

High-speed Analysis of Proteins by Microchip Isoelectric Focusing with Linear-imaging UV Detection

Fumihiko KITAGAWA,[†] Shiori AIZAWA, and Koji OTSUKA

*Department of Material Chemistry, Graduate School of Engineering, Kyoto University,
Katsura, Nishikyo, Kyoto 615-8510, Japan*

High-speed analyses of proteins by microchip isoelectric focusing (MCIEF) were investigated on straight channel chips. By employing a commercially available microchip electrophoresis instrument equipped with a linear-imaging UV detector, sample proteins focused in a separation channel could be detected without a mobilization step. Typically, standard proteins were focused within 210 s at different positions in a separation channel with its total length of 38.8 mm on the basis of their *pI* values. The linear relationship between the focused position of each protein and its *pI* value was observed in the pH range from 6 to 10. The formation process of a pH gradient in the microchannel and the effects of the applied voltage and the channel length on the MCIEF separation were also investigated.

(Received February 27, 2009; Accepted April 24, 2009; Published August 10, 2009)

Introduction

In the electrophoretic analysis of proteins, several separation modes such as zone electrophoresis, gel electrophoresis and isoelectric focusing (IEF) have been employed.¹ Among them, capillary IEF (cIEF) provides qualitative information of the analytes, because the detection time depends on the isoelectric point (*pI*) of the proteins. In the cIEF analysis, sample proteins dissolved in a separation matrix containing carrier ampholytes are filled into a capillary, and then a pH gradient is formed by applying a voltage to anolyte and catholyte reservoirs. The proteins are focused at positions corresponding to their *pI* values in the pH gradient. When a polyacrylamide-coated capillary is used in cIEF to suppress the electroosmotic flow (EOF) and the adsorption of proteins onto the inner surface of the capillary, the focused zones should be finally mobilized to a detection point. To mobilize the focused samples, several methods have been developed, *e.g.*, chemical mobilization by adding appropriate salts into either reservoir, and hydraulic mobilization by applying pressure or vacuum. Although it takes only ~5 min in the focusing step, it takes 20–40 min to mobilize the focused zones. Such a long mobilization time would cause a band broadening of the focused zone, resulting in a decreased efficiency.

To reduce the analysis time in cIEF, whole-column imaging detection techniques have been employed.^{2–11} In the most simple scheme of whole-column detection for cIEF, a transparent capillary is illuminated by an expanded beam. Transmitted light or fluorescence from the capillary is monitored for UV absorbance or fluorescence detection, respectively, by a photodiode array, CCD camera, or imaging sensor. By utilizing the whole-column imaging techniques, the mobilization step in cIEF can be avoided. On the other hand, microchip electrophoresis (MCE) is suitable for imaging the concentration

profile throughout the separation column compared to capillary-based electrophoresis because the separation is carried out in a shorter channel. In microchip IEF (MCIEF), whole-channel imaging detection schemes based on both fluorescence^{12–18} and UV absorption^{19–21} have been applied. In addition to eliminating the mobilization step, a reduced separation time, which is an essential nature of MCE, can realize a high-speed IEF analysis of proteins. For employing fluorescence detection, sample proteins should be derivatized prior to the MCIEF analysis, though higher sensitivity can be obtained. Fluorescence labeling may cause changes in the *pI* values compared to those of native proteins. For whole-channel UV detection, a commercial available MCE system equipped with a linear-imaging UV detector (MCE-2010) has been introduced by Shimadzu (Kyoto, Japan).²² The linear-imaging UV detector allows for the MCE analysis of various compounds without any derivatization.^{21,23–31} Consequently, a UV detection scheme eliminates the labeling of sample proteins. Recently, Vlčková *et al.* reported on the application of MCE-2010 to the MCIEF analysis of proteins on a conventional cross-channel chip.²¹ On this chip, however, a short sample loading channel may disturb the formation of the pH gradient and the focusing of analytes in a long separation channel due to sample leakage at the cross injector.

In this study, we investigated the application of the MCE-2010 system to MCIEF for realizing high-speed analyses of proteins without a mobilization step and fluorescence labeling. To avoid sample leakage, simple straight channel chips consisting of an optical slit for linear-imaging UV detection were used. In MCIEF analysis employing MCE-2010, the focusing process of proteins and the formation process of the pH gradient in the microchannel were studied. Furthermore, the effects of the applied voltage and the separation channel length on the analytical performances were investigated to obtain a fast and efficient separation of proteins.

[†] To whom correspondence should be addressed.
E-mail: fkitagawa@mbox.kudpc.kyoto-u.ac.jp

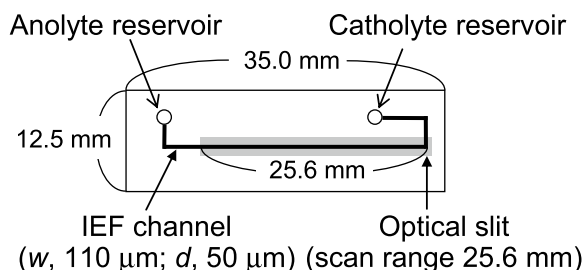
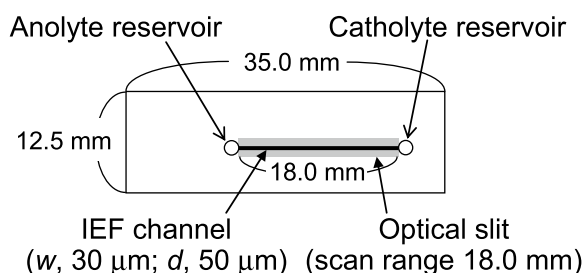
(a) long channel chip**(b) short channel chip**

Fig. 1 IEF microchips used in this study.

Experimental

Chemicals

Carbonic anhydrase II (from bovine erythrocytes, *pI* 5.9), myoglobin (from equine skeletal muscle, *pI* 6.8, 7.3), ribonuclease A (*pI* 9.5), 3-methacryloxypropyltrimethoxysilane (MPTS), acrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and *m*-nitrophenol were obtained from Sigma-Aldrich (Tokyo, Japan), phosphoric acid, sodium hydroxide and lentil lectin (*pI* 8.2, 8.6) from Wako (Osaka, Japan), ampholyte (eCAP cIEF Ampholyte 3-10) and IEF gel (eCAP cIEF Gel) from Beckman Coulter. (Hydroxypropyl)methyl cellulose (HPMC, METOLOSE 60SH-50) was kindly supplied by Shin-Etsu Chemical (Tokyo, Japan). All aqueous solutions were prepared with deionized water, purified by using a Direct-Q System (Nihon Millipore, Japan), and filtered through a 0.45-μm pore membrane filter prior to use.

Instrumentation

MCIEF experiments were carried out with an MCE-2010.²² A monochromatic UV light from D₂-lamp is focused onto the entire region of the separation channel on the quartz microchip. A diode array detector is located along the separation channel. Two quartz microchips with a straight channel (long channel chip: depth, 50 μm; width, 110 μm; total length, 38.8 mm; scan range, 25.6 mm; short channel chip: depth, 50 μm; width, 30 μm; total length (scan range), 18 mm), were supplied from Shimadzu (Fig. 1). There are two platinum electrodes on the chip to apply voltages between the anolyte and catholyte reservoirs fabricated at the end of the straight channel.

Procedure

Prior to MCIEF analyses, the microchannels were coated with linear polyacrylamide to suppress the EOF and the surface adsorption of proteins.³² Briefly, 0.4% MPTS containing 4%

acetic acid was filled in the microchannels. After a reaction at room temperature for 1 h, the MPTS solution was withdrawn. The silanized channels were washed with water and then filled with a deaerated 3% (w/v) acrylamide solution containing 0.1% TEMED and 0.1% ammonium persulfate under a nitrogen atmosphere. After 30 min, the excess of polyacrylamide was pumped away and the channels were rinsed with water. In MCIEF analyses, sample proteins were dissolved in an IEF gel matrix containing 2% ampholyte. The prepared sample solution was filled into the entire channel. The anolyte and catholyte reservoirs were filled with 3 μL of a 10 mM phosphoric acid solution and a 40 mM sodium hydroxide solution, respectively. To suppress the EOF in the channel, HPMC was added to these electrolytes at a 2% concentration. The applied electric field strength was 450 V/cm. The focusing process of the standard proteins was observed continuously by using a photodiode array at a detection wavelength of 280 nm. In the measurements of a pH profile in the microchannel, the absorbance of *m*-nitrophenol was recorded both at 250 and 270 nm.

Results and Discussion

MCIEF process of proteins

In MCIEF analysis employing the linear-imaging UV detector, the data obtained is different from that of the conventional single-point detection scheme. The linear-imaging detector allows a observation of the real-time focusing process of amphoteric compounds. Figure 2a shows the real-time monitoring of the MCIEF of myoglobin on a long channel chip. Two peaks were observed at 50 s after starting application of the voltage. After 150 s, the two peaks were focused to give a single peak of the major isozyme of myoglobin (*pI* 7.2) at a position of 10.5 mm. Finally, a small peak of the minor isozyme (*pI* 6.8) appeared at 8.5 mm. Figure 2b demonstrates the changes in the peak position with the separation time. Closed circles and open squares indicate that the peak appeared from the anolyte and catholyte reservoir sides, respectively. As can be seen, the focusing of myoglobin was finished in 140 s. When other standard proteins were analyzed under the same condition, the focusing times were evaluated to be 120–210 s. It should be emphasized that the peak position remained almost constant after finishing the focusing, indicating that the EOF was well suppressed by the polyacrylamide coating in the microchannel and the use of the anolyte and catholyte containing HPMC. Such an unmovable feature of the focusing position is very important, since the *pI* values of proteins can be determined from a calibration curve for the focusing position *versus* the *pI* value in MCIEF. These results clearly demonstrate that a faster IEF analysis of proteins can be achieved on a microchip with a straight channel.

Measurement of pH gradient formation process

By utilizing the linear-imaging UV detector, the process of forming the pH gradient in the microchannel during the MCIEF analysis will be elucidated. The changes in the pH within the microchannel were estimated by measuring the absorbance of a pH indicator, *m*-nitrophenol, at two different wavelengths.³³ The pH of a solution can be calculated by the following equation:

$$\text{pH} = \text{pK}_a + \log \left\{ \frac{[\text{In}^-]}{[\text{HIn}]} \right\}, \quad (1)$$

where HIn is a neutral form of *m*-nitrophenol and In[−] is its deprotonated form. The *pK_a* value of *m*-nitrophenol is known to

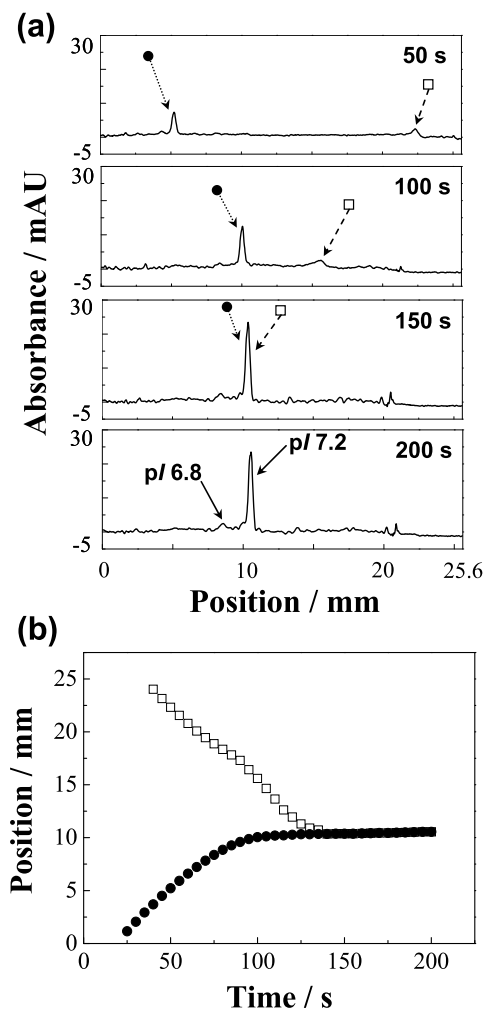


Fig. 2 (a) MCIEF analysis of myoglobin on a long channel chip. (b) Changes in the peak position with the separation time. Sample, 59 $\mu\text{g/mL}$ myoglobin in neat cCAP cIEF Gel containing 2% ampholyte; anolyte, 10 mM phosphoric acid containing 2% HPMC; catholyte, 40 mM sodium hydroxide containing 2% HPMC; electric field strength, 450 V/cm; detection, 280 nm.

be 8.2 and the $[\text{In}^-]/[\text{HIn}]$ ratio can be determined from Beer's law. When the detection wavelengths are selected at 250 and 270 nm, the absorbance is expressed as follows, respectively:

$$A_{250} = \varepsilon_{250\text{HIn}}l[\text{HIn}] + \varepsilon_{250\text{In}^-}l[\text{In}^-], \quad (2)$$

$$A_{270} = \varepsilon_{270\text{HIn}}l[\text{HIn}] + \varepsilon_{270\text{In}^-}l[\text{In}^-], \quad (3)$$

where ε is the molar absorptivity and l is the optical-pass length. At a wavelength of 250 nm, ε of HIn and In^- are $\varepsilon_{250\text{HIn}}$ and $\varepsilon_{250\text{In}^-}$, respectively. Similarly, at 270 nm, the molar absorptivities represent $\varepsilon_{270\text{HIn}}$ and $\varepsilon_{270\text{In}^-}$, respectively. Finally, the pH can be estimated by substituting Eqs. (2) and (3) into Eq. (1).

$$\text{pH} = \text{p}K_a + \log \left\{ \frac{(A_{250}\varepsilon_{270\text{HIn}} - A_{270}\varepsilon_{250\text{HIn}})/(\varepsilon_{270\text{HIn}} - \varepsilon_{250\text{HIn}})}{(A_{270}\varepsilon_{250\text{In}^-} - A_{250}\varepsilon_{270\text{In}^-})} \right\}. \quad (4)$$

The molar absorptivities of $\varepsilon_{250\text{HIn}}$, $\varepsilon_{250\text{In}^-}$, $\varepsilon_{270\text{HIn}}$ and $\varepsilon_{270\text{In}^-}$ are 328, 6660, 3270 and 335, respectively. Thus, the pH at around 8.2 in the microchannel can be determined by measuring the

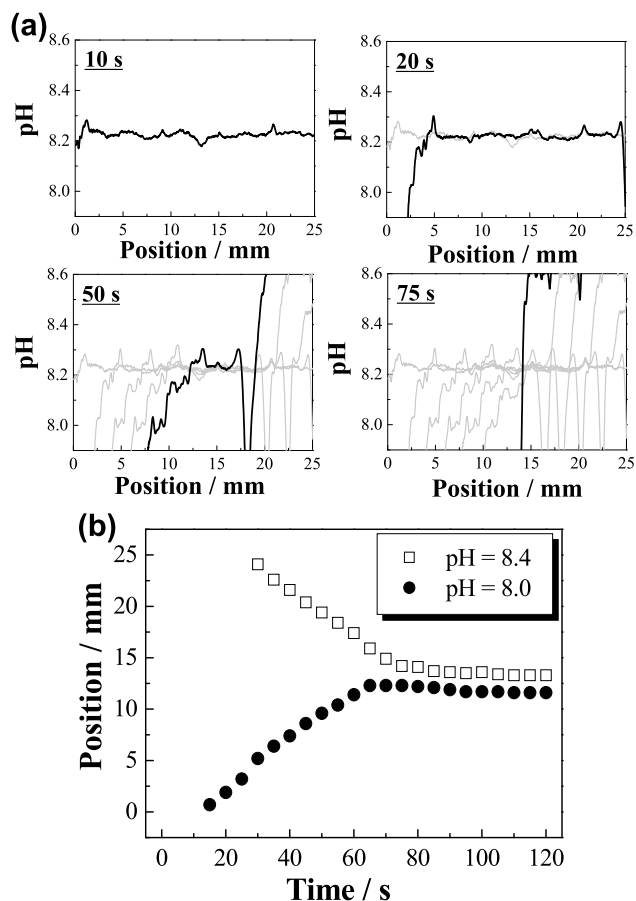


Fig. 3 (a) pH profiles in the microchannel during the MCIEF process. (b) Changes in the channel position giving pH 8.0 and 8.4 with the separation time. Sample, 0.5 mM *m*-nitrophenol in cIEF Gel containing 2% ampholyte; detection, 250 and 270 nm. Other conditions as in Fig. 2.

absorbance of *m*-nitrophenol at 250 and 270 nm on the basis of Eq. (4). Since the focusing time of the standard proteins with their pI values of 5.9 and 9.5 was faster and that of 8.2 was the slowest, the gradient formation around pH 8.2 would be the rate-determining step in the whole formation process of the pH gradient.

A solution of 0.5 mM *m*-nitrophenol dissolved in cIEF Gel containing 2% ampholyte was prepared and filled into the microchannel. During the application of a separation voltage, the absorbance of *m*-nitrophenol in the whole microchannel was recorded at 250 and 270 nm. The obtained pH profiles in the microchannel are shown in Fig. 3a. At 10 s, the pH in the scan range of the microchannel (total length of 25.6 mm) was almost constant. Then, a decrease in the pH was observed at the anodic side due to the migration of protons. After 50 s, the pH at positions ranging from 0 to 10 mm was further decreased, and an increase in the pH was observed at the cathodic side. At a position of 18 mm, a steep pH decrease appeared, suggesting that *m*-nitrophenol was transiently focused at this position. After 75 s, only a steep pH change was observed at positions from 13.8 to 14.6 mm. It should be noted that the concentration of *m*-nitrophenol in the pH gradient ranging from 8.0 to 8.4 remained sufficient to measure the pH at 0–120 s, since the migration rates of protons, hydroxide ions, and ampholytes were faster than the pH indicator. Thus, the pH value could be measured in the time range. Figure 3b demonstrates the changes

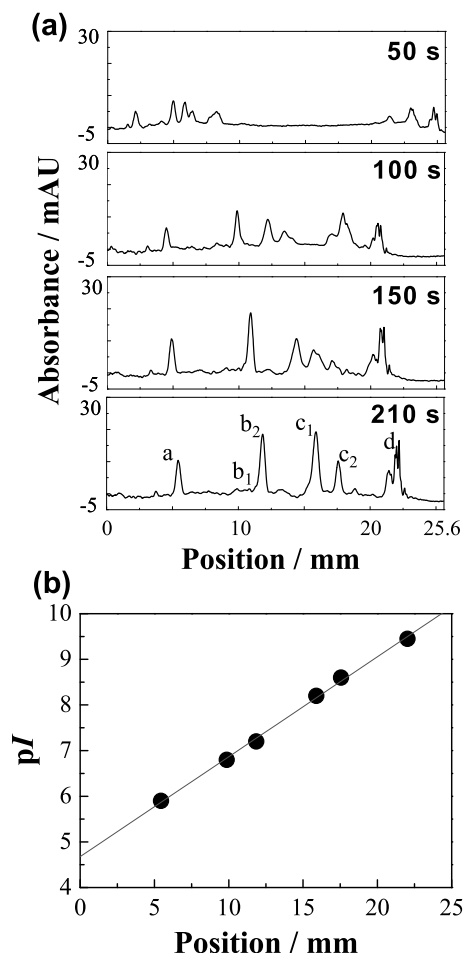


Fig. 4 (a) MCIEF separation of the standard proteins on the long channel chip. a, 28 $\mu\text{g/mL}$ carbonic anhydrase II; b₁, b₂, 56 $\mu\text{g/mL}$ myoglobin; c₁, c₂, 170 $\mu\text{g/mL}$ lentil lectin; d, 150 $\mu\text{g/mL}$ ribonuclease A. (b) Calibration curve for the focusing position *versus* the *pI* value of the standard proteins. Separation conditions as in Fig. 2.

in the position giving pH 8.0 and 8.4 with the time. This clearly shows that the pH change was completed in 75 s on the long channel chip. Thus, the time requiring the formation of a continuous pH gradient throughout the microchannel (75 s) was faster than the focusing time of proteins (120–210 s). Consequently, it was confirmed that the analysis time in MCIEF was determined by the migration rate of proteins, not by the formation rate of the pH gradient in the microchannel.

MCIEF separation of proteins

Figure 4a shows the process of the MCIEF separation of the four standard proteins on the long channel chip. By applying a voltage, several peaks appeared, and finally they were focused to six peaks in 210 s. The shapes of the five peaks at *pI* values from 5.9 to 8.6 were good, whereas the peak shape of ribonuclease A (*pI* 9.5) was poor. This is because the *pI* of ribonuclease A was close to the limit of the pH range of the ampholyte used in this study (*pI* 3–10). In Fig. 4b, the peak positions at 210 s are plotted against the *pI* values of the standard proteins. A good linearity was obtained with regression slope, intercept and correlation coefficient values of 0.219 mm^{-1} , 4.68, and 0.998, respectively. This indicates that the *pI* value of an unknown protein can also be determined by simple interpolation in MCIEF. The run-to-run repeatability ($n = 4$) of the peak

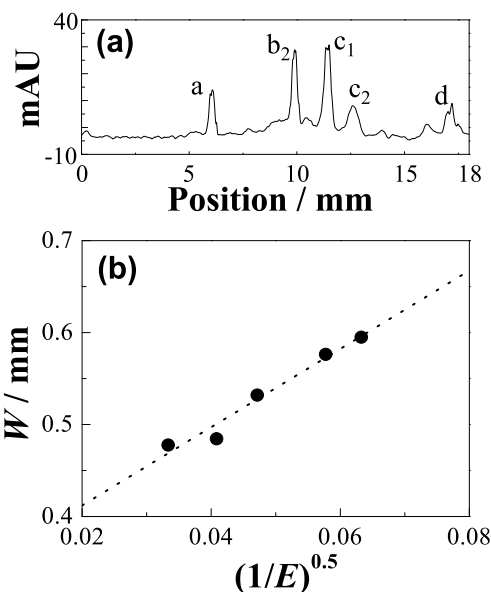


Fig. 5 (a) MCIEF separation of the standard proteins on the short channel chip at the separation time of 130 s. a, 110 $\mu\text{g/mL}$ carbonic anhydrase II; b₂, 210 $\mu\text{g/mL}$ myoglobin; c₁, c₂, 620 $\mu\text{g/mL}$ lentil lectin; d, 550 $\mu\text{g/mL}$ ribonuclease A. (b) Effect of the applied voltage on the peak width of myoglobin. Separation conditions as in Fig. 2.

position was measured, and the RSD values were acceptable, less than 5.4%. The variation caused by this RSD value was less than 0.07 in the *pI* unit. The RSD values of the peak height were good, less than 1.9%. The limit of detection ($S/N = 3$) of myoglobin was 3 $\mu\text{g/mL}$. Since the volume of the separation channel was 0.21 μL , the detectable amount of myoglobin was 0.63 ng. Thus, good linearity and repeatability were obtained, even in the MCIEF analysis of proteins, while the sensitivity was insufficient due to a short optical pass length in UV detection.

MCIEF on short channel chip

To improve the separation performance, a short channel chip was applied to MCIEF. The short channel length is expected to give a faster IEF separation. In the MCIEF analysis of myoglobin, actually, the focusing time was reduced from 140 s (long channel) to 47 s (short channel) at an applied voltage of 450 V/cm. However, the resolutions of the four standard proteins on the short channel chip were less than those on the long channel chip, as shown in Fig. 5a. This is due to a steeper pH gradient formed in the short channel compared to the long channel. In the short channel chip, the width of the separation channel (30 μm) was also smaller than that in the long channel chip (110 μm). Hence, the dissipation ability of Joule heating in the short channel chip is superior to that in the long channel chip, so a higher electric field can be applied to the separation channel. In IEF, the peak width can be expressed by the following equation:³⁴

$$W = 4 \sqrt{\frac{D}{E(-d\mu/dpH)(dpH/dx)}}, \quad (5)$$

where W , D , E , dpH/dx , and $d\mu/dpH$ are the peak width at the baseline in mm, the diffusion coefficient of analytes, the applied electric field strength, the pH gradient, and the mobility change of analytes against the matrix pH. This equation clearly shows

Table 1 Effect of an applied voltage on the MCIEF separation of myoglobin

$E/V\text{ cm}^{-1}$	Focusing time/s	W/mm	R_s^a
900	26	0.48	1.97
600	40	0.49	1.90
450	47	0.53	1.64
350	67	0.58	1.25
250	85	0.60	1.18

a. The resolutions were calculated by the equation $R_s = 1.18 \times (x_2 - x_1) / (W_{1/2,1} + W_{1/2,2})$, where x and $W_{1/2}$ are the peak position and the peak width at the half peak height, respectively.

that the application of a higher separation voltage provides a high efficiency. When the applied voltage was varied from 250 to 900 V/cm on a short channel chip, the peak width of myoglobin became narrow from 0.60 to 0.48 mm, as summarized in Table 1. Consequently, the focusing time was reduced from 85 to 26 s, whereas the resolution was increased from 1.18 to 1.97. Thus, the application of a high voltage is effective to obtain high-speed separation with high efficiencies in MCIEF.^{35,36} To make sure, we checked the effect of Joule heating on the efficiency. In Eq. (5), the peak width, W , should be proportional to the $E^{-1/2}$. As shown in Fig. 5b, a good linear relationship was obtained under an electric field ranging from 250 to 900 V/cm. This indicated that band broadening by Joule heating did not occur under the present condition.

Conclusion

In this study, a high-speed MCIEF analysis of proteins employing linear-imaging UV detection was demonstrated. The application of a linear-imaging UV detector in MCIEF could eliminate the need for sample derivatization and a mobilization process after focusing. By utilizing a short channel chip, the MCIEF separation of four proteins was achieved in 130 s at 450 V/cm, while the fastest focusing of myoglobin was obtained in 26 s at 900 V/cm. In MCIEF, therefore, the analysis time could be significantly shortened relative to conventional CIEF (20–40 min). A good linearity and repeatability were also obtained. The obtained results demonstrate that MCIEF is a useful tool for a high-throughput protein analysis system. Such a high-throughput system should promote the elucidation of biological functions based on “omic” approaches including proteomics and peptidomics, which require particularly massive measurements of amphoteric biopolymers. Also, a high-speed MCIEF can be employed in a routine analysis of proteins in a wide variety of industrial products for supporting product-development and quality control.

Furthermore, the focusing process of proteins and the formation process of a pH gradient could be monitored in MCE-2010. We elucidated that the time requiring the formation of a continuous pH gradient throughout the microchannel was faster than the focusing time of proteins, indicating that the analysis time in MCIEF was determined by the migration rate of proteins. The visualization ability for not only the concentration, but the pH profile in the microchannel will accelerate to elucidate detailed mechanisms in several electrophoretic techniques, e.g., IEF, dynamic pH junction,³³ sweeping in a sharp pH gradient induced by inserting a metal wire into the separation column,³⁷ and so on.

Acknowledgements

The authors thank Shimadzu (Kyoto, Japan) for their technical assistance. F. K. is grateful for a Grant-in-Aid for Scientific Research (No. 20750060) from the Japan Society for the Promotion of Science (JSPS). This study is a part of joint research, which is focusing on the development of a basis of technology for establishing COE of Nano-medicine, carried out through Kyoto City Collaboration of Regional Entities for the Advancing Technology Excellence assigned by Japan Science and Technology Agency (JST). This work was supported in part by a Grant-in-Aid for 21st Century Program, COE for a United Approach for New Materials of Science, from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. This research was also supported in part by the Global COE Program “International Center for Integrated Research and Advanced Education in Materials Science” (No. B-09) of the MEXT, administrated by the JSPS.

References

1. T. Pritchett and F. A. Robey, “Capillary Electrophoresis of Proteins in Handbook of Capillary Electrophoresis”, ed. J. P. Landers, 2nd ed., **1997**, Chap. 9, CRC Press, Boca Raton.
2. T. Wang and R. A. Hartwick, *Anal. Chem.*, **1992**, *64*, 1745.
3. S. C. Beale and S. J. Sudmeirer, *Anal. Chem.*, **1995**, *67*, 3367.
4. X.-Z. Wu, J. Wu, and J. Pawliszyn, *Electrophoresis*, **1995**, *16*, 1474.
5. J. Wu, A. H. Watson, and A. R. Torres, *Am. Biotechnol. Lab.*, **1999**, *17*, 24.
6. X.-Z. Wu, N. S. K. Sze, and J. Pawliszyn, *Electrophoresis*, **2001**, *22*, 3968.
7. X.-Z. Wu, J. Wu, and J. Pawliszyn, *LC-GC*, **2001**, *19*, 526.
8. J. A. Olivares, P. C. Stark, and P. Jackson, *Anal. Chem.*, **2002**, *74*, 2008.
9. N. S. K. Sze, T. Huang, and J. Pawliszyn, *J. Sep. Sci.*, **2002**, *25*, 15.
10. Z. Liu and J. Pawliszyn, *Anal. Chem.*, **2003**, *75*, 4887.
11. N. Li, K. Kessler, L. Bass, and D. Zeng, *J. Pharm. Biomed. Anal.*, **2007**, *43*, 963.
12. H. C. Cui, K. Horiuchi, P. Dutta, and C. F. Ivory, *Anal. Chem.*, **2005**, *77*, 1303.
13. C. Li, Y. Yang, H. G. Craighead, and K. H. Lee, *Electrophoresis*, **2005**, *26*, 1800.
14. C. Das, Z. Xia, A. Stoyanov, and Z. H. Fan, *Instrum. Sci. Technol.*, **2005**, *33*, 379.
15. B. Yao, H. H. Yang, Q. L. Liang, G. Luo, L. D. Wang, K. N. Ren, Y. D. Gao, Y. M. Wang, and Y. Qiu, *Anal. Chem.*, **2006**, *78*, 5845.
16. C. Das and Z. H. Fan, *Electrophoresis*, **2006**, *27*, 3619.
17. K. Ren, Q. Liang, B. Yao, G. Luo, L. Wang, Y. Gao, Y. Wang, and Y. Qiu, *Lab Chip*, **2007**, *7*, 1574.
18. K. Shimura, K. Takahashi, Y. Koyama, K. Sato, and T. Kitamori, *Anal. Chem.*, **2008**, *80*, 3818.
19. Q. L. Mao and J. Pawliszyn, *Analyst*, **1999**, *124*, 637.
20. J. Ou, T. Glawdel, R. Samy, S. Wang, Z. Liu, C. L. Ren, and J. Pawliszyn, *Anal. Chem.*, **2008**, *80*, 7401.
21. M. Vlčková, F. Kalman, and M. A. Schwarz, *J. Chromatogr., A*, **2008**, *1181*, 145.

22. H. Nakanishi, T. Nishimoto, A. Arai, H. Abe, M. Kanai, Y. Fujiyama, and T. Yoshida, *Electrophoresis*, **2001**, 22, 230.
 23. Z. Q. Xu, T. Hirokawa, T. Nishine, and A. Arai, *J. Chromatogr., A*, **2003**, 990, 53.
 24. M. Ludwig, F. Kohler, and D. Belder, *Electrophoresis*, **2003**, 24, 3233.
 25. F. Xu, M. Jabasini, B. Zhu, L. Ying, X. Cui, A. Arai, and Y. Baba, *J. Chromatogr., A*, **2004**, 1051, 147.
 26. T. Miyado, Y. Tanaka, H. Nagai, S. Takeda, K. Saito, K. Fukushi, Y. Yoshida, S. Wakida, and E. Niki, *J. Chromatogr., A*, **2004**, 1051, 185.
 27. F. Kitagawa, S. Aizawa, and K. Otsuka, *Anal. Sci.*, **2005**, 21, 61.
 28. E. Guihen and J. D. Glennon, *J. Chromatogr., A*, **2005**, 1071, 223.
 29. Z. Q. Xu, Y. Nakamura, and T. Hirokawa, *Electrophoresis*, **2005**, 26, 383.
 30. A. R. Stettler and M. A. Schwarz, *J. Chromatogr., A*, **2005**, 1063, 217.
 31. E. Guihen, G. D. Sisk, N. M. Scully, and J. D. Glennon, *Electrophoresis*, **2006**, 27, 2338.
 32. S. Hjertén, *J. Chromatogr.*, **1985**, 347, 191.
 33. J.-B. Kim, P. Britz-McKibbin, T. Hirokawa, and S. Terabe, *Anal. Chem.*, **2003**, 75, 3986.
 34. J. Wu and J. Pawliszyn, *Anal. Chem.*, **1992**, 64, 219.
 35. Y. Li, D. L. DeVoe, and C. S. Lee, *Electrophoresis*, **2003**, 24, 193.
 36. A. E. Herr, J. I. Molho, K. A. Drouvalakis, J. C. Mikkelsen, P. J. Utz, J. G. Santiago, and T. W. Kenny, *Anal. Chem.*, **2003**, 75, 1180.
 37. W. Wei, G. Xue, and E. S. Yeung, *Anal. Chem.*, **2002**, 74, 934.
-