Through-a-chip partial filling affinity capillary electrophoresis for estimating binding constants of ligands to receptors

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Abstract

In this paper, we describe the development of a microfluidic/capillary electrophoresis (CE) technique employing partial filling affinity capillary electrophoresis (PFACE) to estimate binding constants of ligands to receptors using as model systems carbonic anhydrase B (CAB, EC 4.2.1.1) and vancomycin from Streptomyces orientalis. Using multilayer soft lithography (MSL), a microfluidic device (MD) consisting of fluid and control channels is fabricated and fitted with an external capillary column. Multiple flow channels allows for manipulation of a zone of ligand and sample containing receptor and non-interacting standards into the MD and subsequently into the capillary column. Upon electrophoresis the sample components migrate into the zone of ligand where equilibrium is established. Changes in migration time of the receptor are used in the analysis to obtain a value for the binding interaction. The manipulation of small volumes of solution on the MD minimizes the need of time-consuming pipetting steps.

Keywords: Affinity capillary electrophoresis; Microfluidic device; Carbonic anhydrase B; Vancomycin; Binding constants

1. Introduction

The past two decades has seen tremendous advances in the design, development and use of microelectromechanical systems (MEMS) [1–10]. Applications for microfluidic devices (MDs) have proliferated at a speed reminiscent of the use of microelectronics after the invention of the integrated circuit. MDs have been shown to have great potential in a variety of biological applications including biomolecular separations [11,12], enzymatic assays [13,14], immunohybridization reactions [15,16], and the polymerase chain reaction [17,18]. The use of nanoliter reaction volumes and parallel high-throughput sample processing are potential advantages of MDs, making them ideally suited for chemical analysis, screening applications, and other cases where reagents are limited. A major challenge associated with realizing the desired scales in MDs is to simultaneously reduce the number of pipetting steps needed to load the devices while amortizing the sample volume over several reaction or separation steps [19].

To address the issue of sample loading and manipulation a variety of valve-type techniques have been developed. The recent pioneering work of Quake et al. and his development of microvalves is a milestone in microfluidics and shows great promise in MDs [19–24]. In this technique called multilayer soft lithography (MSL) they combined soft lithography with the capability to bond multiple patterned layers of elastomer. Layered structures are constructed by binding layers of elastomer each of which is separately cast from a micromachined mold. The elastomer is made up of a two-component silicone wafer. The bottom layer (fluid or flow channels) has an excess of one of the monomers, whereas, the upper layer (control channels) has an excess of the other monomer. Layered structures are constructed by binding layers of elastomer each of which is separately cast from a micromachined mold. The elastomer is made up of a two-component silicone wafer. The bottom layer (fluid or flow channels) has an excess of one of the monomers, whereas, the upper layer (control channels) has an excess of the other monomer. After the layers are cured, the upper layer is removed from its mold and placed on top of the lower layer, where a seal is formed. Reactive molecules at the interface between the two layers irreversibly bond upon curing. Where the control channels pass above the fluid channels, a valve is formed.

Capillary electrophoresis (CE) is a powerful separation technique that has gained widespread use in biological laboratories because of its versatility and ease of use. It is an excellent tool for many types of bioanalyses and is an unparalleled experimental tool for biophysical studies of interactions in biologically
relevant media. Separations are based on the principles of the electrically driven flow of ions in solution. Selectivity can be manipulated by the alteration of electrolyte properties such as pH, ionic strength, and electrolyte composition, or by the incorporation of electrolyte additives. CE has great promise when used in a microfluidic format and many papers and reviews have detailed the applications thereof [25–45].

One important application of CE is affinity CE (ACE) which has been successfully used to estimate binding parameters between ligands and receptors [46–69]. Since the first papers in 1992 [49–53] demonstrating the use of ACE to measure affinity parameters between biological species, its use in probing a variety of receptor–ligand interactions has greatly expanded and includes, but is not limited to, protein–drug, protein–DNA, peptide–peptide, carbohydrate–drug, and antibody–antigen. For example, Kaddis et al. has used ACE to estimate binding constants for the substrate and activator of *Rhodobacter sphaeroides* adenosine 5′-diphosphate-glucose pyrophosphorylase [46]. Li et al. used capillary isoelectric focusing (cIEF) and ACE to determine binding constants between antibodies to the prion protein [47]. Finally, Lewis et al. described the screening of antimicrobial targets using ACE [48].

ACE uses the resolving power of CE to distinguish between free and bound forms of a receptor as a function of the concentration of free ligand in the electrophoresis buffer. In a typical form of ACE a sample of receptor and standard(s) is exposed to an increasing concentration of ligand in the running buffer causing a shift in the migration time of the receptor relative to the standard(s). We recently developed a more specialized form of ACE called partial filling ACE (PFACE) [64–68]. Here, the capillary is partially filled with ligand and sample plug of receptor is introduced into the capillary and electrophoresed. During electrophoresis the zones of samples overlap within the capillary and equilibrium is established prior to the point of detection. PFACE reduces the amount of sample required for the assay and expedites the speed of analysis.

Herein, we demonstrate the use of through-a-chip PFACE to examine the interaction of CAB to arylsulfonamides and vancomycin to small peptides. In this work sample injection and electrophoresis buffer are manipulated through a microfluidic chip constructed from poly(dimethylsiloxane) (PDMS) and into a capillary column one end of which is embedded into the chip. Upon application of a voltage sample zones migrate through the capillary where equilibrium between receptor and ligand is established and is detected using an external UV–vis detector. Subsequent analysis of the change in migration time of the receptor referenced to two non-interacting standards yields a value of the binding interaction.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were analytical grade. Fig. 1 shows the structures of ligands 1–6 for CAB and vancomycin used in this study. CAB, dimethylformamide (DMF), horse heart myoglobin (HHM), 4-carboxybenzenesulfonamide (4-CBSA) (6) and N-acetyl-d-Ala-d-Ala (7) were purchased from Sigma (St. Louis, MO, USA) and were used without further purification. Carboxyfluorescein (FAM)-d-Ala-d-Ala-d-Ala (8) was custom synthesized by Anaspec (San Jose, CA, USA) and was used without further purification. CAB (1 mg/mL), DMF (1 mg/mL), HHM (1 mg/mL) and NADH (1 mg/mL) were each prepared by dissolving the lyophilized proteins or small molecule markers in buffer (192 mM glycine–25 mM Tris, pH 8.3). Compounds 1–5 (shown in Fig. 1) were gifts from S. Mallik (North Dakota State University, Fargo, ND, USA).

2.2. Apparatus

A Nikon Eclipse TE2000-U microscope (A.G. Heinze, Lake Forest, CA, USA), solenoids and fluid/air controllers (Fluidigm, San Francisco, CA, USA), Spectra 100 UV visible detector (Thermo Separation Products, Piscataway, NJ, USA) and LabVIEW 7.0 computer software (National Instruments Corporation, Austin, TX, USA) are used for fluid manipulation and electrophoresis control, detection, and data collection, respectively. The capillary tubing (Polymeric Micro Technologies, Phoenix, AZ, USA) was of uncoated fused silica with an outer diameter of 365 μm, an internal diameter of 78 μm, a length from inlet to detector of 57.0 cm, and a length from detector to outlet of 6.5 cm.

2.3. Procedures

For CAB: using solenoid pumps with connecting tubing for sample introduction, the microfluidic device was first flushed with electrophoresis buffer (5 min) to remove any impurities that may have remained from the fabrication process. Solutions of increasing concentration of ligand 1 and a sample containing CAB (1 mg/mL), DMF (1 mg/mL) and HHM (1 mg/mL) were injected at low pressure (0.5 psi) for 10 and 2.7 s, respectively. Electrophoresis buffer was then pressure injected (0.5 psi for 10 s) into the electrophoresis channel to push the sample plug past the electrode and into the beginning of the capillary column. The electrophoresis was carried out using a Tris–Gly buffer and repeated at increasing concentrations of 1 for 8 min. For Van: similar to above except NADH was used as an internal standard.

2.4. Design and fabrication

Pneumatically actuated MDs were fabricated at the Caltech Nanofabrication lab from two patterned flexible polymer layers—the “flow layer” (containing the channels which carry reagents) and the “control layer” (containing the channels which cause pneumatic valve closure when pressurized). Each layer is fabricated from two-part-cure silicone (Dow Corning Sylgard 184) cast on a photosensor mold. The master molds are made by spin-coating photosensor onto a silicon wafer and then patterned with high-resolution transparency masks (typically 3386, 8000, or 20,000 dpi). The fluid layer mold consisted of a photosensor that can be reflowed by baking at high temperatures (Shipley SJR 5740). The mold is spin-coated with 20:1 part A:B
Onto this mold about 0.5 cm 5:1 part A:B Sylgard is cast to form the control layer. Each layer is cured for 30 min in an oven (80°C). Control channel interconnect holes are punched through the thick layer (after release from the mold) by using a manual-punching machine with a hollow ram and hole plug ejector-pin system (Technical Innovations, Brazoria, TX, USA), and the layer aligned and sealed (channel-side down) onto the flow layer (still on the mold). The sample inlet, capillary and electrode holes are approximately 889 and 508 μm in diameter, respectively, which facilitates the interface between the MD and the exterior sample inlet vials and tubing, capillary and electrode. Bonding is realized by curing the devices together for an additional 45–60 min (80°C) preventing leakage between the two layers. The resulting two-layer devices are cut from the mold and mounted onto cleaned glass microscope slides, and bonded overnight at 80°C.

3. Results and discussion

3.1. Injection and manipulation of solution

Our first goal was to determine the feasibility of injecting two separate plugs of solution into a microfluidic channel and subsequently into a capillary column fitted at the end of the channel. It is important that the amount of sample injected into the microchip and then into the capillary column be comparable to that delivered by commercial instruments (using either hydrodynamic or electrokinetic injection). In partial filling techniques an additional factor is that the amount of ligand injected be greater than that of sample since during electrophoresis the zone of sample must migrate through the ligand zone to cause equilibrium between receptor and ligand.

Fig. 2 is a picture of the microfluidic chip used in our experiments. A schematic of the injection process is shown in Fig. 3. In Fig. 3A inlets a–c of the microfluidic chip were connected to

Fig. 1. Arylsulfonamide ligands 1–6 for carbonic anhydrase B (CAB) and vancomycin used in this study.
solutions of blue and red dye, and electrophoresis buffer, respectively. A solution of red dye was first injected into the chip to illustrate the plug length of the ligand. It was determined that a pressure of 0.5 psi applied for 10 s equated to a volume of 10.4 nL. This volume of solution is comparable to that used in open-tubular CE for a typical injection of sample of ligand using partial filling techniques [64–66]. A solution of blue dye was then injected into the chip (Fig. 3C) and into the electrophoresis channel. The volume of blue dye was determined to be 2.8 nL comparable to a typical sample injection using a commercial instrument. For example, a one second injection at low pressure (0.5 psi) using a 50 μm (inner diameter) capillary and a BeckmanCoulter P/ACE CE instrument, equates to 1.2 nL of solution.

The zones of solution were subsequently manipulated into the capillary column by pressure injection of a solution of electrophoresis buffer (Fig. 3D). Fig. 3E is a picture of the two dye solutions within the microfluidic channel prior to manipulation into the capillary column (not shown). No leakage of solution around the electrode or capillary was observed on manipulating the dyes into the capillary column. This result is significant as any versatile injection scheme in a MD must be able to manipulate a solution from one point to another on the chip.

### 3.2. ACE experiments

In our first series of experiments, we examined the binding of an arylsulfonamide ligand (1) to CAB. CAB is a zinc protein of the lyase class that catalyzes the equilibration of dissolved carbon dioxide and carbonic acid. It is strongly inhibited by sulfonamide-containing molecules. We chose the CAB system for several reasons: (1) because its pI is 5.9, at pH 8.3, there is little propensity to adsorb onto the walls of the capillary column, (2) we have data describing its electrophoretic behavior in other circumstances, (3) it is commercially available and inexpensive, (4) ligands for it can be easily synthesized, and (5) many ligands bind to it with values of $K_b$ between $10^5$ and $10^7$ M$^{-1}$.

The same injection sequence was used here as that described in the dye experiments and injection volumes are comparable to those determined with the red and blue dyes respectively. Here, solutions of 1 and sample containing CAB, HHM and DMF are injected into the channel. HHM and DMF are non-interacting
standards that do not interact with either 1 or CAB. Finally, a running electrophoresis buffer is manipulated into the channel pushing the zones of ligand and sample through the channel and into the capillary column found near the end of the channel. Upon electrophoresis, the sample flows into the zone of 1 where a dynamic equilibrium between 1 and CAB is established. Fig. 4 shows a representative set of electropherograms of CAB in a capillary partially filled with increasing concentrations (0–30 µM) of 1 in the running buffer. At the point of detection, separate peaks are observed for DMF, CAB and HHM. The complex that forms between CAB and 1 is more negatively charged then CAB and, therefore, the peak for the complex (CAB–1) shifts to a greater migration time on increasing the concentration of 1 in the running buffer. A small amount of inactive CAB can be seen in the series of electropherograms at the same migration time as found for uncomplexed CAB.

3.3. Analysis of binding constants

Fig. 5 is a plot of the data for 1. In this form of analysis termed the relative migration time ratio (RMTR) (Eq. (1)), the binding constant is estimated using two non-interacting markers injected with the sample [57]. Here, \( t_r \), \( t_s \), and \( t_s' \)

\[
\text{RMTR} = \frac{t_r - t_s'}{t_s' - t_s}
\]  

(1)

are the measured migration times of CAB compound, and the two non-interacting standard peaks, respectively. In the present experiments, \( t_s \) and \( t_s' \) are the migration times of DMF and Van, respectively. The values of \( \Delta \text{RMTR}_{PEL} \) obtained using Eq. (1) over a range of concentrations of 1 were then used for analysis using a standard hyperbolic equation to generate the binding isotherm (Eq. (2)).

\[
y = \frac{ax}{b + x}
\]  

(2)

Here, \( a \) and \( b \) are defined as maximum \( \Delta \text{RMTR}_{PEL} \) and calculated \( 1/K_b \), respectively. Values for \( y \) and \( x \) are the change in electrophoretic mobility and concentration of 1, respectively. The above data analyses, curve fitting functions, and statistical calculations were performed using Sigma Plot 8.0 (Jandel Scientific, Erkrath, Germany). Eq. (2) allows for the estimation of \( K_b \) on a relative time scale using two non-interacting standards and compensates for fluctuations in electroosmotic flow (EOF), background electrolyte, viscosity and electric field variations in the capillary column.

Using Eq. (2), a \( K_b \) of \( 4.34 \times 10^5 \text{M}^{-1} \) was obtained for the interaction between CAB and 1. This value is comparable to our previous results using ACE (\( 4.70 \times 10^5 \text{M}^{-1} \)) and to values obtained using other assay techniques [34,36]. A correlation coefficient (\( R^2 \)) of 0.992 was obtained for the fitness of the binding isotherm plot. A series of other inhibitors of CAB (2–6, Fig. 1) were examined and the results are detailed in Table 1. These results compare favorably with those obtained using other ACE techniques [34,36,70].

We also examined the glycopeptide vancomycin (Van) from Streptomyces orientalis. Van is a parenteral glycopeptide antibiotic that kills bacterial cells by inhibiting peptidoglycan biosynthesis [71–77]. It functions by binding to the terminal d-Ala-d-Ala dipeptide of bacterial cell wall precursors. Historically, Van has been the drug of choice in treating infections caused by bacterial resistance to other types of antibiotics.
Unfortunately, Van resistant enterococci (VRE) has emerged which is of major concern to the biomedical community. Van resistance is now also common in Enterococcus faecalis, hence, it is becoming increasingly important to develop new Van-group antibiotics, to study their physicochemical parameters, and to examine their activity against VRE.

In these experiments a similar injection scheme was utilized except that NAD was utilized as a non-interacting standard in addition to DMF. Furthermore, the injection volumes are comparable to that of the previously mentioned dye experiments. Two peptides 7 and 8 were used for these studies. In a typical experiment an increasing concentration of 7 was injected into the microfluidic channel followed by a sample containing markers and Van and then manipulated into the capillary column. Upon electrophoresis the zone of sample migrates into the plug of 7 where equilibrium is satisfied. At the point of detection the Van peak shifts to increasing migration time which is then used to obtain a value for $K_b$ (Table 2). Fig. 6 is a representative series of electropherograms of Van exposed to increased concentrations of 7 in the electrophoresis buffer. Upon complexation to 7 the migration time for the Van peak increases the change of which

Table 1
Experimental values of binding constants $K_b$ ($10^3 \text{ M}^{-1}$) of CAB to ligands 1–6 measured by the through-a-chip PFACE technique

<table>
<thead>
<tr>
<th>Ligands</th>
<th>$K_b^a$</th>
<th>$R^2$</th>
<th>$K_b^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.34</td>
<td>0.992</td>
<td>4.70</td>
</tr>
<tr>
<td>2</td>
<td>2.99</td>
<td>0.943</td>
<td>1.93</td>
</tr>
<tr>
<td>3</td>
<td>2.90</td>
<td>0.996</td>
<td>3.22</td>
</tr>
<tr>
<td>4</td>
<td>5.85</td>
<td>0.984</td>
<td>5.88</td>
</tr>
<tr>
<td>5</td>
<td>5.65</td>
<td>0.986</td>
<td>3.40</td>
</tr>
<tr>
<td>6</td>
<td>1.33</td>
<td>0.992</td>
<td>6.90</td>
</tr>
</tbody>
</table>

$a$ The reported binding constants are the average values from at least two experiments for each ligand.

$b$ Previous estimates [71].
Further work will focus on optimizing conditions for assay, of molecular interactions can be characterized in free solution. Commercial instruments is no longer required. Finally, a wide range using valve and pump techniques. Four, need for expense com-

is used in the binding constant analysis. Fig. 7 is a plot of the data for 7.

The work, herein, is significant for several reasons. One, unlike the majority of other microchip CE devices, the current technique does not require labeling of the receptor with a fluo-

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References