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A Prototype Two-Dimensional Capillary Electrophoresis System Fabricated in Poly(dimethylsiloxane)

Xiaoxi Chen, Hongkai Wu, Chengde Mao, and George M. Whitesides*

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

A method for carrying out 2D gel electrophoresis in a capillary format is presented. In this method, separation in the first dimension is carried out in a 1D capillary, with this system physically isolated from the capillaries that provide the separation in the second dimension. After completion of the first separation, the 1D channel is physically connected to the 2D capillaries, and a second separation is carried out in an orthogonal set of parallel capillaries. The ability of poly(dimethylsiloxane) (PDMS) to support the fabrication of 3D microfluidic systems makes it possible to produce membranes that both enclose the gel used in the first separation in a capillary and provide passages for the proteins to migrate into the array of orthogonal capillaries. The elastomeric nature of PDMS makes it possible to make reversible connections between pieces of PDMS. The feasibility of this system is demonstrated using a protein mixture containing fluorescein-conjugated carbonic anhydrase, fluorescein-conjugated BSA, and Texas Red-conjugated ovalbumin. This work suggests one type of design that might form the basis for a microfabricated device for 2D capillary electrophoresis.

Proteomics is an important, emerging area for bioanalytical chemistry, and new tools are needed for it.[1−5] One of the most widely used analytical methods in protein chemistry is gel electrophoresis.[6,7] One-dimensional (1D) gel electrophoresis (in the form of SDS−PAGE) is still used for separations of proteins, but 1D gels are increasingly being replaced by capillary electrophoresis.[8−14] The most important tool in separating very complex mixtures of proteins is still 2D gel electrophoresis.[15−21] There are two reasons for wanting to convert this method to a capillary format: (1) The small size of the capillary requires only small quantities of sample; and (2) the high surface-to-volume ratio of the capillary results in rapid heat dissipation; maintaining an even temperature minimizes heat-induced peak broadening. Reduction of Joule heating also allows the use of high electric fields; these fields give rapid separations with better resolution than can be obtained in gels.[22] DNA separation has largely moved from cast gels to capillaries for certain kinds of separations (e.g., analytical sequencing) for these reasons.[23−26]

We are exploring methods of making capillary electrophoresis systems that can compete with conventional 2D gel electrophoresis. In 2D gel electrophoresis, separation in the first dimension (usually by isoelectric focusing) is done in a strip of gel (Figure 1A). A pair of electrodes is attached to two ends of the gel strip, and a voltage is applied between them (Figure 1A) until the proteins separate.[27,28] Each distinguishable band on the gel strip

* To whom correspondence should be addressed. E-mail: gwhitesides@gmwgroup.harvard.edu.

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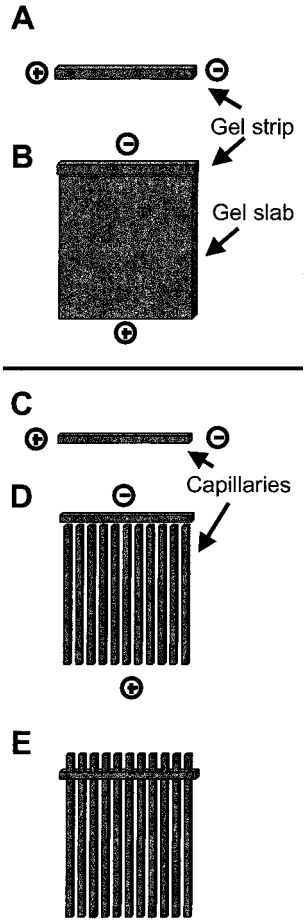

Figure 1. Conventional and capillary formats. In a conventional 2D electrophoresis system, (A) a gel strip is used for the separation in the first dimension, and (B) the gel strip is connected to a gel slab for electrophoresis in the second dimension. In a capillary format, (C) the gel strip is replaced by a capillary, and (D) the gel slab is replaced by multiple parallel capillaries. (E) This diagram suggests the simplest design that connects the capillary used for the first separation to the array of capillaries used in the second separation.

may contain more than one protein; as a result, additional separation according to another property (usually the size of the protein) is carried out by electrophoresis in the second, orthogonal dimension. To accomplish this second separation, the gel strip is brought into contact with the edge of a slab of gel (Figure 1B) (usually containing SDS). An electrode is attached next to the gel strip, and another electrode is attached to the opposite edge of the slab gel. A voltage is applied between them (Figure 1B); the proteins come out of the gel strip and separate in the gel slab.[27,28]

In considering how to convert 2D electrophoresis to a capillary format, we start by assuming that the gel strip would be replaced by a gel-containing capillary (Figure 1C) and the gel slab replaced by a set of closely spaced, parallel capillaries (Figure 1D). This conversion requires solving a number of problems. One of them is the connection between the capillary for the separation in the first dimension (the "1D capillary") and the perpendicular capillaries for the separation in the second dimension (the "2D capillaries"). To transfer the proteins from all sections of the gel inside the 1D capillary simultaneously to the 2D capillaries, multiple openings along the 1D capillary are required. If the 1D capillary is connected with the 2D capillaries when the separation in the first dimension is carried out (Figure 1E shows an example of a simple design), the proteins in the 1D capillary will diffuse to some extent into the 2D capillaries during this first separation. To avoid this loss by diffusion, the multiple openings along the 1D capillary must be sealed during the separation in the first dimension and then connected to the perpendicular 2D capillaries for the separation in the second dimension.

In this work, we have designed and fabricated a prototypical system of microfluidic channels that addresses the problem of converting 2D electrophoresis into a capillary format. The strategy rests on creating a design in which the 1D capillary can be filled and the first-dimensional separation carried out, with this system physically isolated from the array of 2D capillaries. After completion of the first-dimensional separation, the 1D channel is physically connected to the 2D capillaries. This design takes advantage of the ability to fabricate 3D microfluidic systems in poly(dimethylsiloxane) (PDMS)[29,30] and of the fact that the elastomeric nature of PDMS[31] makes it possible to make reversible connections between two pieces of PDMS that do not leak fluid, compared to glass or other polymer materials.[32]

RESULTS AND DISCUSSION

Design of a 2-D Electrophoresis System. Figures 2−4 show the design of a 2D electrophoresis system using microfluidic networks fabricated in PDMS. The design includes seven steps: in steps 1−4 (Figure 2), we fabricate the network for the first-dimensional separation (IEF); in step 5 (Figure 3), this network is disassembled and the IEF gel is imbedded in a PDMS matrix; and in steps 6 and 7 (Figure 4), with the IEF gel and other new components, we reassembled the microfluidic network for the second-dimensional separation. The disassembly and reassembly of the system are easy to perform; each step takes less than 5 min to finish manually.

(1) A PDMS membrane (Figure 2A(b)) containing a horizontal channel and three sets of vertical channels was fabricated as described in the Experimental Section. The horizontal channels are designed for the 1D separation and the vertical channels for the connections between different layers. The PDMS membrane was 200 μm thick. The horizontal channel was 25 mm long and had a square cross section (100 × 100 μm^2); its top was connected to a set of vertical channels (each had a 100 × 100 μm^2 cross section and was 100 μm tall). On either side of the membrane was one set of 200-μm-tall vertical channels (each channel had a 100 × 100 μm^2 cross section). In each set of these vertical channels, two neighboring channels were separated by 100 μm.

(2) A duplicate piece of PDMS membrane was fabricated. These two pieces of membranes were brought together with opposite orientation so that the horizontal channels were next to each other (Figure 2A(b) and (c)). Because PDMS is an elastomer, the two pieces sealed to each other reversibly on making van der Waals contact. Before bringing these two pieces into the

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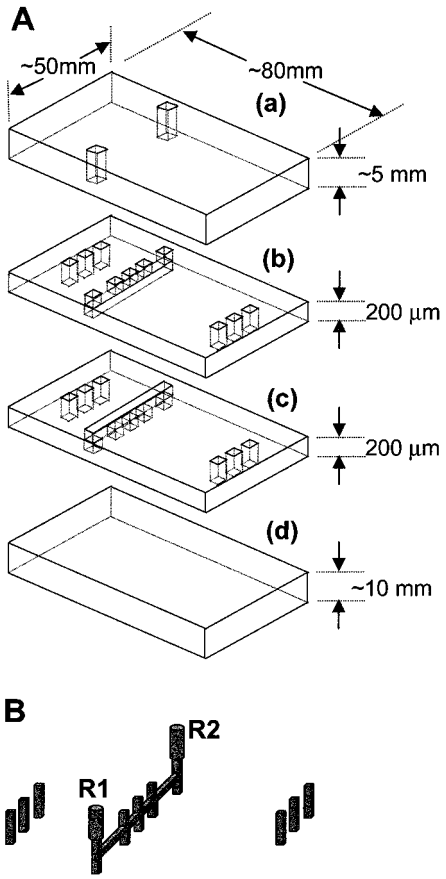

Figure 2. (A) The four PDMS components of the microfluidic system for separation in the first dimension. Component a contains only inlet and outlet ports. There are two PDMS membranes (b and c) that, together, make up a multilevel channel system. Component d is simply a flat slab of PDMS used to close the lower vertical channels in membrane c. (B) This figure shows the layout of channels when the four pieces of PDMS are aligned and assembled. R1 and R2 are two reservoirs prepared in component a before assembly. This system (and the further elaboration of the system in Figures 3 and 4) shows three capillaries in the second dimension; the system actually fabricated had 100 capillaries, and it would have been straightforward to fabricate more.

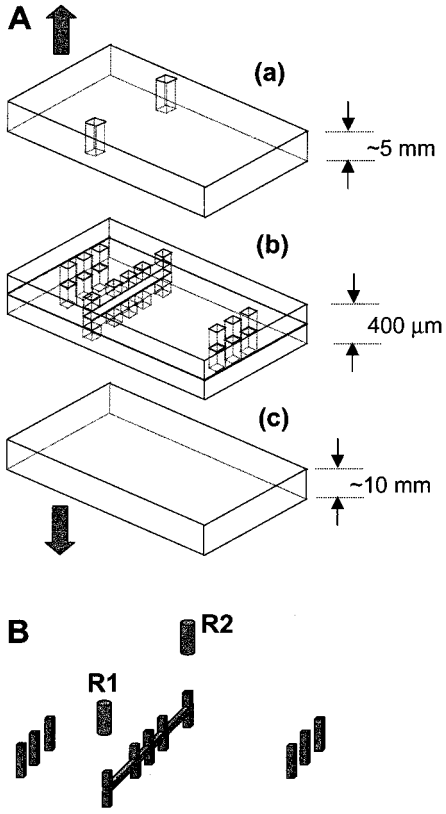

Figure 3. PDMS microfluidic system. (A) After electrophoresis in the first dimension, the PDMS microfluidic system is disassembled into three parts: (a) the top PDMS slab that contains two reservoirs, (b) the composite PDMS membrane that contains the gel-filled channel, and (c) the bottom PDMS slab. (B) This diagram shows the channel layout after electrophoresis in the first dimension and disassembly of the PDMS components. The central two membranes (b and c of Figure 2) are left together.

contact, the two horizontal channels were carefully aligned under a microscope so that one was positioned exactly on top of the other. After making contact, the two horizontal channels combined to make a larger channel ($100 \times 200\ \mu m^2$ cross section). The seal is sufficiently tight that the two pieces of membranes can be treated as one: after they are sealed to another flat surface reversibly, they can be peeled away together, without disrupting the combined horizontal channels.

(3) The composite PDMS membrane was sandwiched between two slabs of PDMS. One was ~5 mm thick (Figure 2A(a)) and contained two holes of ~5-mm diameter to allow the addition of reagents and the insertion of electrodes. The second, ~10 mm thick (Figure 2A(d)), was simply a flat surface used to seal the ends of the vertical channels in the central composite membrane. The pieces sealed to each other reversibly on contact through van der Waals interactions. Two holes on the top PDMS slab were drilled before assembly of the pieces. The ends of the vertical channels in the central composite membrane were sealed by the two PDMS slabs. Figure 2B shows the channel layout for this combined system. R1 and R2 are two reservoirs defined by the two holes in Figure 2A(a). The horizontal channel is for the 1D, IEF separation step described in step 4. The use of the vertical channels is described in steps 6 and 7.

(4) A mixture of soluble gel (details described in the section on the 1D IEF) and protein was added to R1 and pulled into the channel by applying vacuum at R2. The mixture filled the 1D channel and the small vertical channels connected to it. After the gel solidified in the channel, excess gel in the reservoirs was carefully dug out and replaced with buffers. Platinum wires were placed in the two reservoirs. A voltage (1200 V) was applied, and the IEF separation was carried out.

(5) After the 1D separation was complete, the buffers in the reservoirs were removed and the top PDMS slab (Figure 3A(a))—which was thin enough to be bent easily—was peeled away. The composite PDMS membrane (Figure 3A(b)) was then carefully peeled away from the bottom PDMS slab (Figure 3A(c)). At this stage, the composite PDMS membrane was separated from its supporting PDMS slabs. The 1D electrophoresis gel with the included proteins was enclosed in this PDMS membrane and can be transferred to the system required to accomplish the separation in the second dimension. Figure 3B shows the channel layout of the separated system.

(6) Two PDMS slabs (Figure 4A(a) and (c)) containing parallel horizontal channels were fabricated as described in the Experi-

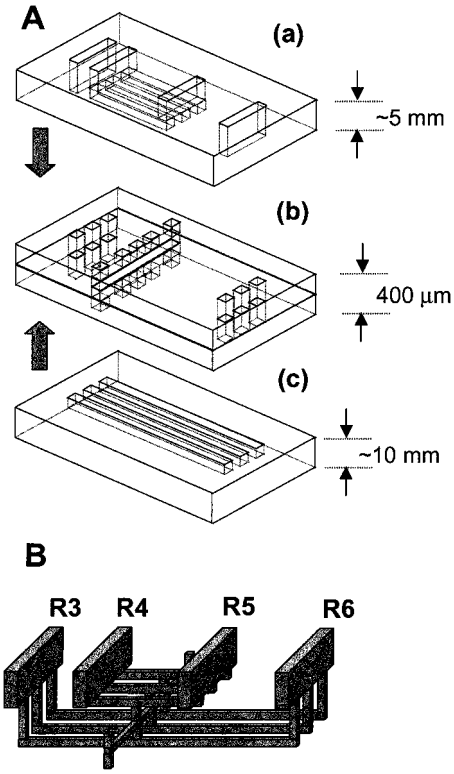

Figure 4. (A) PDMS components used to assemble the microfluidic system for electrophoresis in the second dimension. (a) The top PDMS slab contains a set of parallel channels and four reservoirs. (b) The composite PDMS membrane that contains the gel from the electrophoresis in the first dimension. (c) The bottom PDMS slab contains a set of parallel channels. (B) This diagram shows the channel layout after the PDMS pieces are assembled together for electrophoresis in the second dimension. R3, R4, R5, and R6 are four reservoirs prepared in component a before assembly.

mental Section. The channels were 100 μm deep, 100 μm wide, and separated by 100 μm. The channels in the bottom PDMS slab were 60 mm long, and those in the top PDMS slab were 30 mm long. Four reservoirs were prepared (before assembly) in the top PDMS slab by cutting out rectangular areas (4 mm by 35 mm) of PDMS. Two reservoirs (R4, R5) were fabricated at the ends of the horizontal channels in the top slab of PDMS. The other two reservoirs (R3, R6) were aligned with the ends of the horizontal channels in the bottom slab of PDMS. The composite PDMS membrane containing the gel and mixture of proteins from IEF (step 5) (Figure 4A(b)) was then sandwiched between these two PDMS slabs. Minimal alignment is required before bringing the three parts together. Because the 1D separation channel in the PDMS membranes (Figure 4A(b)) was aligned approximately perpendicular to the horizontal channels in the PDMS slabs (Figure 4A(a) and (c)), each vertical channel across the 1D separation channel connected one horizontal channel on the top with another on the bottom (Figure 4A(a)). The vertical channels in the membrane used for IEF thus provided connectivity between the gel inside the 1D separation channel and the horizontal channels above it, and between the gel inside the 1D separation channel and the horizontal channels below it. The other two rows of vertical channels in the PDMS membranes (Figure 4A(b)) provided connectivity between the lower horizontal channels and the reservoirs (R3, R6) defined in the top PDMS slab. The PDMS pieces were simple placed together and all the contacts were through van der Waals interactions. Figure 4B shows the layout of the connected channels.

(7) By filling reservoir R4 (Figure 4B) with liquid and applying vacuum on reservoir R5 (Figure 4B), a mixture of gel and SDS was injected into the horizontal channels in the top PDMS slab. Using a similar procedure, the same mixture of SDS and gel was made to fill the horizontal channels in the bottom PDMS slab. Platinum wires were placed in two reservoirs R4 and R6, and a voltage was applied to perform the 2D SDS separation in the set of parallel capillaries. The ion current flowing from R4 to R6 brought the proteins out of the IEF gel and allowed SDS electrophoresis to occur in the horizontal channels of the bottom PDMS slab.

Demonstration of Separation in the First Dimension: Isoelectric Focusing. A simple protein mixture containing fluorescein-conjugated bovine carbonic anhydrase II (CA^F), fluorescein-conjugated bovine serum albumin (BSA^F), and Texas-Red-conjugated ovalbumin (Ov^{TR}) was used to demonstrate IEF in the PDMS channel shown in Figure 2B. The values of p*I* of CA, BSA and ovalbumin are 5.9, 4.8 and 5.1, respectively.[33] Conjugation of the fluorescent tags results in shift in the values of p*I* from their native values. The shift in p*I* will depend on the number of fluorescence tags per protein molecule; the IEF band is thus expected to broaden (relative to the native protein) due to heterogeneity in extent (and perhaps position) of labeling.

Beckman cIEF gel was used as the separation medium for IEF. Since cIEF gel is a liquid, we added 1% agarose to facilitate gel solidification inside the channel. After the cIEF gel was heated to 70 °C and the agarose powder dissolved, a protein stock solution containing CA^F, BSA^F, and Ov^{TR} was added to the gel mixture.[34,35] The gel mixture was then injected into the channel in the composite PDMS membrane using a vacuum. The gel solidified on cooling to room temperature. The anolyte solution (10 mM phosphoric acid) was added to reservoir R1 and the catholyte solution (20 mM NaOH) was added to reservoir R2. A dc voltage of 500 V/cm (total voltage, 1250 V) was applied between reservoirs R1 and R2 (Figure 5A). The proteins were allowed to focus for 5 min. Figure 5A shows the fluorescence image of the section of the IEF channel containing the focused proteins. Two bands were observed when a fluorescein fluorescence filter was used, and one band was observed when a Texas Red fluorescence filter was used. The green band near the anode, the green band near the cathode, and the red band were identified as CA^F, BSA^F, and Ov^{TR}, respectively, by performing the same IEF experiments using only one protein in the gel sample.

Figure 5B plots the fluorescence intensity as a function of the distance from the anode for the section of the IEF channel shown in Figure 5A. Green and red lines represent fluorescence intensity obtained at the same positions from the green and red fluorescence images, respectively. CA^F and BSA^F are separated by IEF. Interestingly, the apparent p*I* of the CA^F is smaller than that of the BSA^F, although the p*I* of CA (5.9) is larger than that of BSA

(33) See SWISS-PROT database: http://www.expasy.ch/sprot/sprot-top.html. The value of p*I* of bovine carbonic anhydrase II was taken from the following: Deutsch, H. *Int. J. Biochem.* **1987**, *19*, 101−113.

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(35) For most proteins, a temperature of 70 °C does not cause degradation of covalent bonds (cf. ref 34).

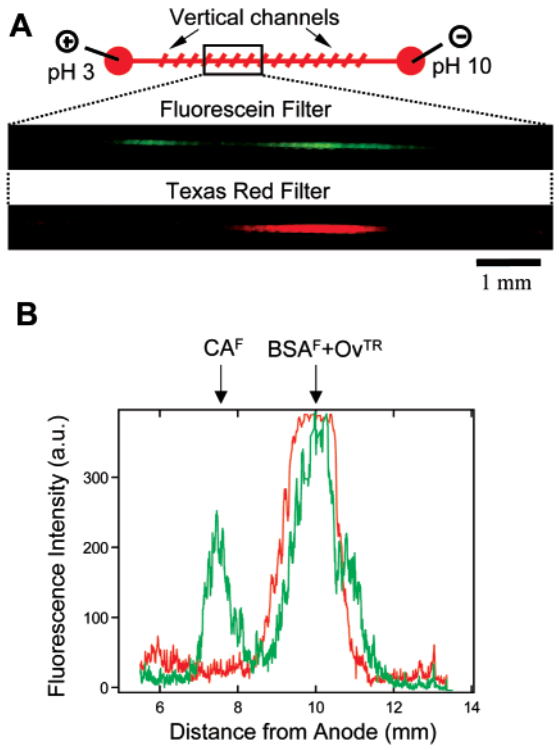

Figure 5. (A) A sketch of the IEF channel. The slanted lines crossing the long IEF channel represent the vertical short channels used to connect to the array of channels used for separation in the second dimension. Below the diagram are the fluorescence images of a section of the IEF channel containing the focused proteins (CA^F, BSA^F, Ov^{TR}). The image on top was obtained using a filter that isolated fluorescein fluorescence, and the image below was obtained using a filter that isolated Texas Red fluorescence. Both images were obtained over the same region of channels. (B) Plots of the fluorescence intensity as a function of the distance from the anode for the section of the IEF channel shown in (A). Green and red lines represent fluorescence intensity obtained at the same positions from the green and red fluorescence images, respectively.

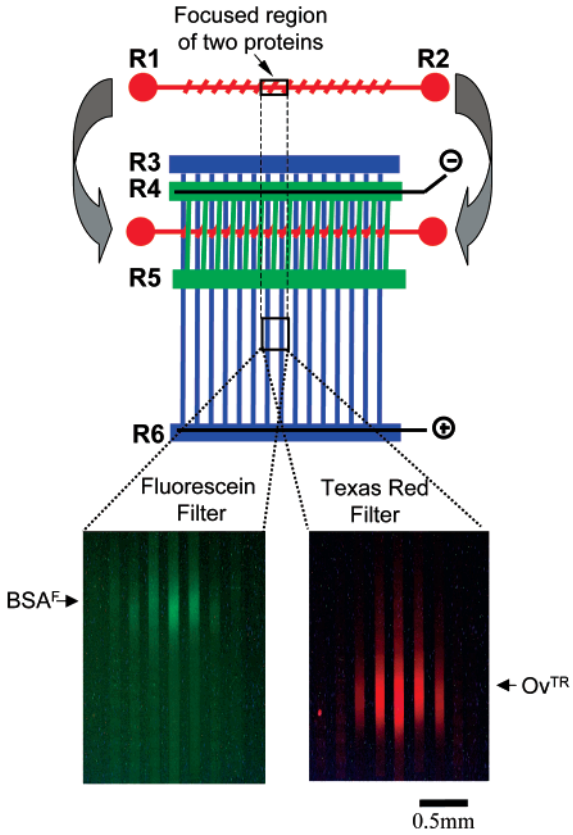

Figure 6. A sketch of the 2-D electrophoresis channels. The long red line represents the IEF channel, the parallel green lines represent the channels in the top PDMS slab, and the parallel blue lines represent the channels in the bottom PDMS slab. Below the sketch are the fluorescence images of a section of the electrophoresis channels containing the separated proteins (BSA^F, Ov^{TR}) that overlapped in the IEF separation. The image on the left was obtained using a filter that isolated fluorescence from fluorescein, and the image on the right was obtained using a filter that isolated fluorescence from Texas Red. Both images were obtained for the same region of the capillary array; this region corresponded to that into which the overlapping peaks of BSA^F and Ov^{TR} migrated. The weaker fluorescence signal in the lower portion of the fluorescein fluorescence image may be due to impurities in the BSA^F sample.

(4.8). This shift is, we presume, due to the effect of conjugation on the values of p*I*: because CA is a smaller protein than BSA (29 versus 69 kDa), the introduction of the negative charges of the fluorescein carboxylate groups may have a larger effect on the p*I* of CA than on that of BSA. The apparent p*I* of the BSA^F and that of Ov^{TR} are similar, and these species therefore were not separated by IEF.

Demonstration of Separation in the Second Dimension: SDS Gel Electrophoresis. After IEF, the composite PDMS membrane containing the IEF channel and gel was peeled away from the top and bottom PDMS slabs (Figure 3) and assembled with the top and bottom PDMS slabs containing parallel channels (Figure 4). The result of this procedure is also sketched in Figure 6, where the long red line represents the IEF channel, the parallel green lines represent the channels in the top PDMS slab, and the parallel blue lines represent the channels in the bottom PDMS slab. Reservoirs R3 and R4 (Figure 6) were filled with 10 mM Tris buffer containing 2% SDS, and a vacuum was applied to reservoirs R5 and R6 (Figure 6) to fill the top and bottom parallel channels with the buffer. The IEF gel was allowed to equilibrate with the buffer for 5 min. This equilibration step is common in conventional 2D slab gel electrophoresis; its purpose is to allow binding of SDS to the proteins in the IEF gel. Shorter equilibration times may result in incomplete SDS binding to the proteins, and longer equilibrium time may result in significant loss of protein from the IEF gel due to diffusion of proteins to the SDS buffer. After the equilibration step, reservoirs R3 and R4 were then replaced with Beckman Coulter eCAP SDS gel and a vacuum was applied to reservoirs R5 and R6 to fill the top and bottom parallel channels with this gel.

A dc voltage of 500 V/cm (total voltage, 1500 V) was applied between R4 and R6 to transfer protein to the channels in the bottom parallel channels and perform SDS electrophoresis. The running time of the separation was 1.5 min. Figure 6 shows fluorescence images of a section of the bottom array of parallel channels. The section shown was downstream from the region of the IEF channel where BSA^F and Ov^{TR} were focused. One "cluster" of protein bands across several parallel channels was observed when a fluorescein fluorescence filter was used, and another "cluster" of protein bands was observed when a Texas Red fluorescence filter was used. These clusters are analogous to the "spots" in 2D gel. The green "spot" corresponds to BSA^F

and the red "spot" corresponds to Ov^{TR}. These two protein conjugates, which were focused in the same spot in IEF, were thus successfully separated from each other in the second dimension by SDS gel electrophoresis. The "spot" of Ov^{TR} migrated more rapidly than the "spot" of BSA^{F}; This difference is expected, since ovalbumin is a smaller protein (MW = 45 000) than BSA (MW = 69 000). The spot sizes of the separation are comparable (~1 mm) to conventional 2D electrophoresis (usually 0.5−2 mm or smaller, depending on the amount of samples used).[9]

CONCLUSIONS

This paper describes a prototype for a design that will carry out 2D gel electrophoresis in a capillary format. The principal focus of the work was in the design and prototyping of the capillary system rather than optimization of the separations or maximization of the efficiency of the gels. The 2D capillary electrophoresis system shown here exploits the properties of PDMS and the ability of soft lithography to fabricate complex 3D channel systems. A key element in this design is the ability to assemble and disassemble the system as required to assemble 1D and 2D separation systems.

This design is a step toward conversion of 2D electrophoresis from slab gel to capillary format. In conventional 2D slab gel methods, handling of the IEF gel strip is often required to bring it to contact with the gel slab used for SDS separation. This process is difficult in a miniaturized format since an ultrathin gel strip would be difficult to handle. In the method described here, the IEF gel is protected during manipulation by the PDMS membranes, which are mechanically much easier to handle. The size of the IEF gel enclosed in the channel of the PDMS membrane, which is 100 by 200 μm in this demonstration, can in principle be made smaller. Due to the high electric field that can be applied to the capillary format, the separation time in our prototype system (minutes) is much less than that in a traditional 2D electrophoresis (hours).

Previous studies have demonstrated 2D separations in which capillary electrophoresis is coupled with another separation method.[36,37] In those systems, the effluent from the first dimension was repetitively injected into the second dimension. In essence, fractions from the first-dimensional separation were analyzed serially in a second dimension. A feature of the design shown in this paper is the ability to extract proteins separated in the first-dimensional separation in parallel. The long horizontal channel in the PDMS membranes encloses and stabilizes the IEF gel; the array of short vertical channels provides passages for protein migration out of the IEF gel into the capillary array used in 2D separation. This type of extraction is not available in a conventional capillary format, where the only way to get the sample out of a capillary is through its two ends.

The "injection" method we used more protein from the IEF to the SDS gels in the capillary format is one design that conserves the characteristic of conventional 2D gel electrophoresis where the focused bands from IEF are injected into the SDS gel simultaneously. There are, however, problems with this approach. Due to the lateral gaps between the vertical channels, proteins in different portions of the IEF gel have longer or shorter paths that they must traverse to migrate into the SDS gel. These differences lead to broader bands in the second dimension than in the first. Minimizing these gaps may alleviate this problem. Another possible reason for broader bands is the failure of PDMS to remove heat from the sample efficiently.

Notwithstanding these problems, the approach demonstrated here suggests the capillary systems that can be assembled and disassembled and provides a strategy for building multicapillary systems. The characteristics of PDMS (and, in principle, other elastomers) offers one practical approach to this type of system. In future work, we intend to optimize the efficiency of separations in our system and to develop it in more useful configurations.

EXPERIMENTAL SECTION

Materials. Fluorescein-conjugated bovine serum albumin and Texas Red-conjugated ovalbumin were purchased from Molecular Probes (Eugene, OR). Bovine carbonic anhydrase II was obtained from Sigma (St. Louis, MO) and conjugated with fluorescein using 5-(and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes) following manufacturer's instructions. SDS 14−200 gel buffer, cIEF polymer solution, and cIEF Ampholyte 3−10 were purchased from Beckman Instruments (Fullerton, CA).

Fabrication of the Flat PDMS Slabs in Figure 2. A 10:1 mixture of PDMS prepolymer and curing agent (Sylgard 184, Dow Corning, Midland, MI) was stirred thoroughly and then degassed under vacuum. The prepolymer mixture was poured onto a plain silicon wafer placed in a Petri dish, degassed under vacuum, and cured at 65 °C for 3 h. After curing, the PDMS slab was peeled away from the silicon wafer. The thickness of the PDMS slabs was controlled by the quantity of PDMS prepolymer applied. Two reservoirs R1 and R2 (Figure 2B) were prepared by drilling the top PDMS slab using a metal tube (5-mm diameter) with sharpened edges at one end.

Fabrication of PDMS Slabs Containing One-Level Channel Systems. The PDMS slabs in Figure 4 were prepared using a rapid prototyping method for creating microfluidic systems in PDMS.[38] Designs of networks of microfluidic channels were created in a CAD program (Freehand 8.0, Macromedia, San Francisco, CA). High-resolution (3386 dpi) transparencies were produced by a commercial printer from the CAD files with the design clear and the background black. Transparencies were used as masks in contact photolithography on negative photoresist (SU-8 10, Microlithography Chemical Corp., Newton, MA) spin-coated onto silicon wafers to create masters. We spin-coated at 580 rpm for 15 s and soft baked at 95 °C for ~2 h to create features of photoresist 100 μm high. After development in propylene glycol methyl ether acetate (Aldrich, Milwaukee, WI), the masters were placed in a desiccator under vacuum for 2 h with a vial containing a few drops of tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (United Chemical Technologies, Bristol, PA); silanization of the master facilitates the removal of the PDMS replica after molding.

A 10:1 mixture of PDMS prepolymer and curing agent was stirred thoroughly and then degassed under vacuum. The prepolymer mixture was poured onto the master placed in a Petri dish, degassed under vacuum, and cured at 65 °C for 3 h. After curing, the elastomeric replica of the master containing a negative

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relief of channels was peeled away from the silicon wafer to yield the desired PDMS slab. The thickness of the PDMS slabs was controlled by the quantity of PDMS prepolymer applied. Four reservoirs R3, R4, R5, and R6 (Figure 4B) were prepared by cutting rectangular areas out of the top PDMS slab using a scalpel.

Fabrication of PDMS Membranes Containing Two-Level Channel Systems. A master composed of two levels of a positive relief of photoresist on a silicon wafer was fabricated using two-level photolithography.[29] In brief, (1) a layer of negative photoresist (SU 8−10), 100 μm thick, was spin-coated (∼580 rpm, 15 s) on a silicon wafer and soft-baked at 95 °C for ∼2 h to drive off solvent. (2) A transparency containing the design of the lower level channel network was used as a photomask and the photoresist was exposed to make the first-layer features. (3) The photoresist was hard-baked at 95 °C for ∼0.5 h and a second layer of negative photoresist, also 100 μm thick, was spin-coated on top of the first layer and soft-baked to drive off solvent. (4) A second transparency containing the design for the second level of the channel system (the vertical channels above the horizontal channel and the upper half of the 200-μm-high vertical channels) was aligned (using the Karl Suss mask aligner) to the first-layer features. (5) The photoresist was exposed and hard-baked. (6) The master was developed in propylene glycol methyl ether acetate to yield two levels of high-relief features. (7) The master was then silanized with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane and was ready for making PDMS membranes.

A few drops of a 10:1 mixture of PDMS prepolymer and curing agent was placed on the master containing two levels of high-relief features. A piece of flat plastic (bottom of a Petri dish) was placed on the PDMS prepolymer. and pressure was applied on the plastic until prepolymer was excluded from between features on the master that were in contact with the plastic. After curing at 65 °C for 3 h, the resulting PDMS membrane, containing two layers of channel system, was then peeled away from the flat plastic and the silicon wafer.

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