

Integration of Isoelectric Focusing with Parallel Sodium Dodecyl Sulfate Gel Electrophoresis for Multidimensional Protein Separations in a Plastic Microfluidic Network

Yan Li,[†] Jesse S. Buch,[†] Frederick Rosenberger,[‡] Don L. DeVoe,^{‡,§} and Cheng S. Lee^{*,†,‡}

Department of Chemistry and Biochemistry and Department of Mechanical Engineering and Institute for System Research, University of Maryland, College Park, Maryland 20742, and Calibrant Biosystems, 7507 Standish Place, Rockville, Maryland 20855

An integrated protein concentration/separation system, combining non-native isoelectric focusing (IEF) with sodium dodecyl sulfate (SDS) gel electrophoresis on a polymer microfluidic chip, is reported. The system provides significant analyte concentration and extremely high resolving power for separated protein mixtures. The ability to introduce and isolate multiple separation media in a plastic microfluidic network is one of two key requirements for achieving multidimensional protein separations. The second requirement lies in the quantitative transfer of focused proteins from the first to second separation dimensions without significant loss in the resolution acquired from the first dimension. Rather than sequentially sampling protein analytes eluted from IEF, focused proteins are electrokinetically transferred into an array of orthogonal microchannels and further resolved by SDS gel electrophoresis in a parallel and high-throughput format. Resolved protein analytes are monitored using noncovalent, environment-sensitive, fluorescent probes such as Sypro Red. In comparison with covalently labeling proteins, the use of Sypro staining during electrophoretic separations not only presents a generic detection approach for the analysis of complex protein mixtures such as cell lysates but also avoids additional introduction of protein microheterogeneity as the result of labeling reaction. A comprehensive 2-D protein separation is completed in less than 10 min with an overall peak capacity of ~1700 using a chip with planar dimensions of as small as 2 cm × 3 cm. Significant enhancement in the peak capacity can be realized by simply raising the density of microchannels in the array, thereby increasing the number of IEF fractions further analyzed in the size-based separation dimension.

Since the concept of micro-total analysis systems (μ TAS) was first proposed,¹ the field has advanced rapidly, with ongoing

developments promising to profoundly revolutionize modern bioanalytical methodologies. Whether termed μ TAS, lab-on-a-chip, or microfluidics, the collection of technologies that define the field is proving to be an important innovation capable of transforming the ways in which bioanalytical techniques are performed. Reduced size and power requirements lead to improved portability, with higher levels of integration possible, for example, on-chip micropumps for sample manipulation or liquid chromatography separations.² Microfabrication methods lend themselves to the formation of complex microfluidic systems, opening the way to highly parallel analytical tools, while realizing low per-unit cost for disposable applications.

Furthermore, the low-volume fluid control enabled by microfluidics allows smaller dead volume and reduced sample consumption, while the low Reynolds numbers that characterize most microfluidic systems lead to highly laminar flow, eliminating the need for considering turbulent effects during instrument design. Many efficient pumping methods, including capillary action and electroosmotic flow, scale favorably in these systems, enabling valveless flow control at the microscale. Similarly, thermal time constants tend to be extremely small due to the large surface area-to-volume ratio, reducing the onset of significant Joule heating during electrokinetic separations and thus allowing higher separation voltages for shorter analysis times and equivalent or better separation resolution for complex mixtures in an integrated format.

Microfluidic systems also hold great promise for realizing multidimensional separations in a single integrated system. Assuming the separation techniques used in the two dimensions are orthogonal, i.e., the two separation techniques are based on different physicochemical properties of analytes, the peak capacity of two-dimensional (2-D) separations is the product of the peak capacities of individual one-dimensional methods and can be employed for the analysis of complex mixtures.³ To this end, several groups have explored the application of microfluidics to multidimensional peptide and protein separations. For example, Rocklin and co-workers have demonstrated 2-D separation of peptide mixtures in a microfluidic device using micellar electro-

* To whom all correspondence should be addressed. Phone: (301) 405-1020. Fax: (301) 314-9121. E-mail: CL143@umail.umd.edu.

[†] Department of Chemistry and Biochemistry, University of Maryland.

[‡] Calibrant Biosystems.

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kinetic chromatography and zone electrophoresis as the first and second dimensions, respectively.⁴ Gottschlich et al. have also fabricated a spiral-shaped glass channel coated with a C-18 stationary phase for performing reversed-phase chromatographic separation of trypsin-digested peptides.⁵ By employing a cross interface, the eluted peptides from the micellar electrokinetic chromatography⁴ or reversed-phase chromatography channel⁵ were sampled by a rapid zone electrophoresis separation in a short glass microchannel. Additionally, Herr and co-workers have coupled isoelectric focusing (IEF) with zone electrophoresis for 2-D separations of model proteins using plastic microfluidics.⁶

In each of these examples, the multiple separation dimensions are performed serially, without the ability to simultaneously sample all proteins or peptides separated in the first dimension for parallel analysis in the second dimension. As an early step toward this goal, a microfabricated quartz device has been proposed by Becker and co-workers with a single channel for the first dimension and an array of 500 parallel channels with submicrometer dimensions as the second dimension positioned orthogonally to the first dimension channel.⁷ In a further step, Chen et al. recently described a 2-D capillary electrophoresis system based on a six-layer poly(dimethylsiloxane) (PDMS) microfluidic system.⁸ The system consisted of a 25-mm-long microchannel for performing IEF, with an intersecting array of parallel 60-mm-long microchannels for achieving sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This six-layer PDMS microfluidic device, however, required the alignment, bonding, removal, re-alignment, and rebonding of various combinations of the six layers to perform a full 2-D protein separation.

It should be emphasized that the extremely high resolution of 2-D PAGE for protein separation is mostly contributed by IEF under denaturing conditions in the first separation dimension. Attempts to perform native 2-D electrophoresis results in 2-D protein patterns with poor reproducibility, smears, and less distinct protein spots.⁹ In this work, non-native IEF is therefore chosen for the first separation dimension and carried out in a single-layer 2-D microfluidic network fabricated in a rigid polymer substrate. Once the focusing is complete, the focused proteins are simultaneously transferred using an electrokinetic method from the first-dimension microchannel into an array of second-dimension microchannels for achieving parallel size-dependent separations on each sampled fraction of focused proteins using SDS gel electrophoresis. The use of non-native IEF further prepares denatured protein analytes for rapid and effective formation of SDS-protein complexes required for performing electrokinetic transfer between the coupled separation dimensions and SDS gel electrophoresis.

Table 1. List of Model Proteins (Conjugated with Fluorescein) Employed in This Study

| proteins | reported pI ^a | distance from focused protein band to reservoir B (cm) | calcd pI ^b | molecular mass (kDa) |
|----------------------|--------------------------|--|-----------------------|----------------------|
| parvalbumin | 4.10 | 0.14 | 3.98 | 12.3 |
| ovalbumin | 4.50 | 0.23 | 4.61 | 45 |
| trypsin inhibitor | 4.55 | 0.23 | 4.61 | 21.5 |
| bovine serum albumin | 4.60 | 0.23 | 4.61 | 66 |
| actin | 5.20 | 0.33 | 5.31 | 43 |

^a The pI values were provided by Molecular Probes. ^b The pI values were calculated using $pI = 3 + (\text{distance from focused protein band to reservoir B} \times 7.0 \text{ pH units/cm})$.

EXPERIMENTAL SECTION

Materials and Reagents. Fluorescent protein conjugates summarized in Table 1 and Sypro Red were purchased from Molecular Probes (Eugene, OR). Pharmalyte 3–10 was acquired from Amersham Pharmacia Biotech (Uppsala, Sweden). Tris-(hydroxymethyl)aminomethane (Tris) and ultrapure urea were obtained from Bio-Rad (Hercules, CA). All other reagents, including ammonium hydroxide, bovine serum albumin, dithiothreitol (DTT), fluorescein, hydrofluoric acid, iodoacetamide (IAM), phosphoric acid, poly(ethylene oxide) (PEO, average MW 600 000), potassium hydroxide, and SDS, were acquired from Sigma (St. Louis, MO). All aqueous solutions were prepared using water purified by a Nanopure II system (Dubuque, IA) and further filtered with a 0.22- μm membrane (Costar, Cambridge, MA).

Protein Sample Preparation. Each model protein (Table 1) with a concentration of 1 mg/mL was completely denatured and reduced in a 100 mM Tris buffer (pH 8.0) containing 8 M urea and 0.1 M DTT for 2 h at 37 °C under a nitrogen atmosphere. Proteins were alkylated to maintain their reduced states during the IEF separations by adding excess IAM with a final concentration of 50 mM. The reaction was allowed to proceed for 30 min at room temperature in the dark. A PD-10 size exclusion column (Amersham Pharmacia Biotech) was employed for buffer exchange, and proteins were eluted in a solution containing 10 mM Tris at pH 8.0.

Fabrication of Plastic Microfluidic Network. The fabrication of a silicon template was performed by following standard procedure involving photolithography and chemical etching. Briefly, the template starting substrate consisted of a 10-cm-diameter (100) oriented p-type silicon wafer coated with 2.4 μm of thermally grown silicon dioxide (WaferNet, San Jose, CA). Standard photolithography was performed using a transparency film mask and a contact mask aligner to pattern inverse microchannel features into a thin layer of photoresist. The wafer was then placed in a 5:1 buffered hydrofluoric acid solution to remove the exposed silicon dioxide. The remaining silicon dioxide was then used as a hard mask for silicon patterning, allowing the bulk wafer to be etched anisotropically in 45% (w/v) potassium hydroxide solution to form a positive relief mold containing three-dimensional structures for fabrication of the desired plastic microchannel designs.

Polycarbonate (PC) disks, 9.0 cm in diameter, were cut from a 1.5-mm-thick PC sheet (Sheffield Plastic, Sheffield, MA) for

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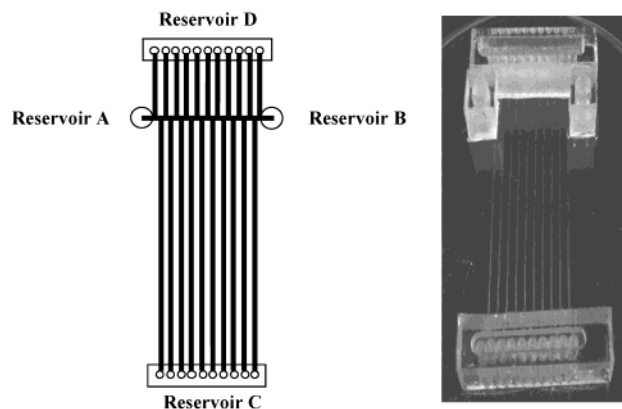


Figure 1. Schematic and image of 2-D protein separation platform using plastic microfluidics.

imprinting microchannels with the silicon template. The PC substrate was placed over the silicon template, and the whole assembly was sandwiched between two glass plates. A hydraulic press was employed to apply a pressure of 300 psi at 160 °C for 5 min. After the pressure was released, the PC substrate containing imprinted microchannels was cooled and subsequently removed from the press. The open microchannels were aligned with another PC substrate containing drilled holes for fluid access. To achieve thermal bonding between two PC substrates, the whole assembly was again sandwiched between two glass plates and placed inside the press under 900 psi and 138 °C for 10 min, creating an irreversible seal for the microchannels. The surface of the device retained optical quality following the thermal-bonding step. The entire fabrication process was performed in a class-1000 cleanroom to prevent any particulate contamination of the polymer microfluidic device.

The 2-D plastic microfluidic network employed for rapidly separating protein analytes with high resolution based on their differences in isoelectric point (pI) and molecular mass is shown conceptually in Figure 1. The newly fabricated devices were cleaned with methanol and rinsed with 10 mM Tris at pH 8.0 for 10 min. To reduce protein adsorption, the PC substrates were flushed with a bovine serum albumin solution (5 mg/mL in 10 mM Tris at pH 8.0) prior to performing 2-D protein separations.

2-D Protein Separations Using Single-Separation Medium. Two different approaches, including the introduction of single or multiple separation media into the microfluidic network, were employed to study the optimization of 2-D protein separations. For the first approach, the PC device was filled with a gel solution containing 1.5% PEO and 2% Pharmalyte 3–10. A dynamic introduction and focusing method demonstrated in our previous studies¹⁰ was utilized for only introducing protein analytes into the first separation dimension provided by the microchannel (1 cm in length, 100 μm in width, and 40 μm in depth) connecting reservoirs A and B (see Figure 1). Briefly, a mixture of protein–fluorescein conjugates (see Table 1), which were denatured, reduced, and alkylated, was prepared using the catholyte solution (30 mM ammonium hydroxide at pH 10.5) with a final concentration of 10 ng/ μL for each model protein and placed in reservoir A. A solution of 10 mM phosphoric acid at pH 2.8 was employed

as the anolyte and placed in reservoir B. Sample loading and protein focusing were performed at an electric field of 500 V/cm for 90 s using a CZE 1000R high-voltage power supply (Spellman High-Voltage Electronics, Plainview, NY).

The separation voltage was turned off as soon as the dynamic protein introduction and focusing was complete in the IEF microchannel. Reservoir D (see Figure 1) was then filled with a 10 mM Tris buffer containing 2% SDS. A positive voltage to create an electric field of 500 V/cm between reservoirs C and D was applied at reservoir C for 30 s for rapid electrokinetic injection and filling of SDS within the IEF microchannel, followed by the incubation of focused proteins with SDS for 5 min. Since non-native IEF was used as the first separation dimension, SDS–protein complexes formed very rapidly while avoiding significant diffusion band broadening of focused proteins. Additionally, the high viscosity of the gel medium also restricted diffusional band broadening during the incubation process. The rapid formation of SDS–protein complexes not only established the foundation for performing electrokinetic protein transfer but also prepared protein analytes for size-based separation in the second dimension.

All model proteins resolved in the second separation dimension were monitored using noncovalent, environment-sensitive, fluorescent probes such as Sypro Red (excitation, 550 nm; emission, 630 nm).¹¹ To introduce the probes, the 2% SDS solution originally present in reservoir D (Figure 1) was replaced with a 10 mM Tris buffer containing 0.05% SDS and $1 \times$ Sypro Red. The newly labeled negatively charged SDS–protein complexes were then electrokinetically injected into the lower microchannel array by applying a positive electric field of 500 V/cm from reservoir C to reservoir D, with reservoirs A and B electrically floating. Using this approach, each plug of focused proteins residing in the IEF microchannel between a given pair of adjacent upper channels was electrokinetically injected into its corresponding second separation dimension microchannel (4 cm in length, 100 μm in width, and 40 μm in depth). Size-based protein separation was measured using a Nikon fluorescence microscope (Melville, NY), which was equipped with a high-sensitivity charge-coupled device camera from Andor Technology (Belfast, Northern Ireland).

2-D Protein Separations Using Multiple Separation Media. IEF involved the use of carrier ampholytes for the creation of a pH gradient in the microchannel. However, the size-dependent separation of SDS–protein complexes was based on their differences in electrophoretic mobility inside a polymer-sieving matrix. Instead of using a single gel-based separation medium, the ability to introduce and isolate two different separation media in the 2-D plastic microfluidic network was developed using a pressure filling method and employed for achieving optimal separation performance. The entire PC device was initially filled with a 1.5% PEO solution. An aqueous solution containing protein analytes and 2% Pharmalyte 3–10 was introduced into the IEF microchannel from reservoir B using pressure while reservoirs C and D were completely sealed (see Figure 1). The gel/solution interface at the intersections of the microchannel array and the channel connecting reservoirs A and B was monitored by adding fluorescein into the PEO solution and using a Nikon fluorescence

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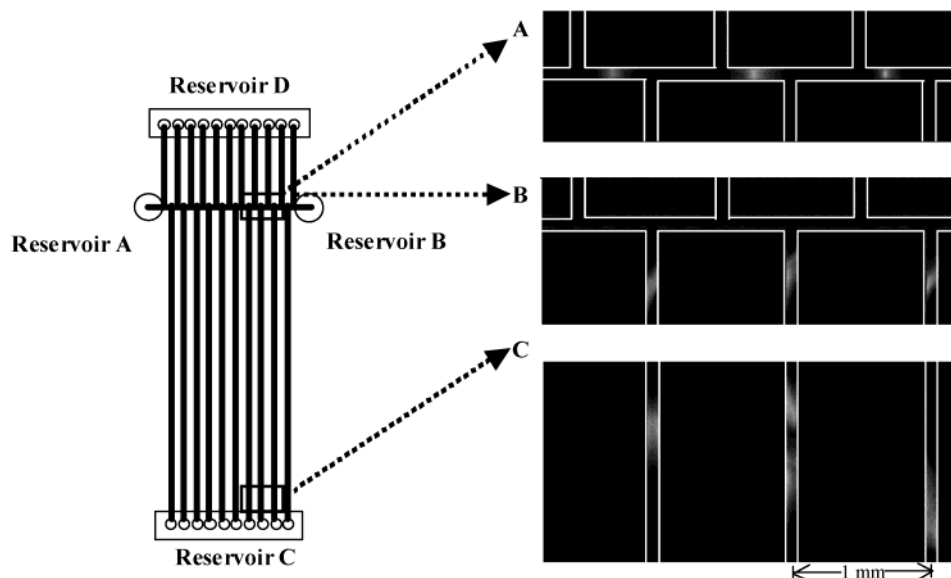


Figure 2. Fluorescent images of on-chip 2-D separation of four model proteins using single separation medium. (A) Non-native IEF with focusing order of (i) actin, (ii) bovine serum albumin and trypsin inhibitor, and (iii) parvalbumin from left to right; (B) electrokinetic transfer of focused proteins; (C) SDS gel electrophoresis. Images were captured at either 90 or 150 s following the initiation of IEF or SDS gel electrophoresis separations, respectively. Images were obtained using either green fluorescence of protein–fluorescein conjugates in IEF or red fluorescence of Sypro Red-labeled proteins during electrokinetic transfer and size-based separation.

microscope equipped with a computer-controlled moving stage. The filling was stopped as soon as the aqueous solution reached reservoir A. The solutions of 10 mM phosphoric acid (pH 2.8) and 30 mM ammonium hydroxide (pH 10.5) were added into reservoirs B and A and employed as the anolyte and the catholyte, respectively. Protein focusing was performed at an electric field of 500 V/cm for 90 s, followed by SDS–protein complexation, electrokinetic transfer, Sypro Red labeling, and size-based protein separation using the same procedures as described previously.

RESULTS AND DISCUSSION

An important aspect of any multidimensional separation platform is its ability to improve the detection of analytes present in low quantities during the analyses of complex protein mixtures. The use of IEF as the first separation dimension provides excellent resolving power with a typical concentration factor of 50–100 times. Pretreatment of the PC devices resulting in the adsorption of bovine serum albumin and carrier ampholytes onto the plastic channel walls eliminates the electroosmotic flow. This is evidenced by the lack of movement of focused protein bands after the focusing is complete. The lack of electroosmosis in chip-based IEF separations is further supported by our previous work using PC and PDMS devices pretreated with bovine serum albumin.¹⁰

The typical protein bandwidth after focusing was $\sim 130 \mu\text{m}$ inside a 1-cm-long IEF microchannel by applying electric field strength of 500 V/cm over a pH gradient from 3 to 10 (see Figure 2A). This yielded to a baseline resolution of ~ 80 peaks or pI difference as small as 0.09 pH unit. By comparing with the results obtained from single IEF separations with peak capacities of approximately 30–50,^{12–14} the combined usage of a PC substrate with the bovine serum albumin pretreatment offers improvement in separation resolution. Further enhancement in separation

resolution can be achieved by raising the applied voltage or using carrier ampholytes in support of narrow pH gradients.¹⁵

2-D Protein Separations Using Single Separation Medium. Dynamic protein introduction and focusing¹⁰ was employed to achieve sample loading in PC devices filled with a single separation medium containing 1.5% PEO and 2% Pharmalyte 3–10. Protein–fluorescein conjugates present in reservoir A (see Figure 1) continuously migrated into the IEF microchannel and encountered a pH gradient established by carrier ampholytes originally present in the channel for focusing and separation. Thus, the sample loading during dynamic sample introduction and analyte focusing is no longer dependent on channel length but is instead controlled only by electrokinetic conditions, including the injection time and the applied electric field strength. This channel length independence hypothesizes the potential for significant channel miniaturization with minimal loss in the sample loading for performing focusing-based electrokinetic separations using microfluidic devices.

Four model proteins consisting of parvalbumin (pI 4.10, MW 12 300), trypsin inhibitor (pI 4.55, MW 21 500), bovine serum albumin (pI 4.60, MW 66 000), and actin (pI 5.20, MW 43 000) were employed for early demonstration of 2-D separations in the plastic microfluidic network. To fulfill the requirements of a comprehensive 2-D separation system, any separation accomplished by the first dimension should ideally be retained upon transfer to the second dimension. To achieve this goal, an electrokinetic-based technique was demonstrated for successfully transferring focused proteins from the IEF microchannel into the second separation dimension (see Figure 2B). The transfer

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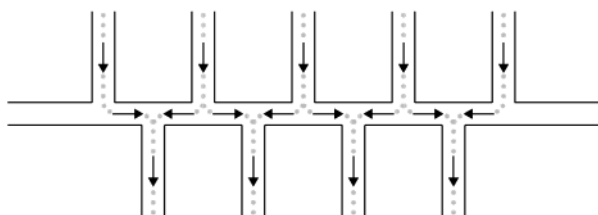


Figure 3. Schematic depicting the paths for electrokinetic introduction of SDS from reservoir D (see Figure 1) to microfluidic network and electrokinetic transfer of focused protein bands between the first and second separation dimensions.

process was facilitated by designing the microfluidic network such that the upper and lower second-dimension channels were arranged in a staggered configuration (see Figure 1). This configuration allowed for the entire contents of the first-dimension IEF channel between the two outermost channels of the upper second-dimension array to be transferred into channels of the lower second-dimension array in a single process (see Figure 3). A key benefit of this approach lies in its ability to minimize band broadening during the transfer. Still, initial studies with simple right-angle intersections such as those depicted in Figure 2B have been found to result in approximately 1.5–2 \times broadening of focused protein bands during transfer. The extent of band broadening during the transfer step is largely dependent upon the electric field distribution within the intersection region, in combination with variations in total path length traveled by proteins at different locations within the intersection.

Through the use of electrokinetic transfer, the extent of urea and focused ampholytes transferred from the first to second separation dimensions is extremely low due to the lack of net charge in both urea and focused ampholytes. Furthermore, the presence of minimal amounts of urea and ampholytes is not expected to have any significant effects on the size-based separation in the second-dimension microchannels. It is well known that the extremely high resolution of 2-D PAGE is mostly contributed by IEF under denaturing conditions. The urea utilized for protein denaturation during the sample preparation and remaining during SDS incubation exhibits essentially no impact in IEF and SDS-PAGE separations.

It has been reported that the formation of SDS-protein complexes is the critical step in determining separation resolution of capillary gel electrophoresis.¹⁶ Once the SDS-protein complexes are properly formed, they remain relatively stable and the presence of SDS in the separation buffer is no longer needed for further stabilization. This is particularly true for rapid protein separation in capillary gel electrophoresis and microfluidics-based devices. Thus, a 2% SDS solution was employed and electrokinetically introduced into the IEF channel for complexation with focused proteins. The SDS-protein complexation was allowed to proceed for 5 min. Shorter reaction times may result in incomplete SDS binding to the proteins, and longer incubation times may contribute to additional protein band broadening due to diffusion.

Instead of using green fluorescence of protein-fluorescein conjugates for acquiring the images of focused protein bands (see Figure 2A), protein analytes migrated within the intersection region (see Figure 2B) and resolved during the size-based

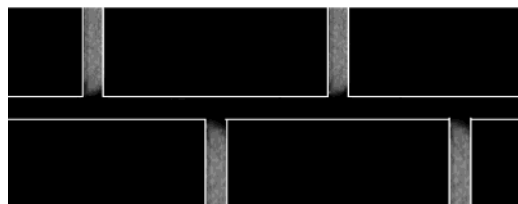


Figure 4. Image of plastic microfluidic network filled with multiple separation media.

separation (see Figure 2C) were monitored using noncovalent, environment-sensitive, fluorescent probes such as Sypro Red.¹¹ As shown in Figure 2A, the first-dimension focusing was unable to resolve trypsin inhibitor and bovine serum albumin with a pI difference of only 0.05 pH unit. However, following transfer to the second dimension and size-based separation, all four model proteins were successfully resolved.

The Sypro Red probes are nonfluorescent in water but highly fluorescent in detergent, in which they take advantage of SDS binding to proteins to build a fluorescence-promoting environment. In comparison with covalently labeling proteins, the use of Sypro staining not only presents a generic detection approach for the analysis of complex protein mixtures such as cell lysates but also avoids additional introduction of protein microheterogeneity as the result of labeling reaction. However, SDS concentration in the electrophoresis buffer has a significant effect on protein labeling using Sypro dyes. When the SDS concentration is above its critical micellar concentration, 8.3 mM (\sim 0.24% in water and somewhat less in buffer solutions), the major portion of the staining dye attaches to the SDS micelles instead of the SDS-protein complexes.

To efficiently and rapidly label SDS-protein complexes using Sypro dyes and to sensitively detect the resolved SDS-protein complexes, Csapo and co-workers¹⁷ determined the optimal SDS concentration in the electrophoresis buffer to be \sim 0.05%. Their results were confirmed in our studies and further supported by the work of Bousse et al.,¹⁸ in which an on-chip SDS dilution step was required between the separation channel and laser-induced fluorescence detection. The dilution step reduced the SDS concentration from 0.25 to \sim 0.025%. Such dilution thus broke up the SDS micelles, thereby allowing more dye molecules to bind to the SDS-protein complexes. This rearrangement prior to the fluorescence detection led to an increase in the peak amplitude by 1 order of magnitude.

Furthermore, comparison of Sypro dyes with silver staining in SDS gels has shown that this class of fluorescent dyes detects polypeptides with sensitivity similar to that obtained by silver staining.^{11,19,20} Binding of the dyes to the protein is stoichiometric and fluorescence is related to the amount of dye binding; therefore, the dynamic range is 3 orders of magnitude greater than for silver staining.¹⁹ Our current protein detection sensitivity, involving the use of a fluorescence microscope, is \sim 0.5 ng/ μ L.

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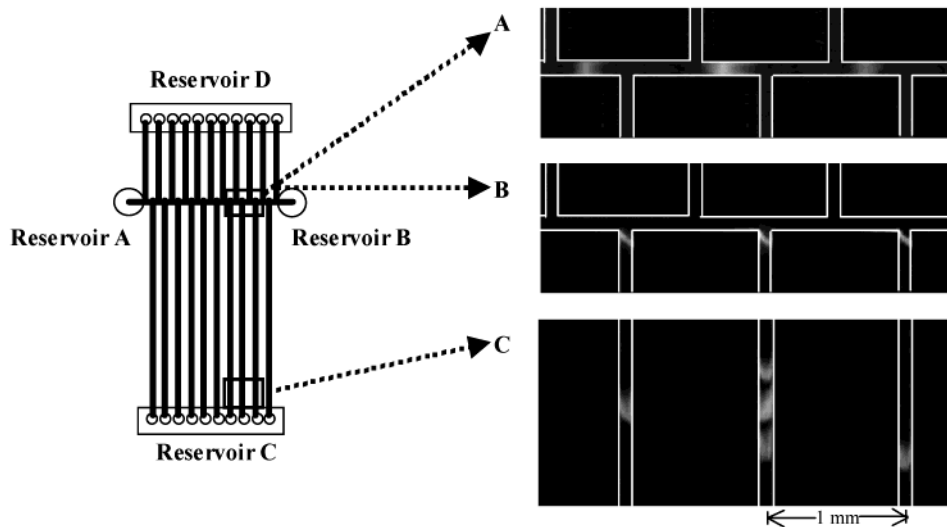


Figure 5. Fluorescent images of on-chip 2-D separation of five model proteins using multiple separation media. (A) Non-native IEF with focusing order of (i) actin, (ii) bovine serum albumin, ovalbumin, and trypsin inhibitor, and (iii) parvalbumin from left to right; (B) electrokinetic transfer of focused proteins; (C) SDS gel electrophoresis. Images were captured at 90 s following the initiation of IEF or SDS gel electrophoresis separations. Images were obtained using either green fluorescence of protein–fluorescein conjugates in IEF or red fluorescence of Sypro Red-labeled proteins during electrokinetic transfer and size-based separation.

This can be further reduced by using the dynamic introduction and focusing approach as demonstrated in our previous studies.¹⁰ Finally, it has been shown that proteins detected by Sypro staining are compatible with matrix-assisted laser desorption/ionization mass spectrometry analysis.^{20,21}

2-D Protein Separations Using Multiple Separation Media. In this work, a two-step pressure filling method has been developed to introduce and isolate two different separation media in the 2-D plastic microfluidic network. The filling process was monitored by adding fluorescein into the PEO gel solution and using a fluorescence microscope equipped with a computer-controlled moving stage. The fluorescent image of gel/solution interface (see Figure 4) at the intersections of the IEF microchannel and the microchannel array illustrated the capability for introducing multiple separation media, including the ampholyte solution containing protein analytes and the PEO gel required for performing IEF and size-based separations, respectively. While a slight degree of diffusion by small molecules such as fluorescein/ampholyte was evident in the microchannels, conventional IEF separations performed in the first-dimension microchannel exhibited negligible degradation of separation resolution due to the limited protein diffusion and the zone-sharpening effect that occurred during IEF (see Figure 5A). Further increase in sample loading can be achieved using dynamic analyte introduction and focusing as reported previously¹⁰ and employed in PC devices containing a single separation medium.

In analogy to stacking gel as employed in SDS–PAGE, the gel/solution interface in the microfluidic system provides a mechanism for protein stacking during electrokinetic transfer between the coupled separation dimensions (see Figure 5A and B). Instead of acquiring additional band broadening as observed when a single separation medium is used (see Figure 2A and B),

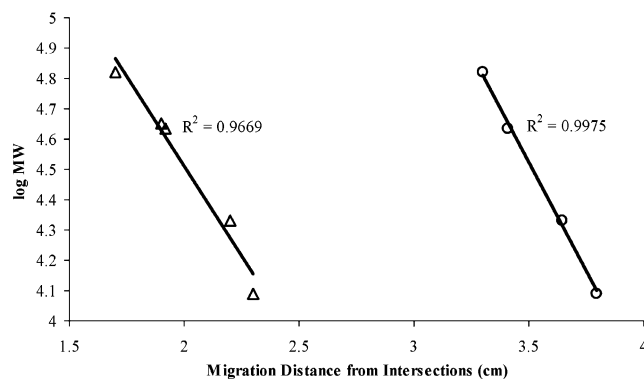


Figure 6. Standard curves of logarithmic molecular mass versus migration distance from intersections during SDS gel electrophoresis studies performed using single (○) or multiple (△) separation media. These migration distances were taken from the images presented in Figures 2C and 5C.

the bandwidth of focused proteins was reduced by a factor of ~ 2 as the result of protein stacking at the gel/solution interface. By reducing the size of the injected bandwidth in the second dimension, significant enhancement in size-based resolving power was evident for baseline resolution of bovine serum albumin, ovalbumin, and trypsin inhibitor (see Figure 5C). It should be noted that the length of the lower microchannel array was reduced from 4 cm in the single separation medium case to 2.5 cm when using multiple separation media. Furthermore, the standard curves for molecular mass estimation were constructed by plotting the logarithmic molecular masses of model proteins examined in this study against their migration distances from the intersections measured during SDS gel electrophoresis (see Figures 2C and 5C). As shown in Figure 6, a good linear correlation was demonstrated in both SDS gel electrophoresis studies over the range of 12 300–66 000.

A comprehensive 2-D protein separation was completed in less than 10 min, with the majority of time consumed in the required SDS–protein complexation reaction. Furthermore, a peak capacity

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of ~ 170 in the second dimension of size-based separation was estimated by assuming average bandwidth of $150 \mu\text{m}$ over a span of 2.5-cm channel length. Further improvements in peak capacity may be realized using longer channels in larger chips during the size-based separation. Because the separation mechanisms in IEF and SDS-PEO gel electrophoresis were completely orthogonal, the overall peak capacity in the current 2-D protein separation platform was anticipated to be around 1700 (10 fractions from IEF \times 170 from SDS-PEO gel electrophoresis). Significant enhancement in the peak capacity of the multidimensional separation platform can be realized by further raising the density of microchannels in the array, thereby increasing the number of IEF fractions analyzed in the size-based separation dimension. Due to the use of parallel separations in the second dimension, there is no accompanying increase in the analysis time.

CONCLUSION

The demonstrated on-chip combination of non-native IEF with SDS-PEO gel electrophoresis offers electrokinetic focusing for concentrating dilute protein samples while providing excellent resolving power in a multidimensional separation platform. Instead of sequentially sampling and analyzing eluants from the first separation dimension as previously reported in the literature,⁴⁻⁶ an electrokinetic transfer technique is employed to simultaneously introduce all the focused protein bands from the first to second separation dimensions, enabling parallel and high-throughput size-based separation in a solution containing SDS and PEO sieving

matrix. The ability to introduce and isolate multiple separation media in the plastic microfluidic network further provides protein stacking during electrokinetic transfer between the coupled separation dimensions.

The single-layer 2-D microfluidic network fabricated in a PC substrate not only allows straightforward implementation and automation of multidimensional protein separations in a low-cost disposable platform but also provides excellent resolving power toward the analysis of complex protein mixtures. A comprehensive 2-D protein separation is completed in less than 10 min with an overall peak capacity of ~ 1700 . The reproducibility of this technology can be further improved upon by the development of an automated system capable of performing all processes involved in the analysis in an autonomous fashion. Furthermore, the resolving power and sensitivity of the analysis can be greatly enhanced by utilizing microfluidic networks containing a higher density of second-dimension channels as well as employing more sensitive multichannel laser-induced fluorescence detection systems such as those previously reported in the literature.^{22,23} Ultimately, this work demonstrates the potential for microfluidic technology to transform conventional 2-D PAGE into an automated, portable, rapid, and reproducible protein bioanalytical technology.

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