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Backscattering Interferometry: An Alternative Approach for the Study of Hydrogen Bonding Interactions in Organic Solvents

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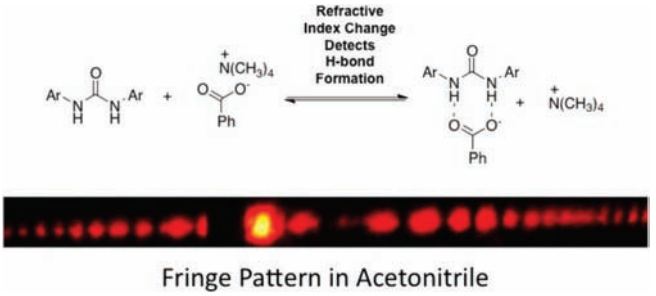
ABSTRACT



Intermolecular interactions involving hydrogen bonds are responsible for catalysis and recognition. Traditional methods used to study hydrogen-bonding interactions are generally limited to relatively large volumes and high substrate concentrations. Backscattering Interferometry (BSI) provides a microfluidic platform to study these interactions in nonaqueous media at micromolar to nanomolar concentrations in picoliter volumes by monitoring changes in the refractive index.

Intermolecular interactions, especially those involving hydrogen bonds, drive the structure and function of macromolecules that catalyze reactions responsible for maintaining living systems.[1] In organic chemistry, hydrogen bonding partners have been utilized as the backbone of approaches used to drive crystal formation, molecular recognition and catalysis.[2] Traditionally hydrogen bonding interactions are studied using isotope incorporation,[3] NMR,[4] UV−vis spectroscopy,[5] fluorescence,[6] and calorimetry.[7] While these tools are widely used to characterize molecular interactions that arise through the formation of hydrogen bonds, they often require high substrate concentrations and volumes. Given the synthetic effort required to produce the components for the study of systems that utilize hydrogen bonds for recognition and catalysis, it is desirable to be able to study their interactions using small quantities of substrates. Recently backscattering interferometry (BSI) has been shown to effectively determine the binding affinity of several biomolecular

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(1) Taylor, M. S.; Jacobsen, E. N. *Angew. Chem., Int. Ed.* **2006**, *45*, 1520–1543.
(2) (a) Hunter, C. A. *Angew. Chem., Int. Ed.* **2004**, *43*, 5310–5324. (b) Kim, S.-G.; Kim, K.-H.; Kim, Y. K.; Shin, S. K.; Ahn, K. H. *J. Am. Chem. Soc.* **2003**, *125*, 13819–13824.
(3) (a) Brown, A. R.; Kuo, W. H.; Jacobsen, E. N. *J. Am. Chem. Soc.* **2010**, *132*, 9286–9288. (b) Zotova, N.; Broadbelt, L. J.; Armstrong, A.; Blackmond, D. G. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3934–3937.
(4) Tarkanyi, G.; Kiraly, P.; Varga, S.; Vakulya, B.; Soos, T. *Chem.—Eur. J.* **2008**, *14*, 6078–6086.
(5) Noziere, B.; Cordova, A. *J. Phys. Chem. A* **2008**, *112*, 2827–2837.
(6) Hoang, L.; Bahmanyar, S.; Houk, K. N.; List, B. *J. Am. Chem. Soc.* **2003**, *125*, 16–17.
(7) (a) Jadhav, V. D.; Schmidtchen, F. P. *J. Org. Chem.* **2008**, *73*, 1077–1087. (b) Solomonov, B. N.; Novikov, V. B. *J. Phys. Org. Chem.* **2007**, *21*, 2–13.
(8) (a) Bornhop, D. J.; Latham, J. C.; Kussrow, A.; Markov, D. A.; Jones, R. D.; Sorensen, H. S. *Science* **2007**, *317*, 1732–1736. (b) Latham, J. C.; Stein, R. A.; Bornhop, D. J.; McHaourab, H. S. *Anal. Chem.* **2009**, *81*, 1865–1871. (c) Kussrow, A.; Kaltgrad, E.; Wolfenden, M. L.; Cloninger, M. J.; Finn, M. G.; Bornhop, D. J. *Anal. Chem.* **2009**, *81*, 4889–4897.

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interactions ranging from interactions with proteins[8] to nucleic acid hybridization.[9] BSI measures the change in refractive index of an aqueous solution resulting from intermolecular association of two components. The key features of BSI that make it appealing for the study of intermolecular interactions are the small sample sizes (pL to μL range) and concentrations (nM to μM) and the ability to carry out experiments without functionalizing or surface immobilizing one of the binding partners.[8a] To date, BSI has been employed in aqueous systems where one or more of the components are macromolecules. Given the importance of hydrogen bonding in nonaqueous media, we posed the following questions: (1) Can BSI be used to study hydrogen bonding in nonaqueous media? (2) What are the limits of detection in a nonaqueous environment when the binding partners are small molecules? (3) Can BSI be used to distinguish between similar hydrogen bonding partners? Herein we show that BSI can be used to study the interaction of diphenyl ureas and thioureas with benzoate in acetonitrile (MeCN) with quantities several orders of magnitude lower than other commonly utilized techniques.

BSI utilizes a low power He−Ne laser focused perpendicularly onto a microfluidic channel in a glass chip to generate a backscattered interference fringe pattern.[8a] The introduction of two binding partners into the channel creates a change in refractive index, causing a spatial shift in the fringe pattern. The magnitude of this shift depends on the precise fringes interrogated, the concentration of the binding pairs, conformational changes initiated upon binding, changes in water of hydration, and binding affinity.[8] To date, BSI has only been used to study interactions in aqueous solvents. To determine if this technique can be extended to studies in organic solvents, we examined the complexation of tetramethylammonium benzoate (TMAB) with 1,3-diphenyl urea (DPU), 1,3-diphenylthiourea (DPTU), 1,3-bis(*p*-nitrophenyl)urea (DNPU), and 1,3-bis-(*p*-nitrophenyl)thiourea (DNPTU) in MeCN. Additionally, the limits of BSI detection was studied by carrying out experiments with DPU interacting with either TMAB or tetramethylammonium *p*-toluene sulfonate (TMAS).

Urea and thiourea have been widely studied in molecular recognition because of their ability to form strong hydrogen bonds.[10] Hydrogen bonding through urea and thiourea derivatives are used to recognize carboxylic acids, sulfonic acids, and nitrates.[11] Ureas and thioureas also act as acid catalysts in a variety of organic reactions including the Diels−Alder reaction and Claisen rearrangement.[12] In addition, these catalysts are very useful in diastereo- and enantioselective reactions.[3a] As a result, the strength of the interaction between substrate and catalyst is important information for a synthetic chemist hoping to utilize such reactions. The results discussed below exemplify how BSI can be used to determine the affinity of commonly employed urea and thiourea derivatives in a miniaturized format. Throughout the course of this study, we found BSI to be a very interaction-efficient method for the study of small molecule interactions.

Initially end-point BSI experiments of TMAB interaction with DPU and DPTU in MeCN were performed. Since the BSI signal is dependent on the strength of the interaction, experiments were carried out in the low to mid micromolar range. For these experiments, DPU and DPTU were held constant at 10 μM while TMAB was varied from 5 to 60 μM. The BSI experiments were carried out in a steady-state manner, in which samples were mixed and allowed to equilibrate for several hours before examining at 25 °C. Prior to the experiment, the laser and temperature controller were allowed to equilibrate for an hour and the instrument was aligned with respect to the microfluidic channel and the detector to obtain a single frequency Fourier transform. The samples were analyzed by pipetting 1 μL of each concentration directly into the channel well and recording the signal for 45 s. The zero point of DPU/DPTU only and the TMAB-only calibration curve were subtracted from the binding data to obtain the final binding curve (Supporting Information Figure S1). Figure 1 shows the representative BSI plot and curve-fit of DPU complexation with TMAB. The BSI signal levels out at the high concentration of TMAB, showing a saturation binding curve that can be fit to a one-site binding hyperbola to obtain K_D values. Both DPU and DPTU (Supporting Information Figure S2) have similar affinity for TMAB with K_D values of 18.56 and 23.20 μM, respectively (Table 1). Next, experiments were carried out on DNPU and DNPTU as these substrates are

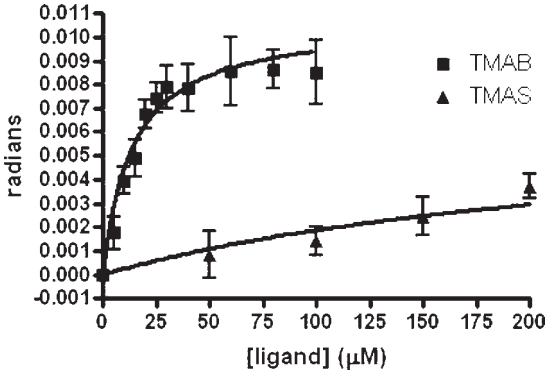

Figure 1. Steady-state BSI data of DPU complexed to TMAB (■) and TMAS (▲). The large signal shift for TMAB compared to TMAS shows that BSI detects concentration-dependent binding.

(9) Pesciotta, E. N.; Bornhop, D. J.; Flowers, R. A. *Chem.−Asian J.* **2011**, *6*, 70–73.

(10) (a) Kelly, T. R.; Kim, M. K. *J. Am. Chem. Soc.* **1994**, *116*, 7072–7080. (b) Schmidtchen, F. P.; Berger, M. *Chem. Rev.* **1997**, *97*, 1609–1646.

(11) (a) Takemoto, Y. *Org. Biomol. Chem.* **2005**, *3*, 4299–4306. (b) Werner, F.; Schneider, H. *Helv. Chim. Acta* **2000**, *83*, 465–478. (c) Smith, P. J.; Reddington, M. V.; Wilcox, C. S. *Tetrahedron Lett.* **1992**, *33*, 6085–6088.

(12) (a) Kelly, T. R.; Meghani, P.; Ekkundim, V. S. *Tetrahedron Lett.* **1990**, *31*, 3381–3384. (b) Etter, M. C. *Acc. Chem. Res.* **1990**, *23*, 120–126. (c) Etter, M. C.; Urbancyzk-Lipowska, Z.; Zia-Ebrahimi, M.; Panunto, T. W. *J. Am. Chem. Soc.* **1990**, *112*, 8415–8426.

expected to have a higher affinity for benzoate.[12c,13] Examination of a higher affinity interaction is important to test the limits of BSI an analytical tool for small molecule interactions and determine the degree in which partners with different affinities can be distinguished from each other. BSI experiments were carried out at lower concentrations than the previous system with DNPU/DNPTU held constant at 1 μM and TMAB varied from 0.5 to 10 μM. Again, final binding curves were obtained after subtracting out the TMAB calibration data. The binding affinity for both DNPU and DNPTU were similar (Table 1) with K_D values of 0.54 and 0.42 μM, respectively. As expected, the data obtained shows the enhanced binding to TMAB due to the presence of the *p*-NO_2 groups. The data in Table 1 and subsequent binding curves (Supporting Information Figures S3 and S4) show similar radian shifts and binding saturation with R^2 values of 0.97 or higher.

Table 1. K_D Values and Standard Error from BSI and ITC[a]

	BSI	ITC
TMAB complexation	K_D (μM)	K_D (μM)
DPU	18.56 ± 4.46	21.75 ± 6.04
DPTU	23.20 ± 4.55	27.43 ± 3.72
DNPU	0.54 ± 0.08	1.26 ± 0.06
DNPTU	0.42 ± 0.08	1.18 ± 0.17
TMAS-DPU	146 ± 40	356 ± 27

[a] Thermodynamic data is available in the Supporting Information.

BSI detects specific binding interactions in a concentration dependent manner. To illustrate this point, experimental results were compared for DPU-TMAB and DPU-TMAS interactions. TMAS is not expected to have a strong affinity for DPU because the sulfonate oxygens are much less electronegative in comparison to the benzoate oxygens, as demonstrated by the lower pK_b for benzoate (10) vs benzenesulfonate (20).[10a] Given the low affinity for DPU-TMAS, performing a BSI experiment under the same conditions as the DPU-TMAB experiment should produce very different results. As expected, Figure 1 shows the negligible signal shift due to DPU-TMAS complexation whereas DPU-TMAB generates a large signal shift that saturates at much lower concentrations. However, when the DPU-TMAS BSI experiment was run at higher concentrations with 50 μM DPU and 0–600 μM TMAS, a saturation binding curve was produced (Supporting Information Figure S5) and fit to obtain a K_D value of 0.146 ± 0.04 mM. This value agrees with previously reported K_D values between sulfonate and (thio)urea derivatives ranging from 0.006 to 22.2 mM, with the lower values attributed to the precense of electron withdrawing groups on the (thio)urea derivatives.[10a,13]

To benchmark the BSI studies, isothermal titration calorimetry (ITC) experiments were performed. Initial experiments were carried out with 5 mM TMAB titrated into the sample cell containing 0.5 mM DPU or DPTU in MeCN at 25 °C. As with the BSI studies, the DNPU/DNPTU ITC experiments were run at slightly lower concentrations, with 2 mM TMAB titrated into 0.2 mM of either DNPU or DNPTU. Additionally, ITC experiments were carried out with 10 mM TMAS titrated into 1 mM DPU. The results from these experiments are shown in Table 1, with DPU having a slightly higher affinity for TMAB than DPTU, with respective dissociative binding constants of 21.75 and 27.43 μM. The opposite trend was observed for TMAB interaction with DNPU and DNPTU, with DNPTU having a slightly lower K_D of 1.18 μM and DNPU with a K_D of 1.26 μM. The BSI K_D values are all within experimental error of the ITC data and exhibit the same trends. The only exception is the interaction between DPU-TMAS, where the K_D values between BSI and ITC are slightly different. This interaction is most likely near the upper detection limit of BSI and ITC, given that smaller signal changes are observed in both methods.

The data described above clearly show that BSI is capable of measuring the affinity of small molecules in nonaqueous media. Although BSI is useful for examining a range of intermolecular interactions, the basis for the signal obtained in these experiments is not fully understood. It is our supposition, that changes in refractive index arise from the release or capture of solvent upon association. To examine this hypothesis, ellipsometry experiments were carried out for the DPU-TMAB interaction to determine the relationship between refractive index and a binding event. These experiments were run using a 60° liquid prism cell and calculating the refractive index at 633 nm, the same wavelength used in the BSI experiments. To obtain measurable changes in the refractive index, the concentrations of the binding pairs were increased, with DPU held constant at 25 μM and TMAB varied from 25 to 200 μM. The final binding data was obtained by subtracting out the zero point of 25 μM DPU from the binding data and then subtracting out the subsequent refractive index

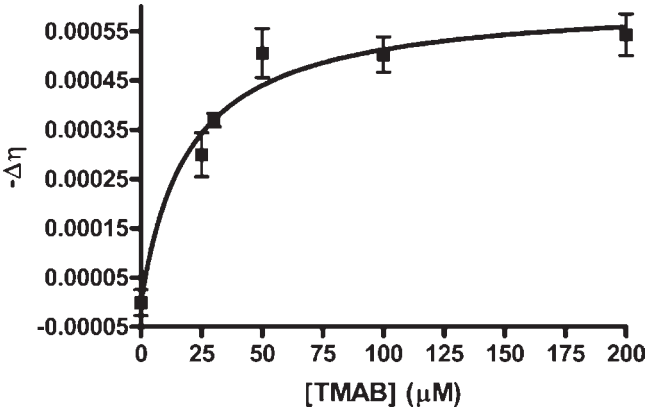

Figure 2. Plot of ellipsometry refractive index measurements at 633 nm. The relative refractive index of equilibrated samples containing 25 μM DPU and 0–200 μM TMAB show a saturation binding curve. Curve fitting to a one-site binding hyperbola yielded a K_D of 19.40 ± 6.23 μM with an R^2 of 0.97.

(13) Wilcox, C. S.; Kim, E.; Romano, D.; Kuo, L. H.; Burt, A. L.; Curran, D. P. *Tetrahedron* **1995**, *51*, 621–634.

calibration curve of the varying concentration of TMAB. Analysis of the ellipsometry data was analogous to BSI since the experiments were performed at steady-state and also measured the change in refractive index. Figure 2 shows the ellipsometry average plot with the curve fit obtaining a K_D value of $19.40 \pm 6.23\,\mu$M. The ellipsometry data correlate with the BSI K_D values, providing evidence that the BSI signal shift is related to variations in refractive index during complex formation.

In conclusion, the present study demonstrates the applicability of BSI as a tool for studying small molecule interactions in nonaqueous solvents. Not only is BSI able to measure the formation of just two hydrogen bonds, but it can also be used to distinguish between TMAB complexation with DPU/DPTU and DNPU/DNPTU with an affinity difference of more than 1 order of magnitude. BSI experiments have the advantage of using smaller volumes and lower concentrations than ITC and ellipsometry. The microfluidic channel used in these BSI experiments has a cross-sectional area of 3600 μm^2 that, when interrogated by a 100 μm diameter laser, provides an optical probe volume of ca. 360 pL. Therefore, at the lowest concentrations used in these experiments, BSI can detect an interaction between ca. 325.2 million molecules. When compared to the concentrations and volumes used for the other techniques in this study, BSI is 6 orders of magnitude more sensitive than ITC and 8 orders of magnitude more than ellipsometry. This makes BSI interaction-efficient, with the ability to detect a relatively small number of discrete interactions in comparison to other free-solution techniques. Furthermore, the straightforward, user-friendly design of BSI provides an elegant technique for screening intermolecular interactions of small molecules in organic solvents making it potentially useful for application in high-throughput screening.

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Supporting Information Available. General methods, experimental protocols, and supporting data. This material is available free of charge via the Internet at http://pubs.acs.org.