Multi-Dimensional Microchip Electrophoresis of Proteins

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Abstract

The popularity of micro-Total Analysis Systems (μ-TAS) is exponentially increasing, especially in the fields of medical and biological analyses. The work presented here includes 2-D separation of proteins in PMMA-based microchips. Sodium Dodecyl Sulfate Capillary Gel Electrophoresis (SDS-CGE) and Micellar Electrokinetic Chromatography (MEKC) were used as the separation scheme for the first and second dimension of electrophoresis, respectively. The separation mechanism in MEKC is based on differences in distribution coefficient between the micellar and the non-micellar (aqueous) phases. SDS-CGE is the adaptation of traditional gel electrophoresis into the microchannels using polymers in solution as sieving matrices. This allows solutes having similar charge-to-mass ratios to be resolved by size. Both dimensions were prepared in a hot-embossed PMMA microchip. Electrokinetic injection and separation were used with field strengths ranging from 100-400 V/cm. To perform this, an automated-programmable power supply was used. Laser-induced fluorescence (LIF) was used as the detection method for dye-labeled proteins ranging from 38 kDa to >100 kDa in size. Each peak in the first dimension (SDS-CGE) is shifted to the second dimension for further separation using MEKC. Finally, a 2D separation platform is reported for protein samples that were efficiently separated in a few minutes.

Keywords: Electrophoresis, Protein, Microchip, Two-dimensional

1. Introduction

Proteomics, namely the large-scale screening of proteins of a cell, organism, or biological fluid, was given its name in the mid 1990s but had actually originated over 20 years ago when the separation of proteins from whole cell extracts was accomplished [1]. However, the studies of protein mixtures of biological origin often impose significant analytical challenges due to their complexity. Recent trends currently employed in laboratories involve the development of miniaturized and integrated total chemical analysis systems (μ-TAS) [2]. They provide shorter analysis time, smaller device size, disposability, lower consumption of chemicals, and lab-on-a-chip versatility [3]. The use of microchannels minimizes band-broadening, providing efficient dissipation of Joule heat, and allows the application of high electric fields [4]. Electrokinetically driven separation techniques eliminate the need for pumps and, therefore, enable a simple instrument design. In addition, the flat flow profile in an electrokinetically driven system reduces band broadening compared to a parabolic (Poiseuille) flow profile in pressure-driven systems. Electrokinetically driven separation techniques demonstrated on microchips include capillary electrophoresis (CE), micellar electrokinetic chromatography (MEKC), electrochromatography, and gel electrophoresis [5].

Many fabrication materials are being explored, including glass, silica, polycarbonate, poly(methyl methacrylate), and poly(dimethylsiloxane) for microchip separations. The use of

polymeric materials has made the fabrication of microchips easier, because once a master is created, polymeric microchips can be made rapidly and inexpensively [3]. Most of the microanalytical devices reported have been fabricated on glass substrates using photolithography, and wet chemical etching processes [6]. Plastics have recently gained ground as an alternative. The advantages of plastic microfluidic devices include low cost, a wide range of materials to choose for assay compatibility, well-developed manufacturing processes (molding, casting, or from embossing), and the potential to be disposable to avoid "carry-over" contamination. Thermo-plastics including polymethylmethacrylate (PMMA), polyolefins, poly(ethylene terephthalate), and polycarbonate, as well as elastomers such as poly(dimethylsiloxane) (PDMS) are among the common substrates used for manufacturing and / or rapidly prototyping microfluidic devices for a variety of applications [7]. An additional advantage of using thermoplastics for this application is their low surface charge, potentially eliminating the necessity of time-consuming coating steps for certain types of separations [6].

Among many approaches to proteomics, multi-dimensional electrophoresis and mass spectrometry are becoming the primary tools because of their ability to separate and detect complex biological samples. Today, the analysis of complex multicomponent mixtures, containing as many as 5,000 single components (SCs), is a challenging task for frontier research fields, such as proteomics. However, mixtures are often so complex in terms of number and similarity between SCs that the separation power offered by a single dimension (1D) separation technique is not sufficient; separation science has therefore entered into the era of multidimensional separations [8]. Assuming unit resolution between neighboring peaks, a nondimensional quantity known as the peak capacity of a 1D separation, n, can be expressed as;

$$n = L / w \sim \sqrt{N} \qquad (1)$$

where L is the separation channel length and w is a measure of the average analyte bandwidth based on the standard deviation of the concentration distribution, σ. Typical estimates of w include 4σ and the full width half-maximum value (for a Gaussian distribution, fwhm ~ 2.35σ). The peak capacity is proportional to the square root of the number of theoretical plates ($N = L^2 / \sigma^2$). The total peak capacity of a 2D system, n_{total}, is estimated to be the product of the peak capacities of each respective separation dimension;

$$n_{total} = n_1 . n_2 \qquad (2)$$

where n_1 and n_2 are the peak capacities of the constituent separation dimensions, as described in eq 1. Giddings outlined considerations for discrete coupling of techniques that include the independence of each successive separation mechanism (orthogonality), rapid peak generation, high resolution, and overall technique compatibility [9]. For example, to isolate any component (single peak) from a mixture containing 20 components with 95% probability of success, a peak capacity of ~ 800 would be needed. The required peak capacity increases linearly with the number of components in a mixture, assuming a constant probability of success. Since peak capacities for highly efficient one-dimensional analyses range from 300 to 500, the development of multidimensional techniques is necessary for analyzing complex mixtures. A difficulty in designing a successful two-dimensional analysis is the coupling of multiple, individual separations. "Heart-cutting" analysis is performed by collecting an interesting portion of the first-dimensional effluent and subsequently injecting it into the second dimension. "Comprehensive analysis" relies on regular interval, constant-volume sampling of the first-dimension effluent. A minimum rate must be maintained to ensure that the first-dimension effluent is adequately analyzed [10]. Although 2D gel electrophoresis has been used as the primary method for protein separation from complex mixtures, its laborious and time-consuming steps involving protein transfer and extraction from the gel can result in

sample loss and make it an unfavorable technique. Comprehensive 2D column analyses have been achieved using automated switching valves, parallel columns in the second dimension, or using optically gated techniques to rapidly sample the first dimension [11]. Size exclusion chromatography (SEC) has been coupled with CE, reversed-phase liquid chromatography (RPLC) with CE, and ion-exchange chromatography with RPLC for the generation of 2D systems [12]. Herr and co-workers have coupled isoelectric focusing (IEF) with zone electrophoresis for 2-D separations of model proteins using plastic microfluidics [13]. An integration of micellar electrokinetic chromatography (MEKC) or isoelectric focusing (IEF) with capillary electrophoresis (CE) has also been described. More recently, a microfluidic device that couples IEF and SDS-PAGE has been demonstrated, with appropriate peak transfer between IEF and SDS-CE [14]. However, in the analysis of a complex biological sample such as serum, interdiffusion between the focused protein peaks during the coupling stage could decrease the efficiency and resolution achieved in the first-dimension (IEF), especially when the sample contains both majority and minority protein species with concentrations that differ by orders of magnitudes [15].

In this work, we present a two-dimensional separation of proteins using a PMMA microchip. Proteins were separated using SDS-CGE in the first dimension followed by MEKC in the second dimension. Each separation dimension and the entire process were optimized using a statistical approach [16].

2. Experimental

2.1. Chemicals

Gel and buffers were purchased from Beckman (Fullerton, CA). The dye-labeled proteins were received from Molecular Probes (Eugene, OR). Other chemicals were purchased from Sigma (St. Louis, MO). A list of proteins used in this study is presented in Table 1.

Table 1: Dye-labeled proteins

No	Protein
1	Wheat germ agglutinin
2	Ovalbumin
3	Protein A
4	Streptavidin
5	Albumin
6	Transferrin
7	Concanavalin A

2.2. Microfabrication

A layout of the basic chip design is shown in Figure 1. Mechanical details are described in the caption. The mold insert was fabricated on brass using a micro-milling machine (Kern MMP, Germany) with the pattern transferred to PMMA using a hot-embossing protocol [17]. Finally, the PMMA microchip was annealed to another thin PMMA sheet in a GC oven.

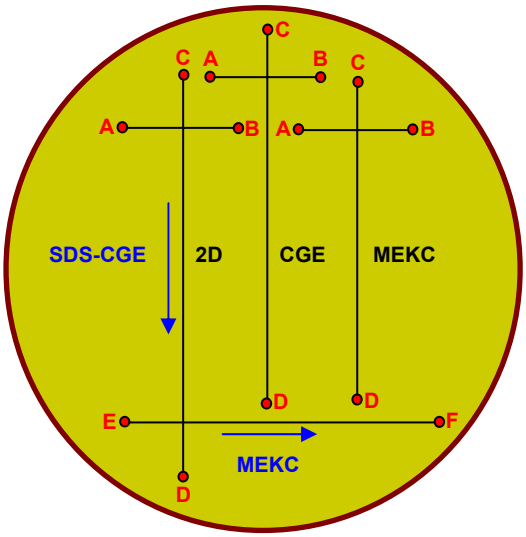

Figure 1: Layout of microchip design on a brass molding die. All channels were 20 µm wide and 120 µm deep. A, sample reservoir; C, E buffer reservoirs; B, D, F waste reservoirs, all were 2 mm in diameter. Pt wires were used to apply voltage to the reservoirs. SDS-CGE channel: separation length 40 mm; MEKC channel: separation length 30 mm, Two-dimension channel: separation length 1D / 2D, 40 mm / 30 mm; Injection length was 10 mm in all channels. Same pattern was copied to PMMA plate using hot embossing. The SDS-CGE and MEKC channels represent the exact copy of each separation dimension shown in the 2D design. These channels were used to run each dimension of separation individually, and to optimize them before combination.

3. Results

3.1 SDS-CGE separation of proteins

Electrophoretic separation of proteins in solution occur according to differences in the mass / charge ratio of the proteins. However, the resolution of this technique is poor as “Joule heating” effects due to the electric current impair the separation. This Joule heating can create a temperature gradient leading to gradients in density, which causes natural convection and the disturbance of the separated zones. Diffusion has a second negative effect on the sharpness of the protein zones. To minimize these effects, electrophoretic protein separations are mainly carried out in supporting media like aqueous gels. Under denaturing conditions using detergents such as sodium dodecyl sulphate (SDS) and a reducing agent simultaneously, the disulfide bridges in the proteins are broken due to the reducing agent and unfolded SDS polypeptide complexes of negative charge are created. The rate of SDS binding to the dissociated polypeptides is constant (1.4 gram per gram polypeptide), resulting in linear SDS complexes of equal charge density. The SDS-protein complexes can be described as free-draining coils and the separation in the gel matrix is then conducted based on molecular weight [18].

In order to get an estimation of the migration time for each protein, and to perform the optimization, many SDS-CGE runs were conducted in the SDS-CGE channels. A typical result is shown in Figure 2. Protein 2 and 3 were not resolved because of their similar molecular weights. The rest of proteins were resolved, but better resolution and peak capacity was needed. Using longer channels increases resolution, but also the analysis time. Furthermore, longer channels are not able to produce the suitable value of peak capacity for proteomic study, which deals with analysis of complex mixtures. To do this, a combination of two different separation techniques (SDS-CGE and MEKC in our work) was investigated due to the orthogonality of the separation process.

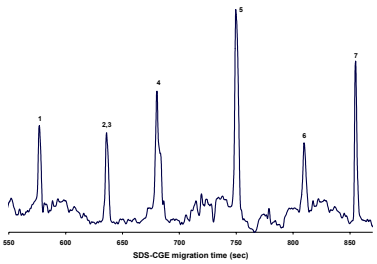

Figure 2: A typical SDS-CGE separation of proteins

3.2 MEKC separation of proteins

Micellar Electrokinetic Chromatography (MEKC) is a mode of Electrokinetic Chromatography (EKC) in which surfactants (micelles) are added to the buffer solution. Surfactants are molecules which exhibit both hydrophobic and hydrophilic character. They have polar “head” groups that can be cationic, anionic, neutral, or zwitterionic and also have nonpolar, hydrocarbon tails. The formation of micelles or “micellization” is a direct consequence of the “hydrophobic effect”. The surfactant molecules can self-aggregate if the surfactant concentration exceeds a certain critical micelle concentration (CMC). The hydrocarbon tails will then be oriented toward the center of the aggregated molecules, whereas the polar head groups point outward. Micellar solutions may solubilize hydrophobic compounds, which otherwise would be insoluble in water. When an anionic surfactant, such as sodium dodecyl sulfate (SDS), is employed the micelle migrates toward the positive electrode by electrophoresis. The electroosmotic flow (EOF) transports the bulk solution toward the negative electrode. The EOF is usually larger than the electrophoretic mobility of the micelle under neutral or alkaline conditions and, therefore, the anionic micelle also travels toward the negative electrode at a retarded velocity. When a neutral analyte is injected into the micellar solution, a fraction of it is incorporated into the micelle and it migrates at the velocity of the micelle. The remaining fraction of the analyte remains free from the micelle and migrates with its own velocity. The migration velocity of the analyte thus depends on the distribution coefficient between the micellar and the non-micellar (aqueous) phase. The greater the percentage of analyte that is distributed into the micelle the slower it migrates [19].

To compare results, a MEKC separation in the second dimension was performed using MEKC channel (Figure 3). As shown, the migration time is shorter with a different elution order indicating some degree of orthogonality to SDS-CGE. Comparing Figure 2 and Figure 3, the elution order differed. A rigorous comparison indicated a ~ 41% reversed in elution order because of the different separation mechanism of SDS-CGE and MEKC. Once again, to obtain better resolution and peak capacity, a combination of MEKC with SDS-CGE was under taken.

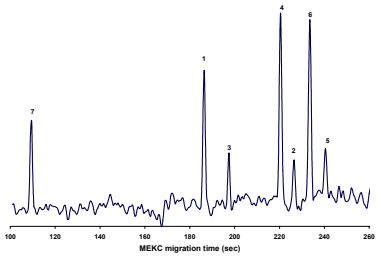

Figure 3: A typical MEKC separation of proteins

3.3 Two-dimensional separation of proteins: combination of SDS-CGE and MEKC

Finally, a combination of two separation techniques was conducted using this microchip. Figure 4 presents a two-dimensional plot for this separation. All proteins were completely separated without the need to increase the length of separation channels to increase plate numbers.

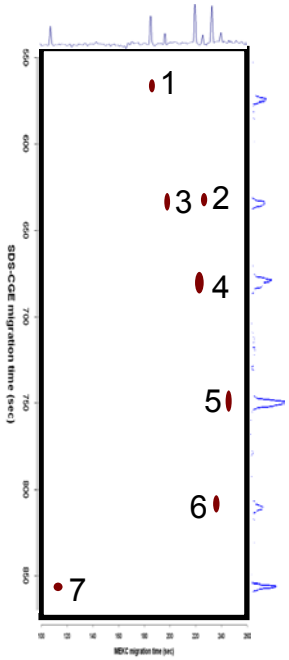

Figure 4: 2D plot of a two-dimensional separation of proteins using a combination of two separation mechanisms. A pulse injection was used to inject samples coming from SDS-CGE into MEKC channel.

4. Conclusion

An improvement in separation of proteins was achieved by a combination of two orthogonal electrophoretic platforms in a polymeric-based microfluidic device. Using pristine PMMA makes these devices excellent disposable and cheap microchips for proteomic study. Further work is needed to obtain better reproducibility and lower detection limit to make this chip more applicable for analysis of complicated biological samples such as serum proteins by producing larger peak capacities.

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