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Journal of Chromatography A, 1049 (2004) 205–209
JOURNAL OF CHROMATOGRAPHY A
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Rapid protein separations in ultra-short microchannels: microchip sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing

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Received 4 May 2004; received in revised form 23 July 2004; accepted 11 August 2004

Abstract

We have developed novel protein gel electrophoresis techniques, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing (IEF) in short microchannels (∼millimeters) that take less than a minute. A photopatterning technique was used to cast in situ crosslinked polyacrylamide gel in a microchannel to perform SDS–PAGE. A fluorescent protein marker sample (M_r range of 20 000–200 000) was separated in less than 30 s in less than 2 mm of channel length. Crosslinked polyacrylamide gel, patterned in channels using UV light, provides higher sieving power and sample stacking effect, therefore yielding faster and higher-resolution separation in a chip. IEF of proteins was also achieved in a microchannel, and several proteins were focussed within tens of seconds in mm-length channels. As resolution in IEF is independent of separation distance, focusing in ultra-short channels results in not only faster separation but also more concentrated bands potentially allowing detection of low-concentration species.
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Keywords: SDS–PAGE; Microchip; Photopolymerization; Protein separation; Isoelectric focusing

1. Introduction

Miniaturization of gel electrophoresis, widely used in biomolecule analysis, has attracted much attention as it holds the promise of significantly reducing the analysis time and the amounts of sample needed, and has the potential to be automated and portable. A microchip-based fluidic architecture may also be easier to interface with a mass spectrometer. A number of articles have appeared on performing IEF [1–7] and SDS-capillary gel electrophoresis (CGE) [8–10] in microchips. Also, protein two-dimensional separation devices, with variety of separation combinations, were demonstrated recently [11–15].

The IEF and SDS–PAGE separations in microchips reported to date use relatively long (a few cm) channels, making their combination result in a chip that is several cm by several cm. This significantly reduces the yield of chips and also results in longer analysis time. Separations of SDS-coated protein on a microchip reported to date [8–10] use liquid gel, and generally require relatively long (∼5 cm) channels. The liquid sieving gel has an advantage that it can be easily replaced after each runs, but at the same time it makes the integration into a higher level system more challenging as it can flow and diffuse into other channels. The use of solid polyacrylamide gel in a channel has been demonstrated for DNA separation [16] and has several advantages over liquid sieving material. Solid, crosslinked gel does not mix with or diffuse into other regions of the channel. In addition, solid gels have better sieving power than liquid gels, which allows faster separation within a short channel length. Patterned gel structures can also be used for other purposes than molecular sieving, such as sample stacking and molecular

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doi:10.1016/j.chroma.2004.08.018

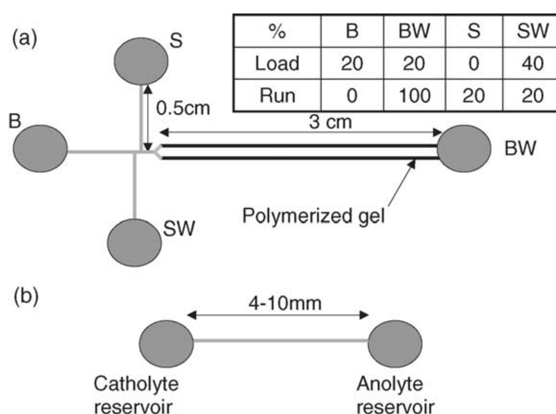
manipulation. It is also possible to do a multiplexing, where different gels can be run in different channels of a single chip at the same time.

In IEF, the resolution of separation does not depend on the length of the channel for a given applied voltage. Therefore, in a shorter IEF channel, higher electric field would result in faster focusing and more concentrated peaks (and possibly more sensitive detection), while the Joule heating due to the higher current would be effectively prevented in a microchip. In addition, the entire IEF channel (with several millimetre length) could be easily imaged instantly by a simple optics without the need for mobilizing peaks or scanning optics over the entire capillary or column [1,7]. This could lead to a much faster and more sensitive protein analysis based on their p*I* values—valuable protein information that is readily available from the public protein database.

In this paper, we report microchip-based SDS–PAGE protein separation, as well as microchip-based IEF protein separation, using very short channels, and demonstrate their advantages over conventional capillary gel separation or microchip protein separation using liquid gel. We have developed photopolymerization techniques to pattern a polyacrylamide gel for SDS–PAGE in a channel using a ultraviolet (UV) lamp and photomask. Compared with separations by liquid sieving gels, faster separation speed, higher separation resolution due to the sample stacking at the gel boundary, lower separation potential, and smaller overall chip size were demonstrated. Isoelectric focusing of proteins were achieved within less than a minute in a short microchannel (∼4 mm). The small size of the separation system in this work also facilitates detection of separated protein bands, since protein bands can be detected quickly by a whole-field imaging technique without the need of eluting the peaks. These advantages can be considered favorably in designing future integrated, portable protein separation system.

2. Experimental

To demonstrate SDS–PAGE and IEF in a short channel, several straight microchannels with different lengths (4 mm–3 cm) were fabricated on poly(dimethylsiloxane) (PDMS) for IEF and glass substrates for separate IEF and SDS–PAGE. Detailed fabrication procedures for PDMS [17] and glass [18] microchip have been reported previously. An inverted microscope (IX-70, Olympus, Melville, NY, USA) was used as an experimental platform, and a cooled charge-coupled device (CCD) camera (CoolSNAP HQ, Roper Scientific, Trenton, NJ, USA) connected to the microscope was used as an imaging and detection device. A 100 W mercury lamp was used as a light source, and the fluorescence filter cubes for blue (∼480 nm) and green (∼520 nm) excitation were used to detect protein molecules. The images from the camera was digitized and analyzed by an imaging software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA) to generate electropherograms.



%	B	BW	S	SW
Load	20	20	0	40
Run	0	100	20	20

Fig. 1. Layout for the microchip for: (a) SDS–PAGE and (b) IEF. The inset in (a) shows the typical sample loading and launching potential values.

Fig. 1 shows the device layout for SDS–PAGE experiment. The depth of the microchannel for SDS–PAGE was 27 μm, with 1–3 cm long separation channel. Glass microchip was coated by 3-(trimethoxysilyl)propyl acrylate in an acidic environment, followed by the photo-polymerization process of acrylamide monomer (∼5%) on the surface of the channel [19]. Then, sieving gel is formed by patterned UV exposure in the channel, for up to 10 min. A mixture of 12% acrylamide/bis-acrylamide solution in 0.375 M Tris–Cl buffer (pH 8.6) with 0.1% of SDS and 0.2% of 2,2′-azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide] (Wako, Richmond, VA, USA) was prepared and introduced into the channel. Upon UV light (350 nm) exposure (UVP high intensity UV lamp B-100AP), this chemical turned into polyacrylamide gel within the channel. The exposure was done at about 5 cm distance from the lamp, and the intensity of the UV light is estimated to be about 20 mW/cm^2. Only the separation channel region (not the sample loading region) was exposed by blocking the UV light with an optical mask. The length of the channel was 1–3 cm, and the gel was polymerised over the entire length of the channel. However, only a small distance (2–4 mm) was needed to get complete separation of protein bands. After the polymerisation, unexposed section of the microchannel was flushed with 0.375 M Tris–Cl buffer containing 0.1% SDS. The same solution was used as a sample buffer and buffers for other reservoirs. A commercially available fluorescence-labeled protein marker sample (product number F-3526, Sigma, St. Louis, MO, USA) was used as a sample. The final total protein concentration in the sample reservoir was about ∼10 mg/ml. The protein sample was introduced into the loading region of the channel by applying a negative potential to the sample reservoir. Loading, launching and separation of protein bands were monitored and recorded in real time by the cooled CCD camera.

For IEF experiment, two naturally-fluorescent proteins (enhanced green fluorescent protein (EGFP, BD Biosciences, Palo Alto, CA, USA) and *R*-phycoerythrin (Molecular Probes, Eugene, OR, USA) and one labeled protein (carbonic anhydrase II, Sigma) were used. Carbonic anhydrase II was

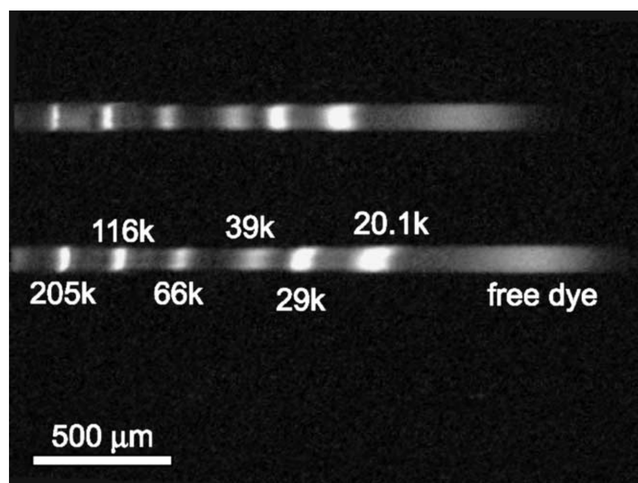
labeled by rhodamine–maleimide, which binds to the cysteine residue of the protein. Carbonic anhydrase at a concentration of 0.1 mM was reacted with 20-fold molar excess of rhodamine–maleimide at pH 7.0 for 12 h at 4 °C. The excess rhodamine–maleimide was separated from labeled protein by size exclusion chromatography. Commercially available carrier ampholytes [cIEF ampholytes (pH 3–10), Beckman Coulter, Fullerton, CA, USA] was used for the experiment at the concentration of 2%. Proteins were added to the carrier ampholytes mixture to make the final concentration of ∼1 μg/ml for each protein species. 40 mM phosphoric acid and 20 mM sodium hydroxide solutions were used as anolyte and catholyte, respectively. For the experiment in PDMS microchannel, 1% methylcellulose solution was first introduced into the microchannel to coat the surface, just before the experiment for about 5 min. In addition, as a dynamic coating agent, methylcellulose was added to carrier ampholytes mixture, as well as catholyte and anolyte, at 0.5–1% concentration.

3. Results and discussion

For miniaturized SDS–PAGE on a chip, a microchip fabricated on a glass substrate was first coated with polyacrylamide to reduce electroosmotic flow, then polyacrylamide gel is formed in a microchannel as a sieving matrix. Commercially available fluorescently-labeled protein marker sample was used, with protein concentration of roughly 10–100 μM for each species. The concentration used here is by no means the detection limit of the fluorescence detection, and it is well known that fluorescence detection could be used to detect the proteins at much lower concentrations. However, the efficiency of separation using the photopolymerized gel can be clearly demonstrated. Fig. 2 is the picture of the microchannel filled with solid polyacrylamide gel while protein samples are being separated. The left edge of the image roughly coincides with the beginning of gel in the channel. This fluorescence image was taken 26 s after launching the sample from the loading area. Six protein peaks are well separated in both channels, within the channel distance less than 2 mm. The plate number of the separation was between 300 and 800, which is comparable to the plate number (∼1000) of slab gel SDS–PAGE, which is remarkable considering the short length of the gel channel. The plate number per length was calculated to be $1.5–4 \times 10^5$ plates/m.

Ability to pattern crosslinked gels in channels also allows us to take advantage of stacking effects analogous to slab gel electrophoresis. The initial peak launched at the sample injection cross region has a width of about ∼250 μm. However, upon entering the crosslinked polyacrylamide gel protein samples are retarded and stacked into a narrower band (10–20 μm), leading to improved resolution and detection limits. Also, the applied field in the gel is about an order of magnitude higher than typical electric field values in standard slab gel SDS–PAGE, allowing faster separation. We varied the electric field applied to the gel from 34 V/cm up to 170 V/cm, and electrophoresis ran at higher fields yielded faster separation result without significant changes in separation resolution. It has been reported, from the thin layer electrophoresis experiment, that fields up to 89 V/cm does not affect the mobility of SDS–protein complexes [20]. Since our channel was much thinner (∼27 μm) compared to the case of the thin-layer electrophoresis (∼180 μm), it is less prone to gel heating problem. However, we occasionally observed the bubble generation in the gel solution when the field was increased over 200 V/cm.

One has to note that the concentration of the acrylamide gel used in this experiment is 12%, and such concentration is typically used for separating low-molecular weight proteins



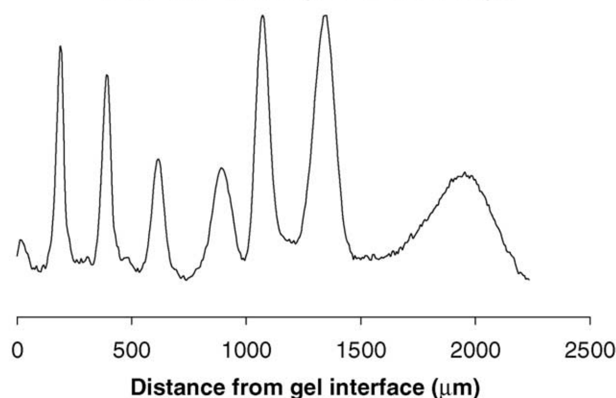


Fig. 2. SDS–PAGE separation of six proteins in 12% gel (acrylamide:bis-acrylamide = 37.5:1), also containing 0.1% SDS and 0.2% photoinitiator. This picture was taken 26 s after the launching, and the electric field was 170 V/cm. Both channels contain the same concentration gels, but the difference in the location of gel boundary in the two channel yields slightly different peak locations. The gel buffer was 0.375 M Tris–Cl (pH 8.6) with 0.1% SDS. Six proteins are myosin (M_r 205 000), beta-galactosidase (116 000), bovine serum albumin (66 000), alcohol dehydrogenase (39 000), carbonic anhydrase (29 000), and trypsin inhibitor (20 100). The rightmost band in the channel is due to the Bromophenol blue dye added to the sample. The width of dye band is much larger than other protein bands, suggesting no stacking effect for small dye molecules.

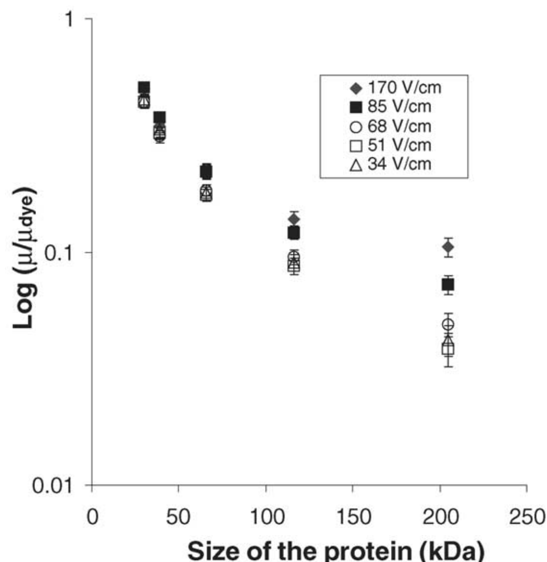


Fig. 3. The plot of mobility vs. electric field. The y-axis is $\log(\mu/\mu_{\mathrm{dye}})$, where μ is the mobility of the proteins, while μ_{dye} is the mobility of the dye (Bromophenol blue) molecule.

in conventional slab SDS–PAGE. Protein samples were retarded significantly in the gel, which stacked the protein band into narrower one and enhancing the separation resolution. However, protein peaks were still mobilized through the gel and were not completely stuck in the gel matrix, mainly because of higher driving electric field. Fig. 3 shows the relation between the measured mobility and the length of proteins separated at various electric field conditions. According to the Ogston model of sieving [21], $\log \mu$ should be proportional to R^2 (R: the radius of gyration of polymer molecule), and $R^2 \sim N$ (length of the polymer) if one can assume SDS-coated proteins as ideal polymers. Therefore, the $\log \mu$ versus N graph should be linear. The graph in Fig. 3 shows significant deviation from linear behavior, and may suggest that the mobility of longer proteins are higher than the values that the Ogston model would have predicted. This means that SDS-coated proteins in higher density acrylamide gels are not well characterized by Ogston model. Rather, high electric field could have caused conformation change of proteins in the gel, therefore allowing them to migrate much easier via Reptation mechanism [22]. Similar dynamic transition (between Ogston and Reptation models) in polymer dynamics has been well established in the DNA electrophoresis [23,24].

In addition to the well-known advantages of miniaturized separation systems, polyacrylamide gel would be easier to connect with other sample preparation or separation systems than liquid sieving matrix, since there is no concern of mixing and diluting the liquid sieving matrix when the separation channel is connected with other fluidic components. It is also possible to pattern polymer gel matrix with diverse properties within the microfluidic channel, and use the patterned gel as a nanoporous material to filter or manipulate biomolecules in the system.

IEF separation of protein samples has been achieved in a microfluidic channel as short as 4 mm typically within ∼45 s. In Fig. 4, three proteins were focused within a microchannel made out of PDMS. EGFP and R-phycoerythrin are naturally fluorescent proteins, while carbonic anhydrase II proteins were visualized by cysteine-specific labeling (rhodamine–maleimide). To visualize all three peaks at once, multiple fluorescence images taken at different excitation wavelengths were merged into a single image electronically. The two peaks observed for the carbonic anhydrase II protein are charge isomers of the same protein, as observed previously [25].

Miniaturized IEF channel provide several advantages both in analytical and practical point of view. In IEF, the separation resolution does not depend of the length of the channel. The focused peak width σ can be given as the following equation [26].

$$\sigma = \sqrt{\frac{D(\mathrm{d}x/\mathrm{d(pH)})}{E(\mathrm{d}\mu/\mathrm{d(pH)})}} \sim \frac{L}{\sqrt{V}} \quad (1)$$

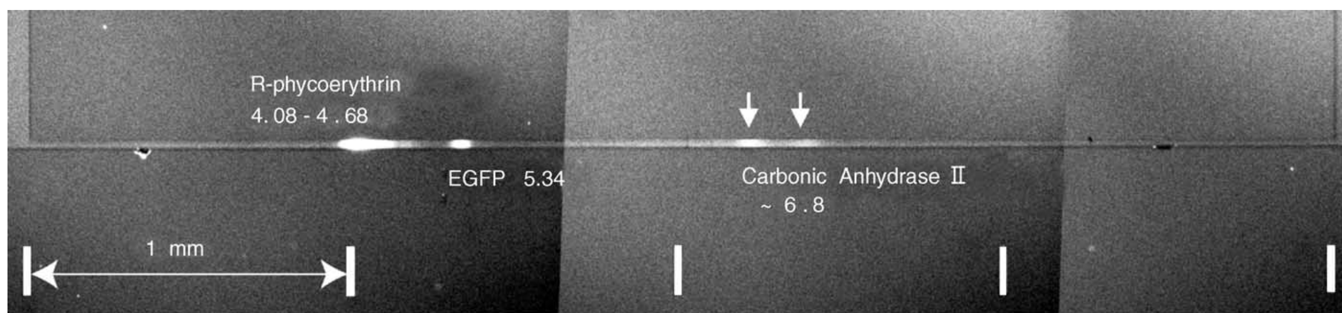


Fig. 4. Isoelectric focusing in 4 mm channel. Electric field was 35.7 V/cm. Carbonic anhydrase II was labeled by cysteine-specific labeling (rhodamine–maleimide), while R-phycoerythrin and EGFP are naturally fluorescent. One percent methylcellulose was added to carrier ampholytes mixture for a dynamic coating. The image was taken 30 s after applying the electric field. Wider channel regions at both ends are filled with catholyte and anolyte, respectively, and pH gradient was established within 4 mm channel. To visualize all three peaks (with different fluorescence wavelengths) as well as the entire 4 mm channel, multiple fluorescence images taken at different excitation wavelengths were combined into a single image electronically. The two peaks (designated with arrows) for the carbonic anhydrase II protein are charge isomers.

where D is the diffusion constant of the protein, E (= V/L, L is the channel length) the electric field, μ the mobility of the protein, and dx/d(pH) ($\sim L$) the reciprocal of the pH gradient. The separation resolution R_s is given as $\sim d/\sigma$, where d is the separation distance in the microchannel between the two peaks of interest. Since d scales as $\sim L$, R_s is only proportional to $V^{1/2}$, independent of the length of the channel L. Therefore, the IEF separation resolution is not sacrificed (at the same potential V) even with decreasing channel length L. However, the time it takes to achieve focusing is decreased, because of higher field and shorter migration length for proteins. Fig. 4 was achieved 30 s after applying the potential, much shorter time compared with ∼45 min in conventional capillary IEF, as well as other short-channel IEF demonstrated [1,3–7]. Higher field strength also means more concentrated focusing bands, which could allow more sensitive detection of the peaks. This would be especially useful in the detection of low-concentration species in the sample. A whole-column detection of the microchannel IEF can be easily achieved with simple microscope optics, eliminating the need of specialized optics (as in previous works [1,3–6]) or mobilizing peaks in the capillary (as in conventional capillary IEF).

4. Concluding remarks

In conclusion, miniaturizing the length of separation channel in SDS–PAGE and IEF techniques leads to a number of improvements over their conventional counterparts. (1) Separation of protein peaks could be achieved very quickly, typically within 30 s. (2) The electric potential to be applied across the channel was greatly decreased, which is another engineering constraint in future integrated systems. (3) Instead of mobilizing or eluting focused peaks, a microscope optic could be used for imaging of the channel in real time for a faster analysis. In the case of polyacrylamide gel in the microchannel, this system has an advantage of using solid gel as a sieving matrix instead of liquid gel. This would be a big advantage when the separation system is coupled with other detection systems (mass spectrometry, for example) that severely limit the buffer solution to be used in the separation systems.

Acknowledgements

We would like to thank George Sartor, Bill Kleist and Boyd Wiedenman for microfabriaction of chips, Ron Renzi for design and fabrication of interconnects, and Dr. Timothy Shepodd and Dr. Brian Kirby for technical advice on polymerization techniques. This work was financially supported by Laboratory Directed Research and Development program at Sandia National Laboratories. Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy under contract DE-AC04-94AL85000.

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