Microfluidic Two-Dimensional Separation of Proteins Combining Temperature Gradient Focusing and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

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ABSTRACT: A two-dimensional separation system is presented combining scanning temperature gradient focusing (TGF) and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in a PDMS/glass microfluidic chip. Denatured proteins are first focused and separated in a 15 mm long channel via TGF with a temperature range of 16−47 °C and a pressure scanning rate of −0.5 Pa/s and then further separated via SDS-PAGE in a 25 mm long channel. A side channel is designed at the intersection between the two dimensions to continuously inject SDS into the gel, allowing SDS molecules to be compiled within the focused bands. Separation experiments are performed using several fluorescently labeled proteins with single point detection. Experimental results show a dramatic improvement in peak capacity over one-dimensional separation techniques.

Two-dimensional (2D) separation methods are commonly used in proteomic analysis due to their ability to resolve proteins and polypeptides in complex protein mixtures.1−3 A requirement of any successful 2D separation system is orthogonality, which means that the selected dimensions possess different, yet compatible, separation mechanisms. Furthermore, the second dimension should not destroy the resolution achieved by the previous one.1−3 2D polyacrylamide gel electrophoresis (2D-PAGE) is the most common form of such systems. In this method, isoelectric focusing (IEF) is coupled with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) for separation of complex protein mixtures based on the differences in their pI and molecular weight.1−4

While 2D-PAGE is widely used in proteomics analysis, it suffers several drawbacks. First, this method is time-consuming and quite labor-intensive due to manual operation, gel preparation, and sample transfer. Second, several steps are required to perform the separation experiments and to transfer the peaks obtained from the IEF into the gel. Third, the results obtained from this method are not quite reproducible between experiments and laboratories2,4 mainly due to the diffusion of the sample peaks inside the gel.

The use of microfluidic technology has provided the opportunity to overcome some of these problems by developing miniaturized systems that require less time and sample volume to operate and can provide higher levels of automation.2,4 In recent years, several microfluidic devices have been developed for 2D-PAGE separation of proteins.6−13 A number of efforts have also been made to combine other types of electrophoresis separation techniques in various configurations2,14−18 such as 2D micellar electrokinetic chromatography-capillary electrophoresis (MEKC-CE)15 and 2D IEF-CE.16 Although these systems provide fast separations with relatively high peak capacities, most of these configurations have complex designs which lead to poor reproducibility and/or require a mechanism to transfer the peaks between the dimensions as the two separation methods cannot function continuously.2,5

In this work, a simple microchannel network design is presented that integrates scanning temperature gradient focusing (TGF) and SDS-PAGE in a continuous format. Scanning TGF is a focusing based separation technique that utilizes an axial temperature gradient and a varying hydrodynamic bulk flow along the separation channel. Analytes enter the channel sequentially and are focused and separated simultaneously based on their charge to mass ratio as they traverse the temperature gradient.19,20 Compared to other CE techniques such as CE2 and CIEF,2 TGF only requires a very short separation channel and does not need a narrow injection band at the beginning of separation.20 It can also generate highly concentrated peaks up to 104-fold21 and thus is a suitable candidate for 2D separation systems.20

In the current design, denatured proteins are first focused and separated in a 15 mm long separation channel via TGF with a temperature range of 16−47 °C and a pressure scanning rate of −0.5 Pa/s. By gradually reducing the average bulk velocity, the focused bands are subsequently transferred toward...
the end of the first dimension, where they are continuously introduced into a 25 mm long channel filled with polyacrylamide gel. A side channel is used at the intersection between the two dimensions to continuously inject SDS into the gel, allowing SDS molecules to be mixed within the focused bands. The proteins are further separated along the second dimension based on their molecular weight using SDS-PAGE.

EXPERIMENTAL SECTION

Materials. Acrylamide/bis-acrylamide (29:1), tris-(hydroxymethyl) aminomethane base (Tris), phenol, glycine, sodium dodecyl sulfate (SDS), urea, dithiothreitol (DTT), and iodoacetamide (IAM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Water-soluble photoinitiator VA-086 were purchased from Wako Chemicals (Richmond, VA). Alexa Fluor 488 conjugated protein samples (bovine serum albumin (66 kDa), sodium dodecyl sulfate (SDS), urea, dithiothreitol (DTT), and hydroxymethyl aminomethane base (Tris), phenol, glycine, iodoacetamide (IAM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fused-silica capillaries with 200 μm i.d. were purchased from Polymicro Technologies (Tucson, AZ, USA). All solutions were made from ultrapure water (Barnstead/Thermolyne, Dubuque, IA, USA). Acrylamide/bis-acrylamide (29:1), tris-glycine buffer were obtained from Microchem (Newton, MA, USA). Milk proteins containing casein and whey inhibitor (21 kDa) were purchased from Molecular Probes (Eugene, OR, USA). Alexa Fluor 488 glycol-monoether-acetate (PGMEA) developer were obtained from Corning (Midland, MI, USA). Sulfuric acid, propylene-glycol-monoether-acetate (PGMEA) developer were obtained from Microchem (Newton, MA, USA). Milk proteins containing casein and whey inhibitor (21 kDa) were purchased from Molecular Probes (Eugene, OR, USA). Alexa Fluor 488 conjugated protein samples (bovine serum albumin (66 kDa), sodium dodecyl sulfate (SDS), urea, dithiothreitol (DTT), and hydroxymethyl aminomethane base (Tris), phenol, glycine, iodoacetamide (IAM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Water-soluble photoinitiator VA-086 were purchased from Wako Chemicals (Richmond, VA). Alexa Fluor 488 conjugated protein samples (bovine serum albumin (66 kDa), sodium dodecyl sulfate (SDS), urea, dithiothreitol (DTT), and hydroxymethyl aminomethane base (Tris), phenol, glycine, iodoacetamide (IAM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fused-silica capillaries with 200 μm i.d. were purchased from Polymicro Technologies (Tucson, AZ, USA). All solutions were made from ultrapure water (Barnstead/Thermolyne, Dubuque, IA, USA).

Fabrication of the Chip. Figure 1 shows the schematic of the 2D separation chip which consists of a glass slide coated with a thin layer of PDMS. The chip consists of a 15 mm channel for TGF separation of analytes followed by a gel filled 25 mm channel for SDS-PAGE. (b) Experimental setup for the 2D separation chip.

Figure 1. Schematic of the 2D separation chip (a). Top layer is PDMS containing the separation channel. Bottom layer is glass coated with a thin layer of PDMS. The chip consists of a 15 mm channel for TGF separation of analytes followed by a gel filled 25 mm channel for SDS-PAGE. (b) Experimental setup for the 2D separation chip.
containing 2% (wt/vol) SDS. To apply the temperature gradient along the TGF channel, thermoelectric heater/cooler units (TE-35-0.6-1, TE Technologies) approximately 5 mm in width were glued onto an aluminum holder with a distance of 2 mm from each other. The holder contained a 4 mm wide slit at the middle to enable channel visualization. The chip was then placed on the holder and taped on the heater/cooler configuration.

RESULTS AND DISCUSSION

Prior to the experiments, the temperature gradient along the TGF channel was measured using a previously reported two-color thermometry technique. Briefly, fluorescein and Alexa fluor 546 dyes were dissolved in 10 mM Tris-HCl buffer at pH 7.1. Although fluorescein is normally a poor candidate for thermometry measurements because of its weak temperature dependent quantum efficiency, it does possess strong pH dependence in the pH range of 6–7. Concurrently Tris-HCl has a strong pH—temperature dependency which allows the pH dependent fluorescent intensity to be converted into a temperature dependent response. Alexa Fluor 546 which has weak dependence on pH and temperature was used as the background dye to avoid intensity measurement errors. A temperature calibration curve was generated by introducing the solution into a microfluidic chip at different temperatures while the ratio of the fluorescent intensity of the dyes was measured using the microscope. Furthermore, the developed calibration curve was used to quantify the TGF temperature profile by measuring the ratio of the fluorescent intensity of the dyes along the TGF channel. 2.5 V was applied to each of the thermoelectric units to achieve a temperature gradient of 16–47 °C which was measured experimentally.

Experiments started with filling the TGF channel with 0.1 M Tris-phenol buffer and establishing a positive pressure difference of around 600 Pa between the buffer and sample reservoir. Electrophoresis separations were then performed by applying a negative high voltage of −2000 V (Labsmith, HV5448-3000) to the sample vial connected to reservoir “A” while keeping reservoir D grounded. The difference in hydraulic head decreased at a predetermined rate to generate a scanning rate of −0.5 Pa/s allowing the samples to be focused and moved along the TGF channel toward the gel entrance where they continuously entered the second dimension channel. A voltage of −1000 V was applied to reservoir C to ensure a high concentration of SDS inside the gel while avoiding the focused peaks to enter the side channel. The average current was measured to be around 100–150 μA during the experiments.

Experiments were first performed using an ideal set of Alexa Fluor 488 conjugated proteins (bovine serum albumin (66 kDa), ovalbumin (45 kDa), parvalbumin (12 kDa), and trypsin inhibitor (21 kDa)). These proteins were denatured without SDS using a previously reported procedure. Briefly, a total of 0.4 mg of protein sample (0.1 mg per protein) was dissolved into 0.5 mL of DI water, and urea and DTT were thoroughly mixed with the sample solution with a final concentration of 8 M and 100 mM, respectively. The reaction was allowed to proceed in the dark overnight at room temperature. Subsequently, iodoacetamide (IAM) was added to the solution to a concentration of 120 mM, and the solution was placed in the dark for 1 h at room temperature. The resulting solution was diluted 10 times with 0.1 M Tris-phenol solution for use in TGF separation experiments. The same procedure was used for preparing other protein mixtures unless stated otherwise.

To investigate the efficiency of the proposed 2D separation system, the separation of labeled proteins was recorded for both the first dimension of TGF (at the end of TGF channel right before the cooler element) and the second in the gel at about 8 mm from the gel entrance. The detection point inside the gel was carefully chosen on the basis of previous experimental evaluations so that each protein peak spends enough time inside the second dimension to be thoroughly mixed with SDS molecules while without significant diffusion prior to detection. Images of an area around 0.5 × 0.5 mm at those locations were taken using the microscope with a 10× objective. Electropherograms were then generated by cropping the images and measuring the fluorescent intensity at a smaller area of 10 × 50 μm inside the channels using a Matlab program developed in-house.

Figures 2 and 3 show the separation results for both 1D TGF and 2D separation techniques for Alexa Fluor 488 conjugated proteins and milk proteins containing casein and whey labeled with Cy3B dye, respectively. The separation performance is improved using the proposed 2D separation for both cases which is able to detect peaks that are not observed with 1D TGF separation. To quantify the improvement, peak-to-peak resolution was calculated using the following equation:

\[ R_s = \frac{\Delta t}{0.85(\Delta w_1 + \Delta w_2)} \]  

where \( \Delta t \) is the distance between the two adjacent peaks and \( \Delta w \) is the width at the half-peak height which is easier to be accurately measured than the baseline width. Tables 1 and 2 list the peak-to-peak resolution results for both 1D TGF and 2D separation techniques for the Alexa Fluor conjugated proteins and milk proteins, respectively, confirming that the overall resolution increases when the 2D design is used.

The separations were finished within 8 min for both cases which can be further adjusted by varying the TGF scanning...
Figure 3. Separation of fluorescently labeled milk proteins using both TGF and the proposed 2D separation method. Peaks order: (1) β-casein, (2) κ-casein, (3) α-casein, (4) β-lactoglobulin, and (5) α-lactalbumin.

Table 1. Peak Resolution Obtained from Separation of Four Alexa Fluor 488 Conjugated Proteinsa

<table>
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<th>parameter</th>
<th>TGF resolution</th>
<th>2D resolution</th>
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<tr>
<td>Peak ID 1–2</td>
<td>n/a</td>
<td>0.77</td>
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<tr>
<td>Peak ID 2–3</td>
<td>0.63</td>
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<tr>
<td>Peak ID 3–4</td>
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<td>1.87</td>
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“aPeaks order is from right to left.

Table 2. Peak Resolution Obtained from Separation of Fluorescently Labeled Milk Proteins Using Both TGF and 2D Separation Methodsa

<table>
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<th>parameter</th>
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<th>2D resolution</th>
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<tr>
<td>Peak ID 1–2</td>
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<tr>
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<tr>
<td>Peak ID 4–5</td>
<td>0.77</td>
<td>0.72</td>
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</table>

“aPeaks order from right to left: (1) β-casein, (2) κ-casein, (3) α-casein, (4) β-lactoglobulin, and (5) α-lactalbumin.

rate. Lower scanning rates will result in longer separation time with better sensitivity while higher scanning rates will speed up the separation but peaks will be less concentrated. Due to the fact that TGF provides highly concentrated peaks during separation (similar to IEF), the proposed method offers high detection sensitivity comparable to SDS-PAGE. As it can be seen from Figures 2 and 3, although the peak intensity slightly decreases in the second dimension due to the presence of the gel media, the sensitivity is still good and all the peaks are detectable.

Reproducibility of the experimental results for the 2D separation system was also examined and is presented in Table 3. Chip-to-chip reproducibility was investigated by comparing the peak-to-peak resolution of the 2D separation experiments for three different chips. As shown in Table 3, the RSDs are less than 8% (n = 3) for Alexa Fluor conjugated proteins and less than 11% for fluorescently labeled milk proteins indicating stable operation of the device and experimental system. Repeatability testing was not able to be conducted because the gel usually became stained which can be neither cleaned nor reused.

<table>
<thead>
<tr>
<th>sample</th>
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<th>chip 1</th>
<th>chip 2</th>
<th>chip 3</th>
<th>chip-to-chip RSD, %</th>
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<tr>
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<tr>
<td>fluorecently labeled milk proteins</td>
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<td>0.68</td>
<td>0.60</td>
<td>9.71</td>
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“Peaks order is from right to left.

CONCLUSION

A PDMS/glass microfluidic chip was developed for continuous two-dimensional separation of proteins, combining temperature gradient focusing (TGF) and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). In this system, denatured proteins are first focused and separated using the scanning TGF method, and the focused bands are further separated on the basis of their size after they are continuously introduced into the polyacrylamide gel which was fabricated using photopolymerization techniques. A side channel is used at the beginning of the second dimension to continuously inject SDS into the gel, allowing defined volumes of SDS to be composed within the focused bands. The temperature gradient along the TGF channel was created using thermoelectric cooler/heater units under the chip surface. The scanning bulk flow was created by adjusting the head difference between the sample and buffer reservoirs using a motorized linear stage. The demonstrated separation resolution for 2D TGF-PAGE is not as high as some of the reported 2D separation methods such as 2D-PAGE, 2D MEKC-CE, and 2D IEF-CE which can be further improved by optimizing the scanning rate. This study focused on exploring the feasibility of 2D TGF-PAGE and therefore did not perform thorough optimization testing. In addition, this method does offer several benefits that other 2D separation methods lack, including (i) its simple design allows for easier and more robust chip fabrication with better reproducibility and allows for reduced reagent consumption which is more environmentally friendly and results in lower cost; (ii) its separation is in a continuous manner eliminating additional actions/steps for sample transfer between dimensions which complicates the operation and often results in low separation resolution; (iii) it allows the focused bands from the first dimension to entirely enter the second dimension...
eliminating sample loss; and (iv) it utilizes single point detection resulting in an easier experimental setup.

Separations of four Alexa Fluor conjugated proteins and fluorescently labeled milk proteins were performed using the developed 2D configuration, and the results were recorded both during the TGF separation (first dimension) and at about 8 mm from the gel entrance. The results show a dramatic improvement in peak capacity and resolution with the proposed 2D system compared with the 1D TGF separation results.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge the support from Natural Science and Engineering Research Council, Canada Foundation for Innovation, and Advanced Electrophoresis Solutions Ltd. through grants to C.L.R.

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