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## Partial-filling affinity capillary electrophoresis

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**Abstract** Partial-filling affinity capillary electrophoresis (PFACE) is used to examine the binding interactions between two model biological systems: D-Ala-D-Ala terminus peptides to the glycopeptide antibiotic vancomycin (Van) from *Streptomyces orientalis*, and arylsulfonamides to carbonic anhydrase B (CAB, EC 4.2.1.1, bovine erythrocytes). Using these two systems, modifications in the PFACE technique are demonstrated including flow-through PFACE (FT-PFACE), competitive flow-through PFACE (CFTPFACE), on-column ligand synthesis PFACE (OCLSPFACE), and multiple-step ligand injection PFACE (MSLIPFACE). In PFACE small plugs of sample are injected into the capillary column and an equilibrium is established between receptor and ligand during electrophoresis. Binding constants are then obtained by Scatchard analysis using changes in the migration time of the receptor/ligand on changing the concentration of the ligand/receptor. Data demonstrating the quantitative potential of these methods are presented. This review focuses on the unique capabilities of the different PFACE techniques as applied to two model biological systems.

**Keywords** Partial filling · Affinity capillary electrophoresis · Binding constants · Receptor · Ligand · Scatchard plot

### Introduction

The characterization of molecular affinity coupled to the structural and quantitative information about the interacting molecules is essential in understanding the functions and molecular mechanisms of biological systems in health

and disease [1]. By elucidating how and to what extent molecules interact, the development of treatments for a host of human diseases including Alzheimer's, Parkinson's, and cancer might be expedited. There are a variety of analytical methods to measure affinity parameters of noncovalent interactions including radioimmunoassays, fluorescence quenching, ammonium sulfate precipitation, and slab gel electrophoresis techniques. However, these techniques are frequently based on the separation and quantitation of free or complexed molecules in an equilibrium mixture. Assuming the amount of bound and free ligand in solution can be distinguished, these techniques give reasonable estimates of binding constants ( $K_b$ ).

Over the past decade affinity capillary electrophoresis (ACE) has been shown to be a versatile technique to study a variety of receptor-ligand interactions including protein-protein, protein-DNA, protein-drug, protein-carbohydrate, peptide-peptide, peptide-carbohydrate, peptide-dye, carbohydrate-drug, and antibody-antigen [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36]. In a typical form of ACE a sample of receptor and noninteracting standard(s) is exposed to an increasing concentration of ligand in the running buffer, thereby, causing a shift in the migration time of the receptor relative to the standard(s). The change in migration time is then used for Scatchard analysis to obtain a value for  $K_b$ .

There are several advantages in using ACE to study biomolecular noncovalent interactions: (1) only small quantities of receptor and ligand are required; (2) purification of the sample is not required as long as CE can distinguish the impurities from the analyte of interest; (3) radiolabeling of the molecules is not necessary; (4) automated CE instrumentation is widely available; (5) data are reproducible and expeditiously obtained; and (6) a wide range of molecular interactions can be characterized in free solution.

In cases where insufficient quantities of material are available variations in ACE using partial-filling techniques have been designed. In this paper we describe the use of PFACE techniques for examining receptor-ligand

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interactions. These techniques are demonstrated using model biological systems. Emphasis is placed on describing the unique capabilities and advantages and disadvantages of each technique described.

## Methods

### Chemicals and reagents

All chemicals were analytical grade. *N*-acetyl-D-Ala-D-Ala, **2**, vancomycin (Van) from *Streptomyces orientalis*, carbonic anhydrase B (CAB, EC 4.2.1.1, containing CAA and CAB isozymes, from bovine erythrocytes), and horse heart myoglobin (HHM), D-Ala-D-Ala, D-Ala-D-Ala-D-Ala, and nicotinamide adenine dinucleotide (NAD) were purchased from Sigma Chemical Company (St. Louis, MO, USA) and used without further purification. 4-Carboxybenzenesulfonamide (CBSA), **4**, 4-nitrobenzenesulfonamide, **5**, *p*-toluenesulfonamide, **6**, and benzenesulfonamide, **7**, were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). ({[4-(Aminosulfonyl)phenyl]methyl}amino)-6-oxohexanoic acid, **3**, *N*-succinyl-D-Ala-D-Ala, **1**, and ({[4-(aminosulfonyl)phenyl]methyl}amino)-ethanol, **8**, were synthesized based on literature procedures [19]. 9-Fluorenylmethoxy carbonyl (Fmoc)-Gly-*N*-hydroxysuccinimide (NHS) ester, Fmoc-Ala-NHS, Gly-Ala-Ala-D-Ala-D-Ala, and D-Ala-D-Ala-D-Ala-D-Ala were purchased from BACHEM California Inc. (Torrance, CA, USA) and used without further purification. For PFACE: stock solutions (1 g L<sup>-1</sup>) of bovine CAB and Van (4 g L<sup>-1</sup>) were each prepared by dissolving the lyophilized protein in buffer (192 mmol L<sup>-1</sup> glycine-25 mmol L<sup>-1</sup> Tris; pH 8.3). For FTPFACE: stock solutions of bovine CAB (1 g L<sup>-1</sup>), Van (4 g L<sup>-1</sup>), and HHM (1 g L<sup>-1</sup>) were each prepared by dissolving the lyophilized protein in buffer (192 mmol L<sup>-1</sup> glycine-25 mmol L<sup>-1</sup> Tris; pH 8.6). For CFTPFACE: stock solutions (1 g L<sup>-1</sup>) of bovine CAB and HHM (1 g L<sup>-1</sup>) were each prepared by dissolving the lyophilized proteins in buffer (192 mmol L<sup>-1</sup> glycine-25 mmol L<sup>-1</sup> Tris; pH 8.3). Ligands **5–8** were also dissolved in buffer. For OCLSPFACE: stock solutions of Van (200 μmol L<sup>-1</sup>), Gly-Ala-Ala-D-Ala-D-Ala (0.01 mol L<sup>-1</sup>), D-Ala-D-Ala-D-Ala-D-Ala (0.01 mol L<sup>-1</sup>), D-Ala-D-Ala (0.01 mol L<sup>-1</sup>), D-Ala-D-Ala-D-Ala (0.01 mol L<sup>-1</sup>), NAD (2.5 mmol L<sup>-1</sup>), and CBSA (3 mmol L<sup>-1</sup>) were each prepared by dissolving in buffer (20 mM phosphate buffer; pH 7.5). For MSLIPFACE: stock solutions of Van (4 g L<sup>-1</sup>), bovine CAB (1 g L<sup>-1</sup>), and HHM (1 g L<sup>-1</sup>) were each prepared by dissolving the lyophilized protein in buffer (192 mmol L<sup>-1</sup> glycine-25 mmol L<sup>-1</sup> Tris; pH 8.3).

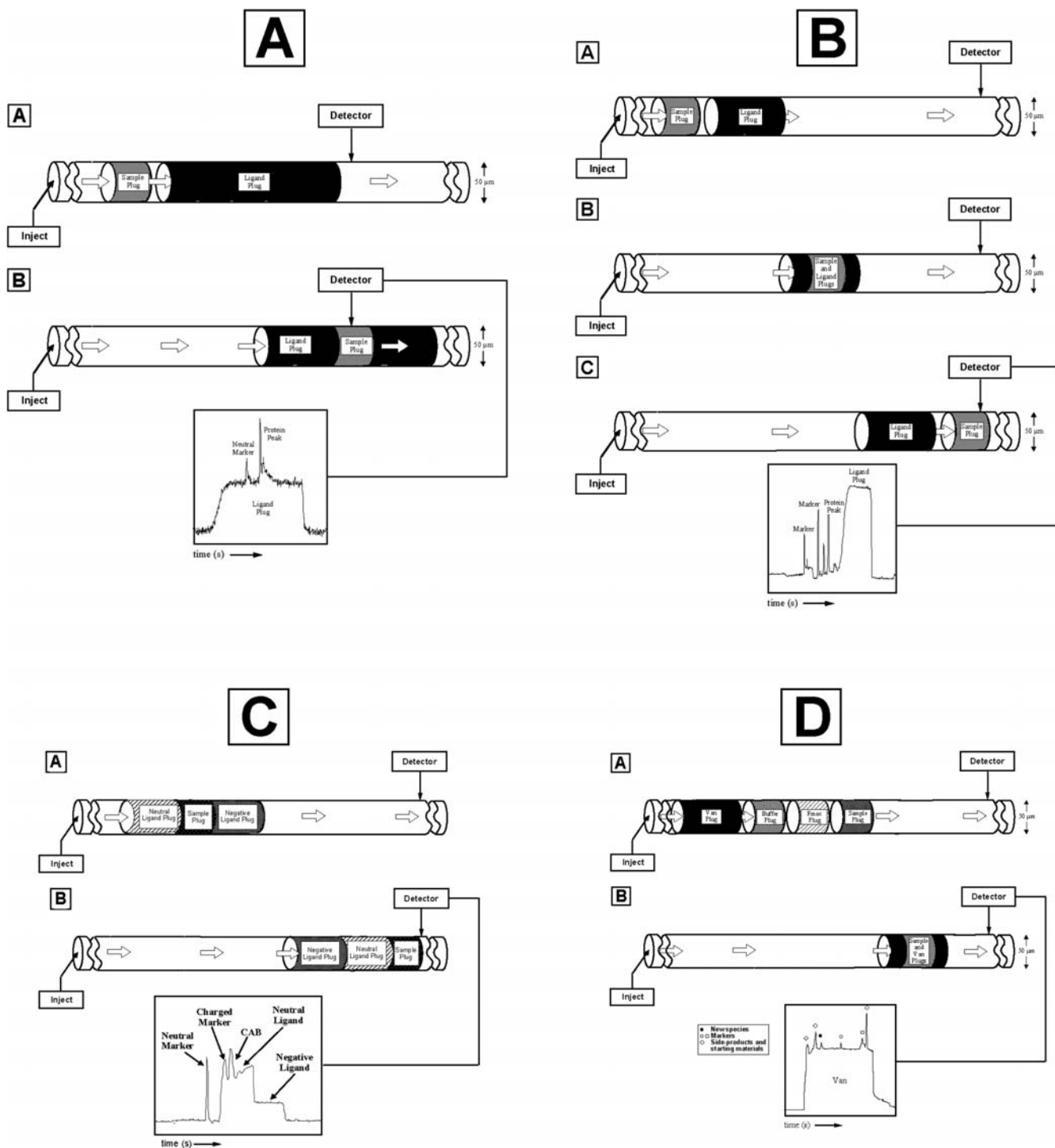
### Apparatus

The capillary electrophoresis (CE) system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Inc.,

Phoenix, AZ, USA) was of uncoated fused silica with an internal diameter of 50 μm, length from inlet to detector of 50.5 cm for FPACE and FTPFACE (60.5 cm for competitive FTPFACE, 40.5 cm for on-column ligand synthesis ACE, 80.5 cm for multiple-step ligand injection ACE), 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: for PFACE: voltage, 25 kV; current, 5.2 μA; detection, 200 nm; temperature, 25±2 °C; for FTPFACE: voltage, 28 kV (25 kV for CAB and **1**); current, 5.2–5.8 μA; detection, 200 nm; temperature, 25±2 °C; for CFTPFACE: voltage, 28 kV; current, 5.8 μA; detection, 200 nm; temperature, 25±0.1 °C; for OCLSPFACE: voltage, 24 kV; current, 35.4 μA; detection, 205 nm; temperature, 25±2 °C; for MSLIPFACE: voltage, 24 kV; current, 4.0 μA; detection, 200 nm; temperature, 25±2 °C.

### Procedures

For PFACE, 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 1 s containing 0.14 g L<sup>-1</sup> of Van and 0.08 g L<sup>-1</sup> 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 μmol L<sup>-1</sup>) for 4.0 min. For FTPFACE, a sample of **3** was vacuum injected into the capillary for 0.1 min at high pressure followed by a sample (3.6 nL) of solution for 3 s containing 0.14 g L<sup>-1</sup> of CAB, 0.14 g L<sup>-1</sup> of HHM, and 0.08 g L<sup>-1</sup> of MO in buffer. The electrophoresis was carried out using a Tris-Gly buffer and repeated at increasing concentrations of **3** (0–80 μmol L<sup>-1</sup>). For CFTPFACE, a sample of **3** (20 μmol L<sup>-1</sup>) was vacuum injected into the capillary for 0.1 min. at high pressure (20 psi), followed by a sample (3.6 nL) of solution containing 0.20 g L<sup>-1</sup> of CAB, 0.313 g L<sup>-1</sup> of HHM, and 0.001 g L<sup>-1</sup> of MO in buffer for 3 s at low pressure (0.5 psi), followed by a sample of **5** for 2.5 min. at low pressure. The electrophoresis was carried out using a Tris-Gly buffer and repeated at increasing concentrations of **5** (0–64 μmol L<sup>-1</sup>) for 5.85 min. The total analysis time of each experiment was 8.5 min. For OCLSPFACE, a sample solution (1.2 nL; a 1 s time of injection equates to 1.2 nL of liquid) containing D-Ala-D-Ala terminus peptides, NAD and **4** was introduced by pressure injection into the capillary equilibrated with buffer (20 mmol L<sup>-1</sup> phosphate; pH 7.5). Separate plugs (2.4 nL each) of Fmoc-amino acid-NHS ester in acetonitrile and buffer (20 mmol L<sup>-1</sup> phosphate; pH 7.5) were next introduced by pressure injection and electrophoresed. In the binding studies a solution of Van (about 491 nL) at increasing concentration (0–80 μmol L<sup>-1</sup>) was introduced after the buffer plug by voltage injection (24 kV, 1.5 min) and the electrophoresis run at 24 kV to complete detection of all species. For MSLIPFACE, the capillary was first filled with buffer solution not containing ligand (Solution A) followed by a sample (7.2 nl; a 1 s time of injection equates to 1.2 nl of liquid) of solution (Solution B) containing 0.10 mg ml<sup>-1</sup> of



**Fig. 1** (A) Schematic of a partial-filling ACE experiment. (B) Schematic of a flow-through PFACE experiment. (C) Schematic of a competitive flow-through PFACE experiment. The sample plug is enlarged to best pictorially represent the technique. (D) Schematic of an on-column ligand synthesis PFACE experiment

Van,  $0.17 \text{ g L}^{-1}$  of MO, and  $0.33 \text{ g L}^{-1}$  of CAB. The sample was subjected to electrophoresis in a solution (Solution C) containing the first concentration ( $50 \mu\text{M}$ ) of **2** for 2.0 min

at 24 kV. A second sample of solution ( $14.4 \text{ nL}$ ; Solution B) containing Van, MO, and CAB was injected for 12 s and subjected to electrophoresis in the next higher concentration of **2** ( $100\text{--}1200 \mu\text{mol L}^{-1}$ ) for 2.0 min at 24 kV. The process of sample injection and ligand electrophoresis was repeated until all concentrations of ligand were run.

### Partial-filling affinity capillary electrophoresis (PFACE)

Although ACE has been shown to be a very effective analytical technique to estimate binding constants of receptors to ligands, there are instances where insufficient amounts of sample are available for analysis by standard ACE procedures. In such cases modifications in the technique are warranted. In 1999, we developed PFACE to measure binding constants of ligands to receptors (Fig. 1A) [2]. In this technique the capillary column is first partially filled with a ligand and then a sample of receptor and noninteracting standard is introduced and electrophoresed. Analysis of the change in the migration time of the receptor relative to the standard, as a function of the concentration of ligand, yields a value for the  $K_b$ .

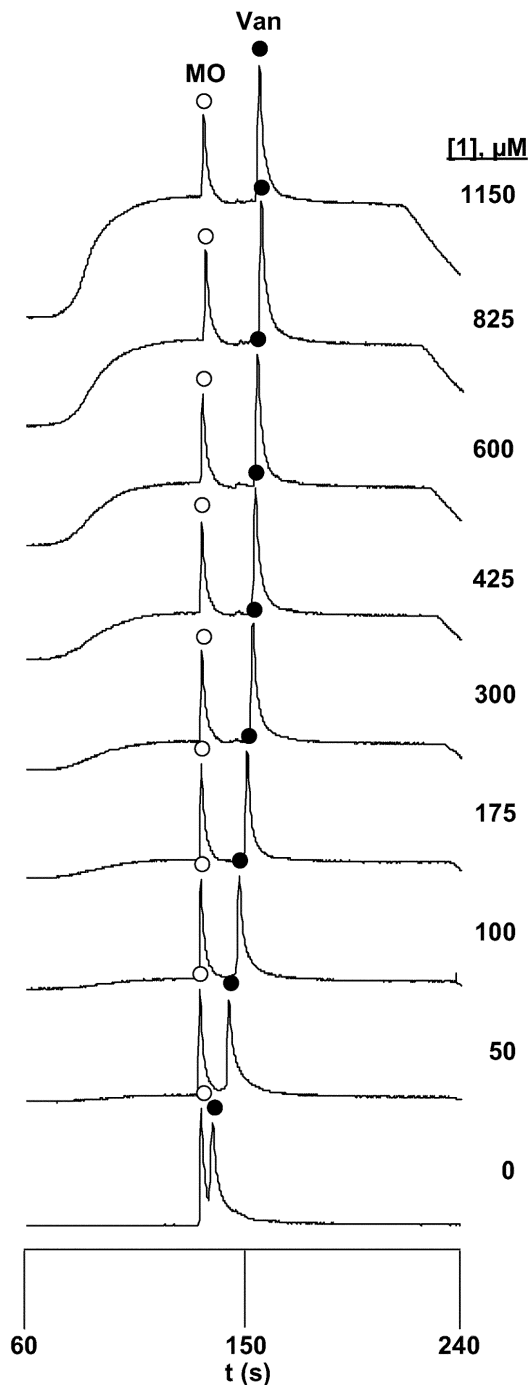
Using this technique we examined the binding of D-Ala-D-Ala terminus peptides to vancomycin (Van) from *Streptomyces orientalis* [2]. Van is a glycopeptide antibiotic that inhibits the growth of Gram-positive bacteria by hindering cell wall peptidoglycan biosynthesis [37, 38, 39]. These drugs bind to the D-Ala-D-Ala portion of peptidoglycan intermediates, inhibiting the transglycosylation reaction required for crosslinking of the cell wall.

In our studies using R-D-Ala-D-Ala peptides (R=*N*-succinyl (**1**) and *N*-acetyl (**2**)) we demonstrated that PFACE could be used to estimate binding constants of peptides to Van [2]. Figure 2 shows a representative series of electropherograms of Van in a capillary partially filled with **1**. Upon increasing the concentration of **1** in the capillary column a shift in the migration time of Van is observed. The Van-**1** complex is more negative than Van and upon binding shifts to the right (longer migration time). The neutral marker, mesityl oxide (MO), is unaffected by the change in ligand concentration and its migration time does not vary significantly during the course of the experiment, hence, MO can be used as a marker in the measurement of  $K_b$ .

As shown in Fig. 2 the change in concentration of **1** in the column is visualized as an increased height in the ligand plateaus. The box-like structure of the ligand peak at all concentrations of ligand denotes both a uniform injection of ligand into the column and a stable concentration of peptide in the capillary column. For the sample plug to elute on top of the ligand boxes care was taken to ensure that the ligand was injected into the capillary for long enough time, otherwise incomplete overlap occurred making analysis of the interaction problematic. Further proof that binding is occurring is shown as an increase in the peak area of the Van peak on increasing the concentration of **1** in the capillary column. Fig. 3 is a Scatchard plot of Van using varying concentrations of **1** in the running buffer using the mobility ratio,  $M$ , as the basis for the analysis [3]. In this form of analysis a noninteracting standard was used in estimating the binding constant using Eqs. 1 and 2.

$$M = (t_{co}/(t_R)) + 1 \quad (1)$$

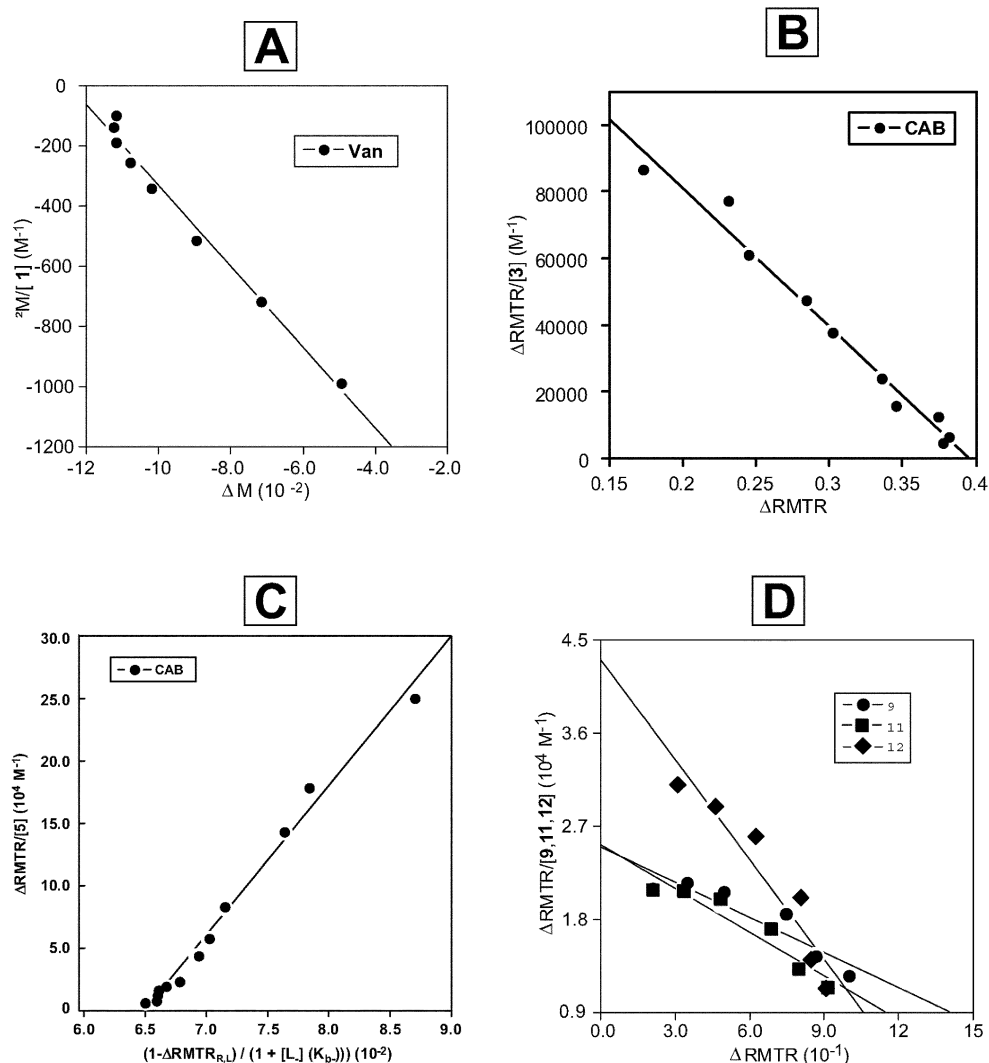
$$\Delta M_{R,L}/[L] = K_b \Delta M_{R,L}^{max} - K_b \Delta M_{R,L} \quad (2)$$



**Fig. 2** A representative set of electropherograms of vancomycin (Van) in  $192 \text{ mmol L}^{-1}$  glycine- $25 \text{ mmol L}^{-1}$  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:  $5.2 \mu\text{A}$ ) using a 60.5-cm (inlet to detector), 50- $\mu\text{m}$  I.D. open, uncoated quartz capillary. Mesityl oxide (MO) was used as an internal standard

Here,  $t_{co}$  and  $t_R$  are the measured migration times of the reference peaks MO and Van, respectively. A Scatchard plot can be obtained using Eq. 2.  $\Delta M_{R,L}$  is the magnitude of the change in the migration ratio as a function of the concentration of ligand. Table 1 lists the values for  $K_b$  us-

**Fig. 3** (A) Scatchard plot of the data for vancomycin according to Eq. 2. (B) Scatchard plot of the data for carbonic anhydrase B according to Eq. 4. (C) Scatchard plot of the data for carbonic anhydrase B according to Eq. 8. (D) Scatchard plot of the data for **9**, **11**, and **12** according to Eq. 4



ing PFACE. These values are in agreement with those obtained by standard ACE techniques and other types of assays.

### Flow-through partial-filling affinity capillary electrophoresis (FTPFACE)

In cases where it is not permissible to use conventional PFACE techniques, e.g., sample and ligand plugs do not elute simultaneously at the point of detection, other means of obtaining binding constants for the interaction must be used. We recently described the use of flow-through PFACE (FTPFACE) to estimate binding constants of ligands to receptors [4]. In this technique we modified the conditions for PFACE by introducing a smaller plug of ligand than that used in standard PFACE. Here the sample containing receptor and standards flows completely through the ligand plug upon electrophoresis. Figure 1B shows a schematic of a FTPFACE experiment.

In this work we examined the binding of carbonic anhydrase B (CAB, EC 4.2.1.1.) to charged arylsulfonamides [3]. CAB is a zinc protein of the lyase class that

catalyzes the equilibration of carbon dioxide and carbonic acid. It is strongly inhibited by sulfonamide-containing molecules. In this experiment a plug of **3** at increasing concentration was injected for 0.1 min into the capillary column at high pressure, followed by a plug of sample containing CAB and noninteracting markers. Upon electrophoresis the sample plug flows into the domain of the ligand plug which is migrating at a slower velocity through the capillary column. A dynamic equilibrium is quickly reached between CAB and **3**. Continued electrophoresis causes the sample plug to flow through the ligand plug and it is detected first. The ligand plug is detected second as a rectangular-shaped box. Figure 4 shows a series of electropherograms of CAB in capillaries partially filled with increasing concentrations of **3**. Complexation between CAB and **3** results in an increasing negative charge on CAB, hence, it migrates later than the uncomplexed form. Figure 3B is a Scatchard plot of the data for CAB obtained using the relative migration time ratio (RMTR) (Eq. 3), a dual-marker form of analysis we have shown to give better results in obtaining a  $K_b$  than other forms of analysis especially in instances where electroosmotic flow (EOF) is variable [1].

**Table 1** Experimental values of binding constants  $K_b$  ( $10^4 \text{ mol L}^{-1}$ ) of ligands **1-16** measured by the different ACE techniques

Ligand	Technique	$K_b$ ( $10^4 \text{ mol L}^{-1}$ )
1	PFACE/MSLIPFACE	1.34/0.99 <sup>a</sup>
2	PFACE/MSLIPFACE	0.39/050 <sup>b</sup>
3	FTPFACE	48.0 <sup>c</sup>
4	FTPFACE	70.0 <sup>d</sup>
5	CFTPFACE	1200 <sup>e</sup>
6	CFTPFACE	250 <sup>f</sup>
7	CFTPFACE	120 <sup>g</sup>
8	CFTPFACE	400
9	OCLSPFACE	1.49 <sup>h</sup>
10	OCLSPFACE	4.16 <sup>i</sup>
11	OCLSPFACE	1.51
12	OCLSPFACE	3.0
13	OCLSPFACE	2.26
14	OCLSPFACE	17.5
15	OCLSPFACE	3.43
16	OCLSPFACE	3.67

<sup>a</sup> Previous estimate [20]:  $K_b=0.93 \times 10^4 \text{ mol L}^{-1}$

<sup>b</sup> Previous estimates [3, 20, 23, 34]:  $K_b=0.49-0.87 \times 10^4 \text{ mol L}^{-1}$

<sup>c</sup> Previous estimate [3, 6, 21, 34]:  $K_b=45-92 \times 10^4 \text{ mol L}^{-1}$

<sup>d</sup> Previous estimate [3, 6, 21, 34]:  $K_b=72-200 \times 10^4 \text{ mol L}^{-1}$

<sup>e</sup> Previous estimate [6]:  $K_b=1250 \times 10^4 \text{ mol L}^{-1}$

<sup>f</sup> Previous estimate [6]:  $K_b=200-250 \times 10^4 \text{ mol L}^{-1}$

<sup>g</sup> Previous estimate [6]:  $K_b=90-110 \times 10^4 \text{ mol L}^{-1}$

<sup>h</sup> Previous estimate [20]:  $K_b=1.4 \times 10^4 \text{ mol L}^{-1}$

<sup>i</sup> Previous estimate [20]:  $K_b=1.9 \times 10^4 \text{ mol L}^{-1}$

$$\text{RMTR} = (t_r - t_s) / (t_s - t_s') \quad (3)$$

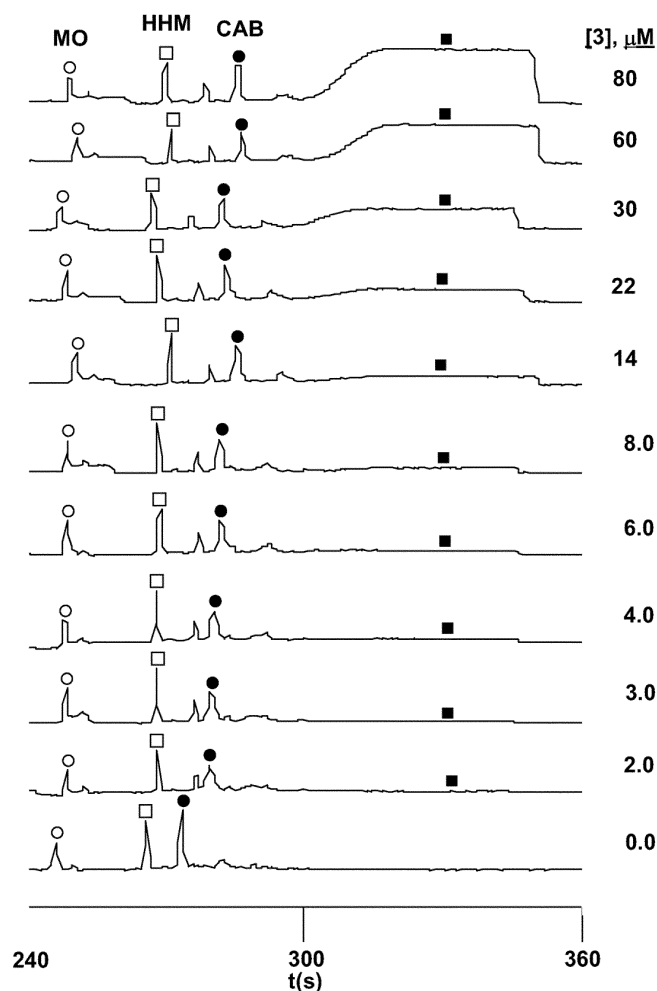
Here,  $t_r$ ,  $t_s$ , and  $t_s'$  are the measured migration times of the CAB peak, and the two noninteracting standard peaks (MO and horse heart myoglobin, HHM, respectively). A Scatchard plot can be obtained using Eq. 4.

$$\Delta \text{RMTR}_{R,L} / [L] = K_b \Delta \text{RMTR}_{R,L}^{\max} - K_b \Delta \text{RMTR}_{R,L} \quad (4)$$

Here,  $\Delta \text{RMTR}_{R,L}$  is the magnitude of the change in RMTR as a function of the concentration of ligand. Equation 4 allows for the estimation of  $K_b$  on a relative time scale using two noninteracting standards and compensates for fluctuations in the capillary column induced by electrophoresis. Table 1 lists the values of  $K_b$  for ligands **3** and **4** to CAB using the FTPFACE technique. These data correlate well to those obtained using other assay techniques.

### Competitive flow-through partial-filling affinity capillary electrophoresis (CFTPFACE)

We have extended the use of the FTPFACE technique by examining the binding of uncharged ligands to CAB [5]. A basic premise of ACE is that the migration time of a receptor will change upon binding to a ligand. This change in migration time can then be related to the noninteracting standards and a new  $\mu$  obtained. Changes in  $\mu$  are then used to obtain a  $K_b$ . Small, neutral ligands, when bound to a protein, do not appreciably change the migration time of the newly formed complex, thereby prohibiting the use of

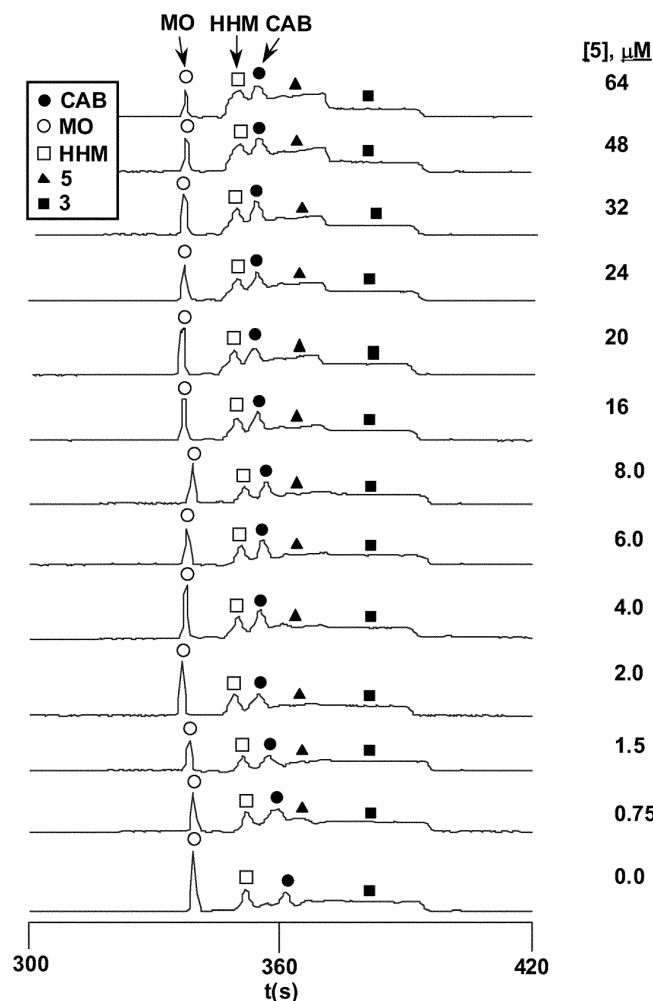


**Fig. 4** A representative set of electropherograms of carbonic anhydrase B (CAB) in  $192 \text{ mmol L}^{-1}$  glycine- $25 \text{ mmol L}^{-1}$  Tris buffer (pH 8.6) containing various concentrations of **3** using the flow-through partial-filling affinity capillary electrophoresis technique. The total analysis time in each experiment was 6.5 min at 28 kV (current:  $5.8 \mu\text{A}$ ) using a 60.5-cm (inlet to detector), 50- $\mu\text{m}$  I.D. open, uncoated quartz capillary. MO and horse heart myoglobin (HHM) were used as internal standards

standard ACE techniques. To remedy this problem we have developed a competitive assay using FTPFACE to examine the binding of neutral arylsulfonamides to CAB. Figure 1C shows a schematic of a competitive FTPFACE experiment.

In this technique the capillary is first partially filled with a negatively charged ligand **3** followed by injections of CAB and noninteracting standards and a separate plug of **5**. Upon application of a voltage, CAB and the standards flow into the plug of **3** where a dynamic equilibrium is established. Ligand **5** then penetrates the CAB-**3** domain and a new equilibrium is established between **3** and CAB. Continued electrophoresis results in the complex and standards flowing through **3** which are detected last.

Figure 5 shows a representative series of electropherograms of CAB in capillaries partially filled with increasing concentrations ( $0-64 \mu\text{mol L}^{-1}$ ) of **5** and electrophoresed



**Fig. 5** A representative set of electropherograms of CAB in  $192 \text{ mmol L}^{-1}$  glycine- $25 \text{ mmol L}^{-1}$  Tris buffer (pH 8.3) containing various concentrations of **5** using the competitive binding FTPFACE technique. The total analysis time in each experiment was 8.5 min. at 28 kV (current:  $5.8 \mu\text{A}$ ) using a 60.5-cm (inlet to detector),  $50\text{-}\mu\text{m}$  I.D. open, uncoated quartz capillary. MO and HHM were used as internal standards

at a constant concentration of **3** ( $20 \mu\text{mol L}^{-1}$ ). The heights of the ligand plateaus, as seen in the series of electropherograms, increase due to the increase in concentration of **5** partially filled in the capillary column.

In this work, we modified a Scatchard analysis used in previous ACE work [6], in which Colton et al. showed that a dissociation constant between a receptor and a charged ligand could be estimated using Eq. 5 [6]:

$$R_f/[L] = (1/K_d) - (R_f/K_d) \quad (5)$$

Here,  $R_f$  was defined as the fraction of the total concentration of R ( $[R]_T$ ) present as R-L where  $R_f = [R-L]/[R]_T$ . In Colton's work [L] is the concentration of a charged ligand. He showed that a similar analysis could be used to estimate binding between a receptor and a neutral ligand ( $L_0$ ) in a competitive binding assay using Eqs. 6 and 7:

$$R_f = [R \cdot L_0]/[R]_T \quad (6)$$

$$R_f/[L_0] = (1 - R_f)/((K_{b0}^{-1})(1 + [L_{\pm}][K_b^{\pm}])) \quad (7)$$

Here,  $K_b^{\pm}$  and  $[L_{\pm}]$  are the known values for the binding constant of a charged ligand and concentration of charged ligand, respectively. Equation 7 was used to obtain binding constants of neutral ligands to a receptor.

In the present work we used Eq. 4 to estimate the RMTR and substituted  $\Delta\text{RMTR}$  for  $R_f$  in Eq. 7 to estimate binding constants between neutral arylsulfonamides and CAB using Eq. 8 [5]. We showed that by using Eq. 8

$$\Delta\text{RMTR}_{R,L}/[L_0] = (1 - \Delta\text{RMTR}_{R,L})/((K_{b0}^{-1})(1 + [L_-]K_{b-})) \quad (8)$$

the value for  $K_{b0}$  could be obtained for the interaction of charged ligands and a receptor. Equation 8 is the general equation used for Scatchard analysis of this competitive binding system.

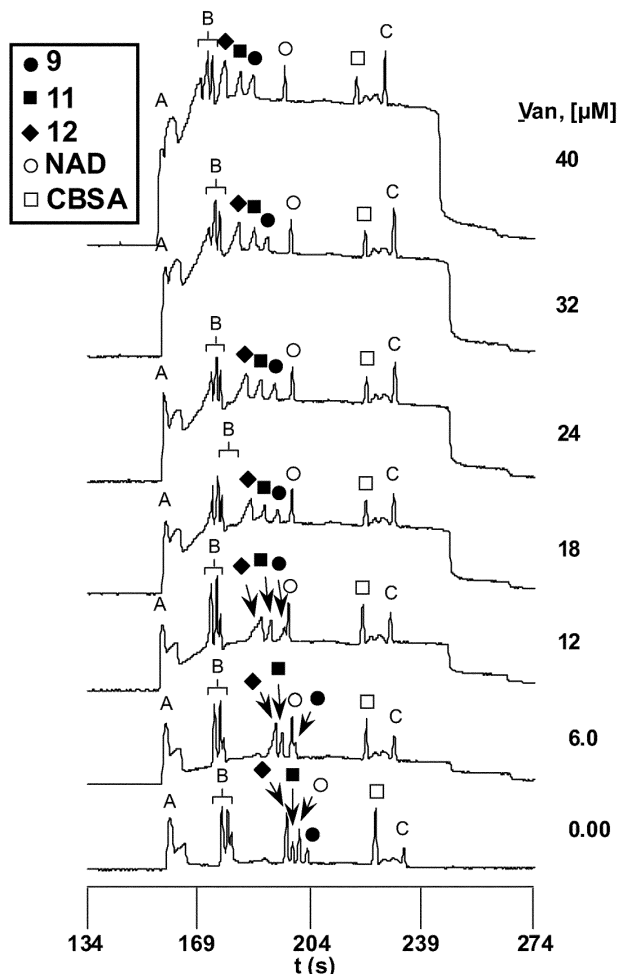
Here,  $K_{b-}$  and  $L_-$  are the known values for the binding constant and concentration of the negatively charged ligand in the running buffer, respectively. In this work  $K_{b-}$  and  $L_-$  are  $4.16 \times 10^6 \text{ mol L}^{-1}$  and  $20 \mu\text{mol L}^{-1}$ , respectively.  $L_0$  is the concentration of the neutral ligand used in the experiment. Subsequent Scatchard analysis of the value of  $\Delta\text{RMTR}$ , measured by FTPFACE as a function of the concentration of  $L_0$ , gives the value for the binding constant ( $K_{b0}$ ) of the neutral ligand to CAB. Figure 3C is a Scatchard plot of the competitive binding assay of **5** for CAB. Table 1 lists the values of  $K_b$  for neutral ligands **5–8** obtained by the CFTPFACE technique.

### On-column ligand synthesis partial-filling affinity capillary electrophoresis (OCLSPFACE)

During the past decade the design and development of new drugs has grown almost exponentially due to new synthetic and analytical approaches to rational drug design. This increased output of new drugs has warranted the development of new analytical techniques and instrumentation that can both expeditiously and accurately analyze many potential drug targets simultaneously. Recently, ACE has been used to examine affinity parameters of ligands to receptors resulting from high-throughput screening and combinatorial approaches to rational drug design [7, 8]. These studies, though, involved the synthesis of ligands prior to their analysis by CE.

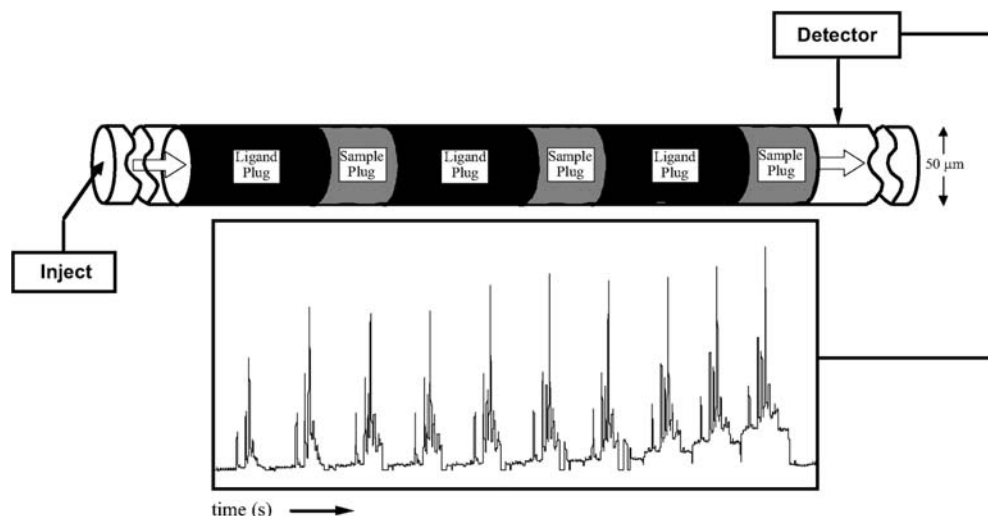
To alleviate the need for synthesis, purification, and characterization of ligands prior to analysis, we devised a technique whereby ACE could be coupled to on-column ligand synthesis, thereby allowing for the analysis of multiple drug targets simultaneously [9]. In this technique 9-fluorenylmethoxy carbonyl (Fmoc)-amino acid-D-Ala-D-Ala peptides are first synthesized using on-column techniques and then analyzed for their affinity to the glycopeptide Van using PFACE. Figure 1D shows a schematic of an on-column PFACE experiment.

In this study a plug of sample containing D-Ala-D-Ala, D-Ala-D-Ala-D-Ala-D-Ala, Gly-Ala-Ala-D-Ala-D-Ala, and two noninteracting markers (nicotinamide



**Fig. 6** A representative series of electropherograms of Fmoc-Gly-D-Ala-D-Ala-D-Ala, **9**, Fmoc-Gly-D-Ala-D-Ala-D-Ala-D-Ala, **11**, and Fmoc-Gly-Gly-Ala-Ala-D-Ala-D-Ala, **12**, in 20 mmol L<sup>-1</sup> phosphate buffer (pH 7.5) containing various concentrations of Van using the on-column ligand synthesis PFACE technique. The total analysis time in each experiment was 5.0 min at 24 kV (current: 35.4  $\mu$ A) using a 40.5-cm (inlet to detector), 50- $\mu$ m I.D. open, uncoated quartz capillary. Nicotinamide adenine dinucleotide (NAD) and 4-carboxybenzenesulfonamide (CBSA) were used as internal standards. A–C are explained in the text

**Fig. 7** Schematic of a multiple-step ligand injection ACE experiment



adenine dinucleotide (NAD), and CBSA (**4**) was injected into the capillary column followed by separate plugs of Fmoc-Gly-*N*-hydroxysuccinimide (NHS) ester and buffer. The capillary was then partially filled with increasing concentrations of Van and electrophoresed. The first two zones of material allow for the formation of three new Fmoc-derivatized species (**9**, **11**, **12**). Continued electrophoresis allows the antibiotic to flow into the plug of sample where an equilibrium is reached between it and the peptides. Figure 6 is the series of electropherograms for peptides **9**, **11**, and **12** in increasing concentration of Van partially filled in the capillary. As can be seen, at increasing concentrations of Van the peaks for the newly formed peptides shift to the left upon complexation to Van. Peaks A and C represent by-products from the reaction. Peak B is remaining unreacted peptides. Figure 3D is the Scatchard plot for the three peptides using Eq. 4. The typical box-like structure for the antibiotic is observed as found in previous PFACE experiments. Table 1 provides the values of  $K_b$  for peptides **9–16** obtained using this technique.

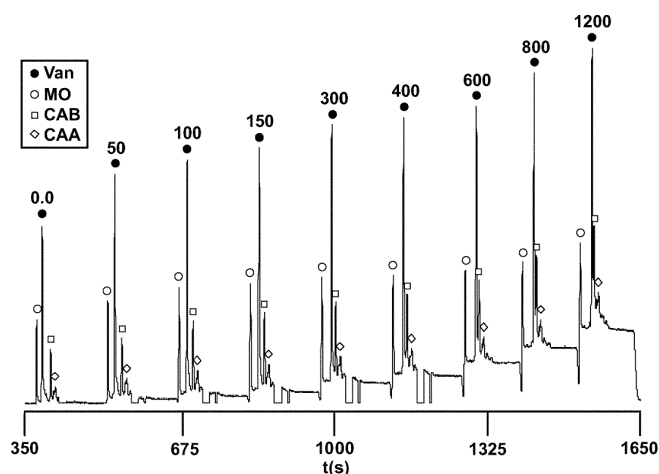
This technique has several advantages as a method for measuring biomolecular noncovalent interactions. First, it requires smaller quantities of both receptor and ligand than in traditional ACE techniques. Second, purified ligand or receptor is not always required as long as the peak(s) of interest can be differentiated from other peaks in the electropherograms. Third, the technique can be modified for high-throughput analysis of ligands if the reaction part of the technique affords products on the timescale of CE.

#### Multiple-step ligand injection partial-filling affinity capillary electrophoresis (MSLIPFACE)

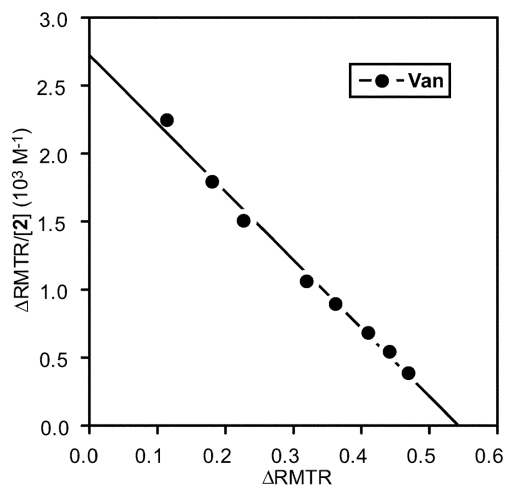
The human genome project and advances in proteomics have resulted in the discovery of many molecular interactions. The small amount of material available, though, sometimes precludes the use of standard analytical techniques to measure the extent of their interactions. Hence, new ACE techniques are needed to determine the strength of these interactions.



To offset the use of large quantities of material we have developed multiple-step ligand injection PFACE to examine the binding between receptors and ligands [10]. Figure 7 is a schematic of the ACE technique. In this technique a plug of Van and noninteracting standards is injected and electrophoresed in buffer containing a given concentration of peptide. The sequence is repeated at increasing concentrations of peptide until all concentrations of ligand are run. Analysis of the change in the RMTR affords  $K_b$ . Figure 8 shows a representative set of electropherograms of Van in increasing concentrations of **2**. Periodic injections of **2** at higher concentration result in the Van peak shifting to the right for any concentration of **2** in the buffer. Figure 9 is a Scatchard plot of the data for Van using Eq. 4. We have also developed a competitive assay us-



**Fig. 8** A representative electropherogram of vancomycin (Van) in  $192 \text{ mmol L}^{-1}$  glycine- $25 \text{ mmol L}^{-1}$  Tris buffer (pH 8.3) containing various concentrations of **7** using the multiple-step ligand injection ACE technique. The total analysis time in each experiment was 27 min at 24 kV (current:  $4.0 \mu\text{A}$ ) using an 80.5-cm (inlet to detector),  $50\text{-}\mu\text{m}$  I.D. open, uncoated quartz capillary. MO and CAB (containing CAA and CAB isozymes) were used as internal standards. The numbers above each set of sample peaks refer to the concentration of **1** in  $\mu\text{M}$



**Fig. 9** Scatchard plot of the data for vancomycin according to Eq. 4

ing multiple-step ligand injection in examining the binding of uncharged ligands to CAB. Table 1 lists the values of  $K_b$  for ligands **1** and **2** obtained using this technique.

This technique is most effective when the mobilities of all species are known and when an equilibrium between receptor and ligand is achieved quickly. The advantages of the technique are two-fold: one, it is faster than conventional ACE techniques, and two, it uses less material than ACE and traditional assays.

## Conclusions

As has been illustrated the use of partial-filling techniques in ACE can greatly expedite the estimation of binding affinities between receptors and ligands. We have developed a number of partial-filling ACE methods that demonstrate the versatility of CE and which afford binding constants comparable to other assay techniques. Partial filling offers a number of advantages over traditional ACE and other assay techniques. First, it requires smaller quantities of both receptor and ligand than in traditional ACE techniques. Second, purified ligand and/or receptor are not always required as long as the peak(s) of interest can be differentiated from other peaks in the electropherogram. Third, the techniques can be modified for high-throughput analysis of many molecules, simultaneously, if the reaction in question affords products on the timescale of CE. Fourth, most of the commercially available instruments are automated making it experimentally convenient to use.

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