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Flow Induced Dispersion Analysis Quantifies Noncovalent Interactions in Nanoliter Samples

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Noncovalent interactions play a key role in many biochemical processes related to for example drug targets or protein−protein interactions.[1] In combinatorial chemistry and in drug development a large number of noncovalent interactions need to be addressed in a limited time frame. Furthermore, often a very small sample amount is available for study which prevents a comprehensive characterization using existing methods. Finally, it is complicated from a technological point of view to integrate standard procedures for addressing noncovalent interactions with binding target isolation and/or synthesis of chemical entities.[1,2]

In this work we present a new approach for quantitative characterization of noncovalent interactions in nanoliter samples. The procedure is based on Flow Induced Dispersion Analysis (FIDA), and the only requirement is a narrow tube, capillary, or channel equipped with a detector having a linear response. The technique may therefore be implemented using standard equipment for e.g. High Performance Liquid Chromatography (HPLC), Flow Injection Analysis (FIA), or Capillary Electrophoresis (CE) (as demonstrated in the present work). In this work UV detection was used, but other detectors based on e.g. fluorescence or electrochemistry should also be applicable.

Large molecules diffuse slowly, and small molecules diffuse comparatively faster. When a molecule interacts with a ligand its apparent diffusivity becomes that of the complex. The apparent diffusivity is thus dependent on the fractions of free and complexed analyte which in turn is given by the noncovalent analyte−ligand equilibrium binding constant. The underlying idea of the present work is that the apparent dispersion of an analyte in a flow system is characterized by the apparent diffusivity of the analyte. It is well-known that peak dispersion may be used to asses the diffusivity of small molecules, macromolecules, and particles.[3−8] However, only a few studies have reported dispersion in systems with several components.[9−12] In the present work we demonstrate that a simple series of experiments involving sample introduction, pressure driven flow, and detection in a thin coated silica capillary can be used to quantify equilibrium binding constants. The selected chemical system is composed of β-cyclodextrin (βCD) as the ligand and α-naphthol or naproxen as analytes. In general the equilibrium corresponding to the formation of a 1 to 1 complex can be written as

$$A + L = AL \quad (1)$$

where A is the analyte, L the ligand, and AL the complex.

Shown in Figure 1 are the peaks corresponding to α-naphthol in the absence and presence of βCD in the run buffer solution, respectively. The sample volume introduced into the capillary in these experiments corresponds to 9 nL. It is immediately apparent that in the presence of βCD the peaks are wider. The peak shape may be fitted to a Gaussian peak shape as described in the Supporting Information; in this way the temporal peak variance σ^2 can be estimated.

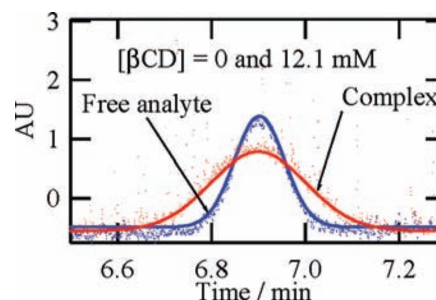

Figure 1. UV trace at 230 nm and 25 °C of α-naphthol (50 μM) in the presence and absence of βCD in a fused silica capillary (i.d.: 50 μm, total length 48.5 cm, length to detector 40.5 cm). The eluent was 67 mM sodium phosphate buffer (pH 7.4) with or without βCD.

According to Taylor−Aris theory the temporal peak variance is related to analyte diffusivity.[13,14] Under optimal conditions for a pressure driven flow in a cylindrical capillary (see Supporting Information) the peak variance is inversely proportional to the diffusion coefficient, D, according to[15]

$$D = \frac{R_c^2}{24\sigma^2} t_R \quad (2)$$

where R_c is the radius of the capillary tube, and t_R is the peak appearance time. In the presence of βCD, the observed variance can be attributed to α-naphthol and the complex between α-naphthol and βCD. We shall assume that the observed variance is a weighted average depending on the fraction of free α-naphthol, x. This assumption is valid when the complexation kinetics is fast compared to the time scale of the experiment.[16] As the complex and free analyte are both nonretained they have identical t_R values. We may thus obtain

$$\frac{t_R}{\sigma^2} = \frac{t_R}{\sigma_A^2} \cdot x + \frac{t_R}{\sigma_{AL}^2} \cdot (1 - x) \quad (3)$$

where σ_A^2 is the observed variance obtained from a Gaussian fit in the absence of an interacting ligand and σ_{AL}^2 is the variance corresponding to the complex AL. Alternatively, eq 2 may be written in terms of diffusion coefficients (Supporting Information). However, the present parameter choice is convenient as it is systems independent (i.e., precise knowledge of channel size and radius is not required) as long as the Taylor conditions are satisfied (see Supporting Information). Corrections due to sample injection and detector variances may be introduced, but as they are small and constant under the present conditions they are neglected.

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The fraction of free analyte, x, can also be expressed according to the equilibrium binding constant, K:

$$x = \frac{1}{1 + K[\mathrm{L}]} \quad (4)$$

where [L] is the concentration of free ligand. Under the present conditions βCD is in a large excess and [L] can therefore be approximated by the total ligand concentration (see Supporting Information).

A combination of eqs 3 and 4 gives the binding isotherm

$$\frac{t_R}{\sigma^2} = \frac{\frac{t_R}{\sigma_A^2} + \frac{t_R}{\sigma_{AL}^2}K[\mathrm{L}]}{1 + K[\mathrm{L}]} \quad (5)$$

Equation 5 can be extended to higher order complexes if necessary.

In a series of experiments the concentration of βCD in the background electrolyte was varied and the peak variances were determined. In Figure 2 is shown t_R/σ^2 as a function of [βCD].

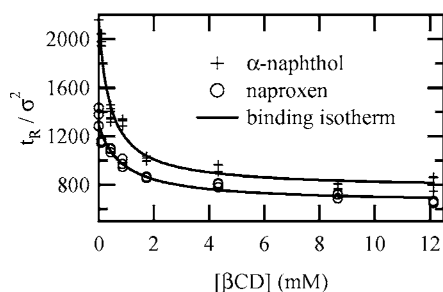

Figure 2. Data corresponding to α-naphthol (+) and naproxen (○) obtained using a fused silica capillary (i.d.: 50 μm, total length 48.5 cm, length to detector 40.5 cm) at 25 °C. The eluent was 67 mM sodium phosphate buffer pH 7.4 with different amounts of βCD.

In Figure 2 the experimental data have been fitted to the binding isotherm (K and t_R/σ_{AL}^2 are the fitting parameters). The experimental points are found to be well described by the model, and the estimated binding constants are in good agreement with literature data considering that other methods and different conditions were used (Table 1). In Table 1, the diffusion coefficients of the analytes and complexes calculated using eq 2 and assuming a capillary radius of 25 μm are also listed.

The diffusion coefficients may be converted into hydro dynamic radii as previously described.[3–7]

To further validate the procedure the equilibrium binding constant corresponding to the anion naproxenate with βCD was determined using standard Affinity Capillary Electrophoresis (ACE) under identical experimental conditions (see Supporting Information). The equilibrium binding constants obtained using the two methods are similar, albeit the results obtained using the ACE procedure appears slightly lower (Table 1). This discrepancy is most likely due to Joule heating in electrophoresis which results in a higher temperature in the capillary (see Supporting Information).

We can thus conclude that FIDA is an effective and general way of obtaining equilibrium binding constants and diffusivities of both free analytes and complexes. The advantages of the FIDA based procedure compared to existing methods include short analysis times, low sample volume requirements, and simplicity of data analysis and instrumentation. To implement the methodology the only requirement is fluid flow based analytical equipment such as HPLC, FIA, or CE instrumentation. Many of these commercially available instruments are equipped with autosamplers making high throughput analysis of noncovalent interactions feasible. In this way the FIDA based procedure for assessing noncovalent interactions is directly available to many research groups. Finally, the methodology is relatively easy to couple to miniaturized chemical procedures including synthesis, sample preparation, and separation making direct analysis of noncovalent interactions of for example synthesized drugs and biological targets possible. Fully integrated procedures may be realized using microfluidic chip based systems.

Table 1. Equilibrium Binding Constants (K) and Diffusion Coefficients of Free Analytes (D_A) and Complexes (D_{AL}), Respectively, Obtained in 67 mM Phosphate Buffer (pH 7.4) at 25 °C

Compound	K^a (M^{-1})	$D_A{}^b$ ($cm^2\ s^{-1}$)	$D_{AL}{}^c$ ($cm^2\ s^{-1}$)
α-Naphthol	2400 (±240)	9.3×10^{-6}	3.3×10^{-6}
	1250[d]	9.2×10^{-6}[e]	
Naproxen	1050 (±185)	5.6×10^{-6}	2.8×10^{-6}
	650 (±28)[f]	5.8×10^{-6}[g]	

[a] Equilibrium binding constants (±standard deviation) obtained using FIDA unless otherwise noted. [b] Diffusion coefficient of the free analyte. [c] Diffusion coefficient of the complex. [d] Literature data.[17] [e] Literature data.[18] [f] Equilibrium binding constant obtained using ACE as described in the Supporting Information (±standard deviation). [g] Literature data.[19]

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Supporting Information Available: Details on experimental procedures and data evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

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