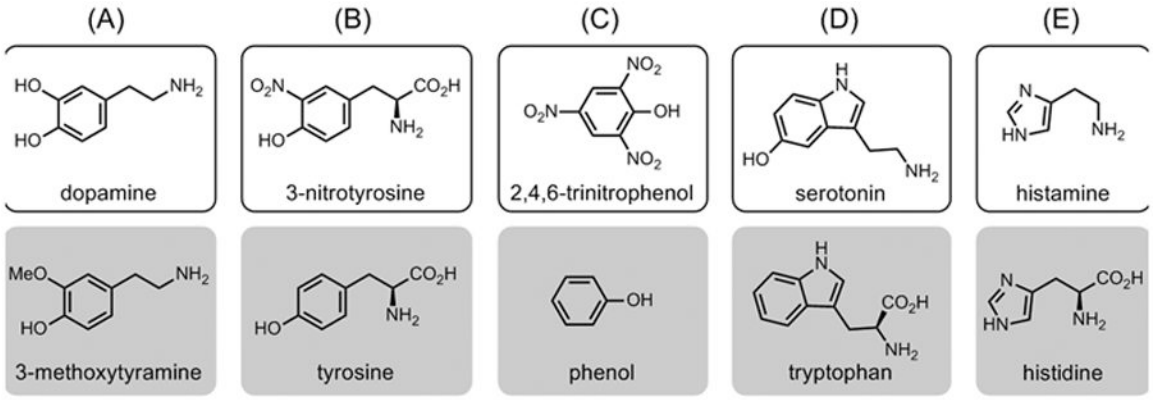

Figure 2.

Analyte (top row) and negative control (bottom row) compounds used for BSI testing. (A) – (E) refer to the data shown in Figure 3.

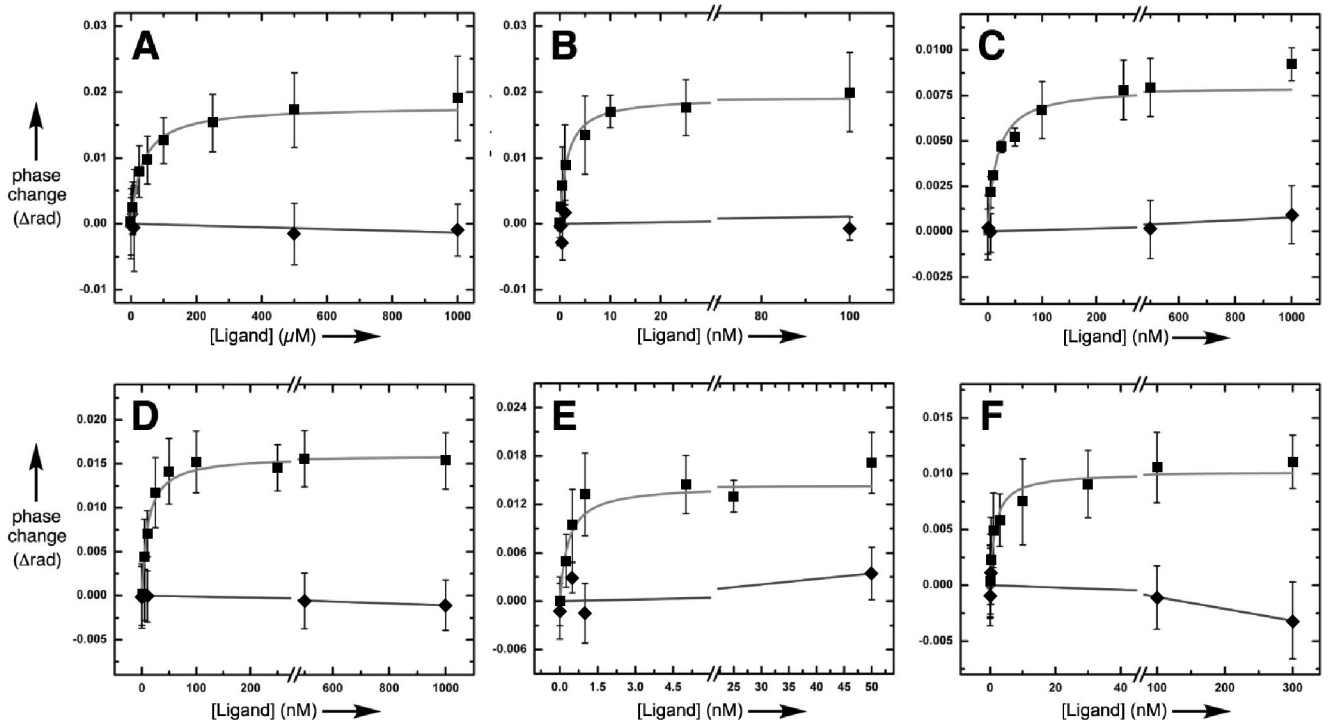

Figure 3.

Representative plots of BSI signal *vs.* ligand concentration for the determination of binding constants for the following pairs of molecules (antibody + small molecule). (squares = “ligand”; diamonds = “control” compound) (A) ligand = dopamine, control = 3-methoxytyramine; (B) ligand = nitrotyrosine, control = tyrosine (C) ligand = trinitrophenol, control = phenol; (D) ligand = serotonin, control = l-tryptophan; (E) ligand = histamine, control = l-histidine; (F) ligand = holo transferrin, control = fully denatured holo transferrin. Each data point represents the average of at least four independent measurements; error bars are plus and minus the full value of standard error in each direction. Repeat determinations of the binding curves gave very similar K_{ads} values.

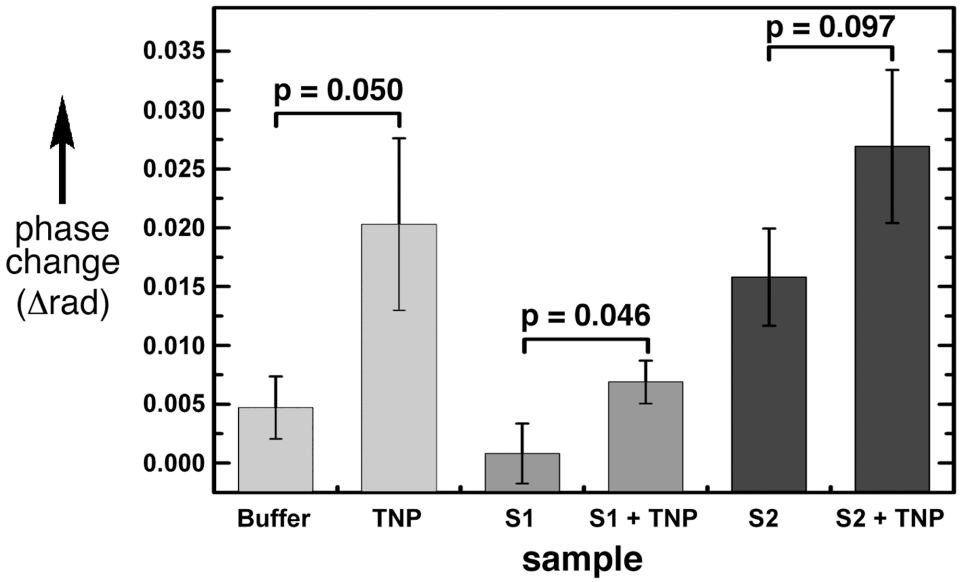

Figure 4.

BSI analysis for TNP in spiked soil samples: positive control = buffer solution of TNP, S1 = garden soil, S2 = beach sand. Each value is the average of three measurements with error bars representing standard deviation.

Table 1

Binding constants determined by BSI from data in Figure 3.

Analyte	Control	$1/K_{ads}$ (BSI)[a]	K_d (Literature)
dopamine	3-methoxy-tyramine	39 ± 6 μM	~100 μM[b]
3-nitrotyrosine	tyrosine	1.3 ± 0.2 nM	---
trinitrophenol	phenol	22 ± 4 nM	~14 nM[c]
serotonin	tryptophan	11 ± 2 nM	---
histamine	histidine	0.3 ± 0.1 nM	~1 nM[b]
holo-transferin	denatured transferrin	1.6 ± 0.4 nM	---

[a] Error limits are derived from the statistical error of curve fitting the curves shown in Figure 3.

[b] Provided on the manufacturer (Abcam) web site.

[c] IC_{50} as determined by SPR in reference [13b] for an antibody from a different commercial source.