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Use of a partial-filling technique in affinity capillary electrophoresis for determining binding constants of ligands to receptors

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Abstract

This work evaluates the concept of a partial-filling technique in affinity capillary electrophoresis (ACE) using two model systems: vancomycin from *Streptomyces orientalis* and carbonic anhydrase B (CAB, EC 4.2.1.1). In this technique the capillary is first partially-filled with ligand followed by a sample of receptor and non-interacting standard and electrophoresed. Analysis of the change in the mobility ratio, *M*, of the receptor, relative to the non-interacting standard, as a function of the concentration of the ligand, yields a value for the binding constant. These values agree well with those estimated using other binding and ACE techniques. Data demonstrating the quantitative potential of this method is presented.

Keywords: Affinity capillary electrophoresis; Binding constants; Partial-filling affinity capillary electrophoresis; Vancomycin; Carbonic anhydrase; Enzymes; Antibiotics

1. Introduction

The ability to accurately determine the extent of interaction between receptor and ligand is basic with respect to rational drug design and development especially in the pharmaceutical industry. In many cases special experimental conditions and instrumentation are required to analyze small quantities of material. Hence, the development of viable and accurate techniques to estimate these interactions is critical. There are a number of techniques currently available to measure binding parameters for non-covalent interactions frequently focusing on the separation and quantitation of free or complexed molecules in an equilibrium mixture [1]. Assuming that there are methods for differentiating and quantitating the amount of bound and free ligand in solution the estimation of a binding constant is straightforward.

Affinity capillary electrophoresis (ACE) has been shown to be a versatile technique for studying biomolecular non-covalent interactions and determining binding and dissociation constants of formed complexes [2–54]. The technique uses the resolving power of capillary electrophoresis (CE) to differentiate between the free and bound forms of a protein as a function of the concentration of free ligand. In a typical form of ACE a sample of receptor and non-interacting standard is exposed to an increasing concentration of ligand in the running buffer thereby

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causing a shift in the migration of the receptor relative to the standard. Subsequent analysis yields a value for the binding constant K_b.

The development and success of new drugs and drug treatments is tied to the ease of which these drugs can be synthesized in large enough quantities for use in clinical trials. In some cases, the lack of such quantities precludes their use in the treatment of some diseases. Although ACE has been shown to be an effective technique to measure binding parameters of ligands to receptors, there are cases where insufficient quantities of ligand make difficult their analyses by traditional ACE techniques. In such cases modifications in the technique are warranted. The use of partial-filling strategies in CE has recently been shown to be an effective method to measure some interactions [55–59]. These studies, though, were limited to enantioseparations of drugs and proteins.

In this paper, we extend the use of a partial-filling technique in ACE by estimating binding constants of ligands to receptors (Fig. 1) using two model systems: vancomycin (Van) from *Streptomyces orientalis* and carbonic anhydrase B (CAB, EC

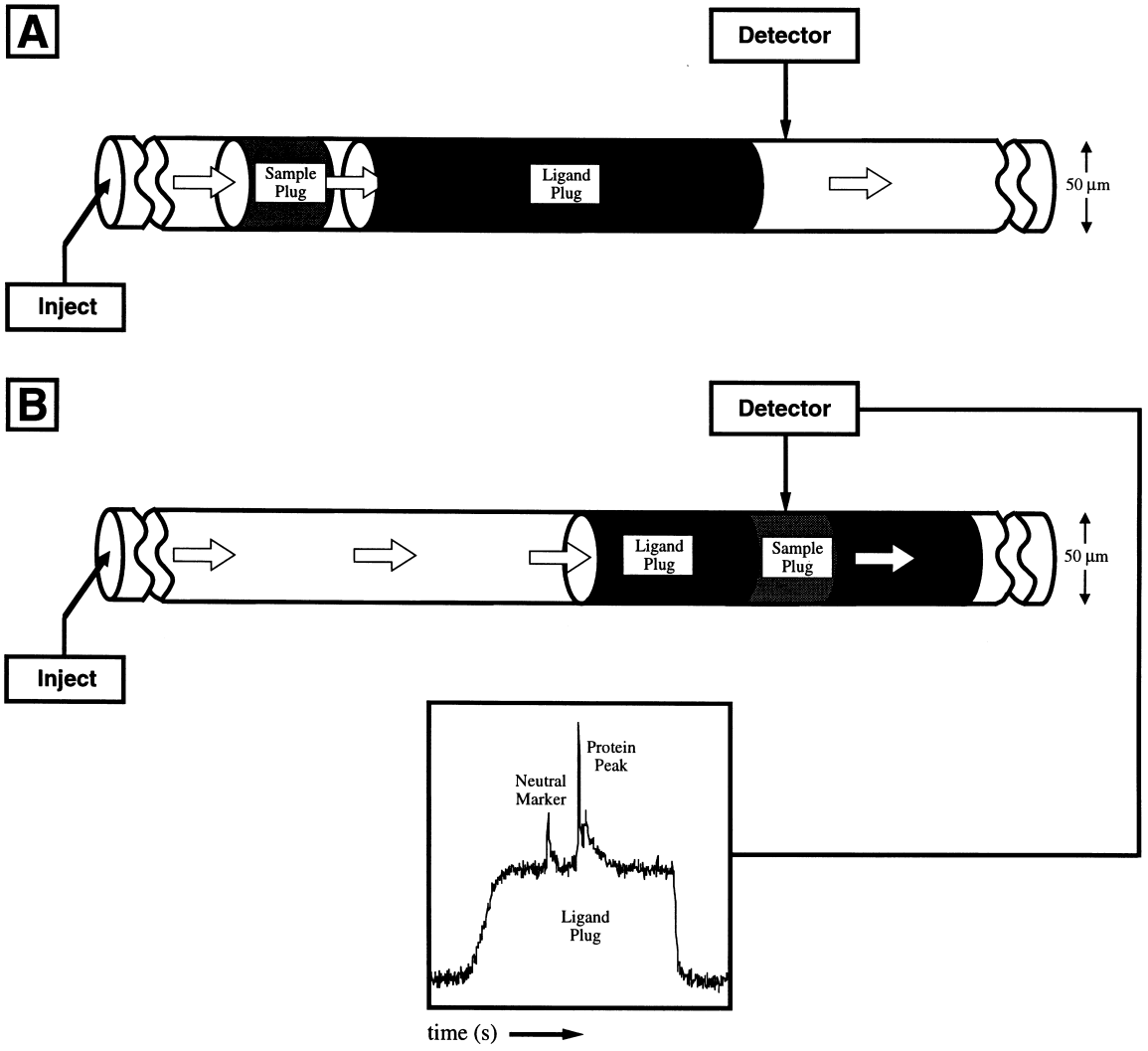


Fig. 1. Schematic of a partial-filling ACE experiment.

4.2.1.1). In this technique the capillary is first partially filled with ligand and then a sample of receptor and non-interacting standard is introduced and electrophoresed. Analysis of the change in the mobility ratio of the receptor, relative to a non-interacting standard, as a function of the concentration of the ligand, yields a value for the binding constant. The major advantage of the technique is the small quantities of material needed with respect to traditional ACE techniques.

2. Experimental

2.1. Chemicals and reagents

All chemicals were analytical grade. *N*-Acetyl-D-Ala–D-Ala **2**, vancomycin from *Streptomyces orientalis*, and carbonic anhydrase B (CAB, EC 4.2.1.1, containing CAA and CAB isozymes, from bovine erythrocytes) were purchased from Sigma (St. Louis, MO, USA) and used without further purification. 4-Carboxybenzenesulfonamide **3**, was purchased from Aldrich (Milwaukee, WI, USA), mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). Compounds **1** and **4** were gifts of J. Gao (Harvard University, Cambridge, MA, USA). Stock solutions (1 mg/ml) of bovine carbonic anhydrase B and vancomycin (4 mg/ml) were each prepared by dissolving the lyophilized protein in buffer (192 m*M* glycine–25 m*M* Tris, pH 8.3).

2.2. Apparatus

The CE system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) was of uncoated fused-silica with an internal diameter of 50 μm, length from inlet to detector of 50.5 cm, and a length from detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold software. The conditions used in CE were as follows: voltage, 25 kV; current, 5.2 μA; detection, 200 nm; temperature, 25±2°C.

2.3. Procedures

For vancomycin, a sample of D-Ala–D-Ala ligand was vacuum injected into the capillary for 15 s followed by a sample (3.6 nl) of solution for 3 s containing 0.14 mg/ml of vancomycin and 0.08 mg/ml of MO in buffer. The electrophoresis was carried out using a Tris–Gly buffer and increasing concentrations of the D-Ala–D-Ala ligand (0–1150 μ*M*) for 4.0 min. For CAB, a sample of arylsulfonamide was vacuum injected into the capillary for 21 s followed by a sample (3.6 nl) of solution for 3 s containing 0.2 mg/ml of CAB and 0.08 mg/ml of MO in buffer. The electrophoresis was carried out using a Tris–Gly buffer and increasing concentrations of the arylsulfonamide ligand (0–50 μ*M*) for 4.0 min.

3. Results and discussion

In the first series of experiments the interaction of vancomycin and two small peptides, *N*-succinyl-D-Ala–D-Ala and *N*-acetyl-D-Ala–D-Ala, was investigated. Extensive studies on molecular recognition between vancomycin and various small peptides with D-Ala–D-Ala terminus and structurally modified variants have been conducted by other techniques [25]. It is known that the three amide linkages and the free terminal carboxyl group are essential for vancomycin binding. In this study a plug of Van and MO were vacuum injected into the capillary and electrophoresed at 25 kV to obtain the mobility ratio for uncomplexed Van normalized to MO. A plug of **1** was then vacuum injected into the capillary for 15 s followed by a plug of Van and MO and electrophoresed at 25 kV. The concentration of **1** was successively increased from 0 to 1150 μ*M* and each concentration of ligand was run in triplicate twice over two separate days.

Fig. 2 shows a representative series of electropherograms of Van in a capillary partially-filled with various concentrations of **1**. Upon addition of increasing concentrations of **1** in the running buffer the Van peak shifts to the right for any given concentration. MO is used as a neutral marker in the analysis and does not interact with **1** in the running buffer under conditions of electrophoresis. Ligand **1** is a small negatively charged molecule and has a more negative electrophoretic mobility than uncomplexed and complexed Van. Hence, it elutes at a

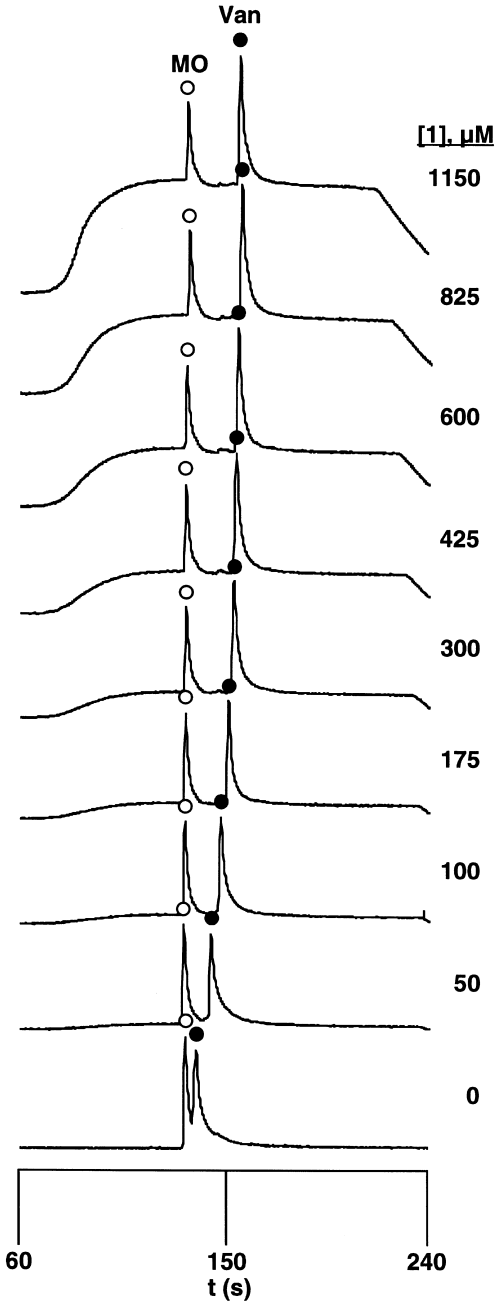


Fig. 2. A representative set of electropherograms of vancomycin (Van) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **1** using the partial-filling ACE technique. The total analysis time in each experiment was 4.0 min at 25 kV (current: 5.2 μA) using a 60.5 cm (inlet to detector)×50 μm I.D. open, uncoated quartz capillary. Mesityl oxide (MO) was used as an internal standard.

greater migration time than both Van and complexed Van. The complexation between **1** and Van resulted in an increasing negative charge and the complex is detected later than the uncomplexed form. As can be seen in Fig. 2, all of the electropherograms had the same elution pattern. Minimal variances in the migration times of Van and MO were observed. Very little peak broadening was observed at the intermediate concentrations and little change in electroosmotic flow (EOF) was observed even at high concentrations of ligand.

The height of the ligand plateaus in Fig. 2 increased due to the increased concentration of **1** partially-filled in the capillary. The box like structure of the ligand peak at any concentration of **1** denotes a constant concentration of ligand in the capillary. A long enough injection time permits Van and MO to elute with the box formed by the ligand. Too short a ligand injection time leads to incomplete overlap of marker and receptor with the ligand box, thereby, hindering analysis of the migration times. Fig. 3 is a Scatchard plot of the data using the mobility ratio *M* as the basis for analysis. In this form of analysis K_b can be estimated using *M* (Eq. 1) [31].

$$M = t_{eo}/t_p + 1 \quad (1)$$

where t_{eo} and t_p are the measured migration times of a reference peak and the receptor peak, respectively. A Scatchard plot can be obtained via Eq. 2.

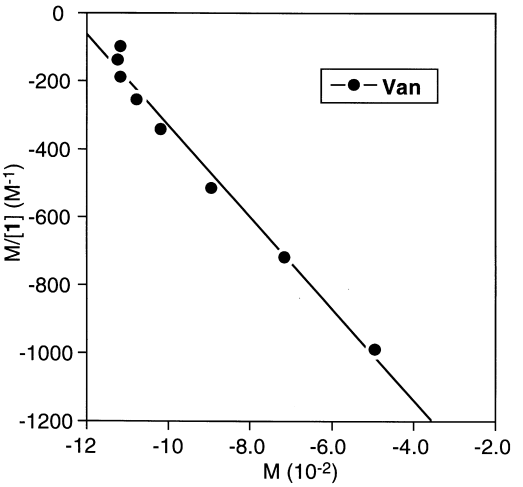


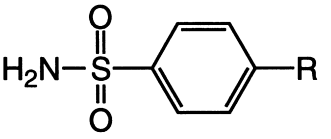
Fig. 3. Scatchard plot of the data for vancomycin according to Eq. 2.

$$M_{P,L}/[L] = K_b M_{P,L}^{max} - K_b M_{P,L} \quad (2)$$

where $M_{P,L}$ is the magnitude of the change in the mobility ratio as a function of the concentration of ligand. Eq. 2 allows for the estimation of K_b on a relative time scale and compensates for fluctuations in voltage and/or capillary length. Table 1 summarizes the binding data for the two ligands and Van. These values agree well with previous ACE studies and with those obtained from other assays for structurally similar compounds [1,6,15,31,60–62]. Of particular interest is the amount of ligand used in any given run. Unlike standard ACE techniques where the receptor is electrophoresed in a running buffer of increasing concentrations of ligand, the partial-filling ACE technique uses much smaller quantities of material (~18 nl of solution compared to approximately 1 μl for standard ACE techniques) for any given run. The ability to obtain binding parameters using small quantities of sample is integral in the development and design of new drugs and is the major advantage of the partial-filling ACE technique over both traditional ACE techniques and other forms of binding assays.

We used vancomycin as a model system for several reasons: (i) it has been extensively studied using other techniques; (ii) there is a body of information on binding constants available for comparison with those estimated by ACE, and (iii) there has been considerable interest in recent years in vancomycin owing to its novel mechanism of bacterial resistance.

A similar type of experiment was conducted with CAB and two charged arylsulfonamides (Fig. 4). CAB catalyzes the conversion of carbon dioxide to bicarbonate. Arylsulfonamides inhibit this reaction from occurring. Fig. 5 shows a representative series of electropherograms of CAB in a capillary partially-filled with various concentrations of **3**. Upon addition of increasing concentrations of **3** the CAB peak shifts to the right for any given concentration. MO is used as a neutral marker in the analysis. The complexation between **3** and CAB resulted in an increasing negative charge and the complex is detected later than the uncomplexed form. Unlike Van, some peak broadening was observed at intermediate concentrations of ligand. This is generally caused by the retardation of migrating molecules due to their frequent interactions with the ligand in the region of intermediate status [25]. Fig. 6 is a Scatchard plot of the data for CAB.



3 R = CO_2^-

4 R = $CH_2NHC(O)(CH_2)_4CO_2^-$

Fig. 4. Structures of compounds **3** and **4**.

Table 1
Experimental values of binding constants K_b ($\cdot 10^4$ M^{-1}) of ligands **1** and **2** and vancomycin measured by the partial-filling ACE technique

Ligand	K_b [a]
1	1.34[b]
2	0.39[c]

[a] The reported binding constants are the average values from six experiments for each ligand.
[b] Previous estimate [15]: $K_b = 0.93 \cdot 10^4$ M^{-1}.
[c] Previous estimates [1,6,15,31,60–62]: $K_b = 0.49–0.87 \cdot 10^4$ M^{-1}.

Binding constants were obtained for two charged arylsulfonamides. Table 2 shows the binding constants obtained by Eq. 2. The values for the binding constants agree well with previous estimates by ACE and other binding techniques [1,10,24,31]. The slight deviation from previous estimates may be the result of changes within the capillary over time, such as ligand and/or protein adsorption to the capillary wall or variations in temperature and current [25].

We used CAB as a model system for several reasons: (i) it does not absorb to the walls of uncoated capillaries; (ii) we have data describing its electrophoretic behavior in other circumstances; (iii) it is commercially available and inexpensive; (iv) ligands for it can be easily synthesized, and (v) many ligands bind to it with values of K_b between 10^5 and 10^9 M^{-1}.

Partial-filling ACE has several advantages as a method for measuring biomolecular non-covalent interactions. First, it requires even smaller quantities

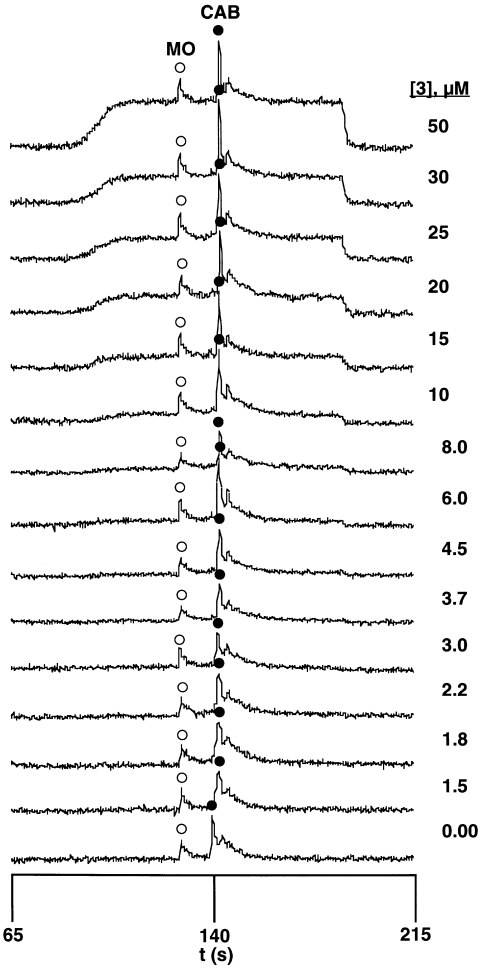


Fig. 5. A representative set of electropherograms of carbonic anhydrase B (CAB) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **3** using the partial-filling technique. The total analysis time in each experiment was 5.0 min at 25 kV (current: 5.2 μA) using a 60.5 cm (inlet to detector)×50 μm I.D. open, uncoated quartz capillary. Mesityl oxide (MO) was used as an internal standard.

of receptor and ligand than in traditional ACE techniques. Second, purification of the sample prior to injection is not necessary as long as the component to be analyzed can be separated from other species. Third, it does not require radiolabeled or chromophoric ligands. Fourth, the commercial availability of automated instrumentation, and the high reproducibility of data, make it experimentally convenient.

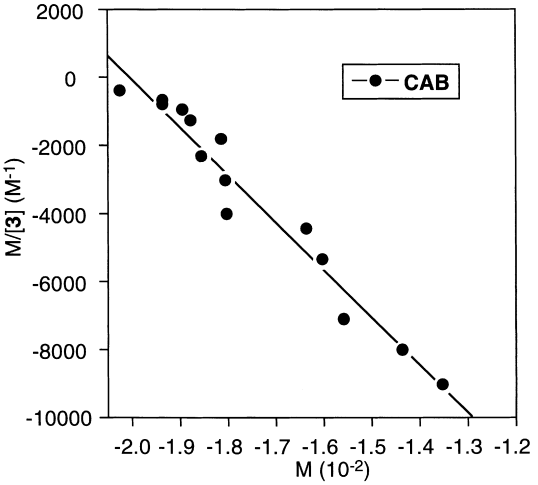


Fig. 6. Scatchard plot of the data for carbonic anhydrase B according to Eq. 2.

4. Conclusions

The partial-filling technique in ACE can effectively be used to estimate binding constants of ligands to receptors. We have shown this by the use of two model systems: vancomycin and D-Ala–D-Ala peptides and CAB and arylsulfonamides. In these experiments the capillary was first partially-filled with ligand followed by a plug of sample containing receptor and a non-interacting marker and electrophoresed. The use of mobility ratios in the analysis yielded a value for the binding constant. The binding constants obtained by this procedure agree well with those obtained by other assay methods and by other ACE techniques. A major advantage of the partial-filling technique over other ACE methods and other assay techniques is the small amounts of

Table 2
Experimental values of binding constants K_b ($\cdot 10^6$ M^{-1}) of ligands **3** and **4** and carbonic anhydrase B measured by the partial-filling ACE technique

Ligand	K_b [a]
3	1.80[b]
4	0.75[c]

[a] The reported binding constants are the average values from five and six experiments for ligands **3** and **4**, respectively.
[b] Previous estimate [1,10,31]: $K_b = 0.72–2.0 \cdot 10^6$ M^{-1}.
[c] Previous estimate [1,10,24,31]: $K_b = 0.45–0.92 \cdot 10^6$ M^{-1}.

material needed to perform the assay. Further work to demonstrate the versatility of the partial-filling technique is in progress.

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