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Poly(methylmethacrylate) Microchip Electrophoresis of Proteins Using Linear-poly(acrylamide) Solutions as Separation Matrix

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Poly(methylmethacrylate) (PMMA) microchip electrophoresis of sodium dodecyl sulfate-protein complexes (SDS-PC) using linear-poly(acrylamide) (L-PA) as a separation matrix was investigated. Prior to electrophoresis, channel walls of PMMA were modified with methylcellulose (MC) to prevent adsorption between channel walls and SDS-PC. Size-based protein separation (SBPS) was successfully performed using the MC-coated microchips with Ferguson plot-fittings. The entangled L-PA solution provided high resolution of peaks of SDS-PC when the concentration of L-PA was increased. Some investigations into the separation mechanism, such as the plot of the logarithm of mobility of each SDS-PC *versus* the logarithm of the molecular weight of the complex exhibiting linear behavior, indicated that the separation mechanism was dependent on mass discrimination, in accordance with Ogston model.

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Introduction

To reveal the functions of proteins and to identify the profiles of proteins have been critical issues in proteomics. The standard technique for protein analysis has been a size-based protein separation (SBPS) such as sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE) for decades. Shapiro *et al*.[1] first adopted this technique for analysis of polypeptide chains. Laemmli[2] improved it using a discontinuous buffer system. Much work has been applied to capillary electrophoresis (CE) to develop more sophisticated analytical systems for SBPS. For example, Zhu *et al*.[3] adopted an entangled polymer, poly(ethylene glycol) (PEG), as a substite of a cross-linked sieving polymer such as poly(acrylamide) (PAA). It was reported that 7 proteins (14.2 – 205 kDa) were separated within 12 min and also successive runs for 50 times were possible.[4] An excellent review on CE-dependent SBPS has been published.[5]

Recently, microchip electrophoresis (ME) has emerged as a miniaturization of CE and has attracted great attention due to its promising potential for faster analysis, even better economy of analyte and better integration of analytical procedures as well as better heat transfer compared with CE. It is hoped that microchip devices should be alternative analytical tools not only for proteome research but also for clinical analysis devices.[6] Much work on SBPS has been done on microchips with high performance. For example, Yao *et al*.[7] compared the performance of CE-dependent SBPS to that of glass-dependent ME. Bousse *et al*.[8] succeeded in separating 11 proteins from 6 to 200 kDa on a soda-lime glass microchip having channels filled with a low viscosity separation medium, poly(dimethyl-acrylamide) (PDMA), as a substite of PAA gel. Tabuchi *et al*.[9] succeeded in 15 s SBPS using a pressurization technique. Other works on ME-dependent SBPS have been summarized in a recent review.[10]

Despite a great deal of work on SBPS using CE or glass-dependent ME, there have been few reports about SBPS in polymeric microchips.[11,12] SDS-protein complexes (SDS-PC) strongly adsorb on channel walls of the microchips *via* hydrophobic interaction,[13] disturbing analytical performances in SBPS. The strategy of channel wall modification have been tailored. For example, Wang *et al*.[11] coupled permanent and dynamic coating to a PDMS microchip by covalently coating it with PAA and then non-covalently rinsing the PAA-coated PDMS with methylcellulose (MC) to integrate isoelectric focusing (IEF) and separation of SDS-PC on the PDMS microchip. Nagata *et al*.[12] demonstrated the separation of 3 proteins on a PMMA microchip that was covalently modified with PEG. Unfortunately, the covalent channel wall modification was difficult and cumbersome to perform. Therefore, further investigation about the modification for SBPS in polymeric microchips is needed.

In this paper, we demonstrate SBPS in poly(methyl-methacrylate) (PMMA) microchip electrophoresis. Suppression of the adsorption of SDS-PC on channel walls of PMMA was

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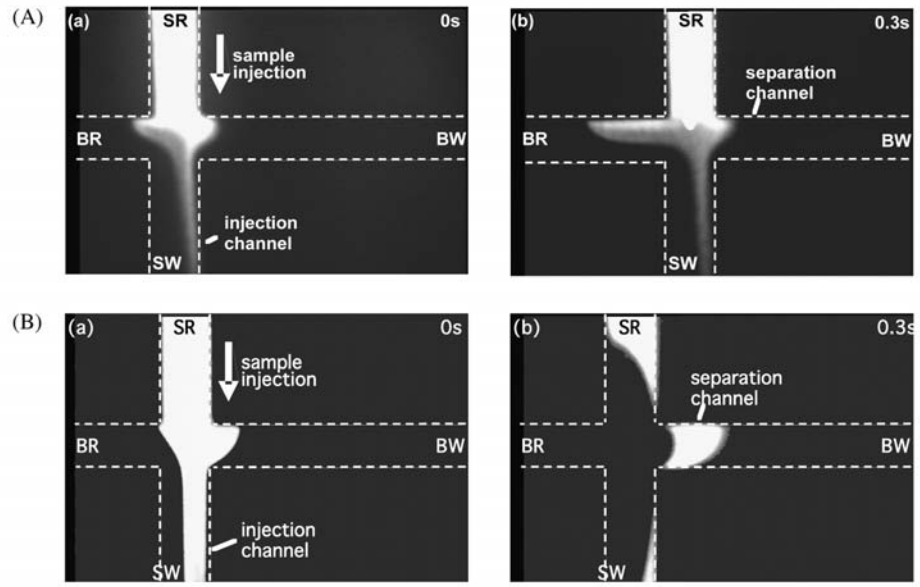

Fig. 1 Images of injection and separation processes of SDS-PC in non-coated channel walls (A) and in MC-coated channel walls (B) at the intersection of injection and separation channels. The white bold arrow is the direction of the motion of injected SDS-PC. (a) Start of separation of SDS-PC, (b) 0.3 s later. SDS-PC were composed of LZ (14.4 kDa), TI (20.5 kDa), CA (29.0 kDa) and each protein was labeled with the fluorescent dye, Cy2. The concentration of each protein was 50 ng/μL. Running buffer composition: 5 mM Tris-HCl, 3.5 mM SDS, and 3% L-PA. The electric field of separation was 950 V.

achieved by two steps: the walls were firstly rinsed with methylcellulose (MC) and then were dried at room temperature. SBPS using an entangled linear-poly(acrylamide) (L-PA) as a separation medium was successfully performed in the MC-modified PMMA microchip electrophoresis with high reproducibility of migration time. Several evaluations about separation performance indicated that Ogston model was available for the mechanism of SBPS on the PMMA microchip. The separation performance was comparable to that of CE or other SBPS devices, therefore, our coating method is promising for protein analysis.

Experimental

Reagents and chemicals

Cy2 bis-reactive dye, Cy5 mono-reactive dye and Sephadex™ G-25 superfine were purchased from GE Healthcare Bio-Sciences Co. (Piscataway, NJ). Lysozyme (LZ) made from chicken egg white, trypsin inhibitor (TI) made from glycine max (soybean), carbonic anhydrase (CA) made from bovine erythrocytes, sodium dodecyl sulfate (SDS), and methylcellulose (MC) (viscosity of 2% aqueous solution at 20˚C, 4000 cP) were purchased from Sigma Chemical (St. Louis, MO). Linear-polyacrylamide (L-PA) (M_W; 600000 – 1000000) was bought from Tokyo Kasei (Tokyo, Japan). Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl) and 2-mercaptoethanol were purchased from Kanto Chemical (Tokyo, Japan). Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA).

Sample preparation and buffer solution

Each protein was dissolved in 200 mM Na_2CO_3 solution (pH 9.3) to a final concentration of 1 mg/mL. An aliquot of 1 ml of the solution was transferred to a vial containing Cy2 or Cy5 (concentration not available) and was incubated for 1 h. The mixture was introduced to a Sephadex G-25 column which had already been swelled with Milli-Q water to remove the dye unbounded to protein. Each Cy2-labeled protein or Cy5-labeled protein was mixed and denatured with the sample buffer (5 mM Tris–HCl, 2% SDS, 0.1 M 2-mercaptoethanol, pH 7.4) at 95˚C for 5 min to a final concentration of 50 ng/μL. Cy2-labelled proteins were used for the experiments shown in Fig. 1. For the experiment shown in Fig. 2, Cy5-labelled proteins were used. Running buffers with 5 mM Tris–HCl were added to the 3.5 mM SDS solution and then were adjusted to the designed concentration of L-PA.

Apparatus

The images of adsorption of SDS-PC were taken using a fluorescent microscope, Axiovert 135TV (Carl Zeiss, Tokyo, Japan) equipped with a 100-W mercury arc lamp, a 10×/0.3 NA objective lens (Carl Zeiss), a No. 9 filter set (Carl Zeiss), and a silicon-intensified target (SIT) camera, C2400-08 (Hamamatsu Photonics, Hamamatsu, Japan). The fluorescent images were recorded on a DV tape (DSR-11, Sony, Tokyo, Japan) through an image processor, Argus 20 (Hamamatsu Photonics). The images were translated into AVI-files by a video capture board (DV Storm-RT; Canopus, Kobe, Japan). Electropherograms were obtained by a μ-CE system, SV1210 (Hitachi High-Technologies, Tokyo, Japan), consisting of a light-emitting diode (LED) detector (ex/em; 650/680 nm) and a high voltage supply. The PMMA microchip was an i-chip 3 (Hitachi High-Technologies) that had 100 μm wide and 30 μm deep channels. The length of the injection channel between the sample reservoir (SR) and the sample waste reservoir (SW) was 10.5 mm, whereas the length of the separation channel from the buffer reservoir (BR) to a buffer waste reservoir (BW) was 44 mm.

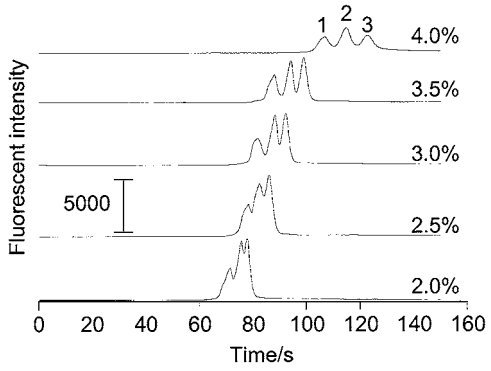

Fig. 2 Protein separation using a entangled polymer, L-PA. L-PA concentrations (%) are shown in the figure. The L-PA's molecular weight (M_W) range: 600 – 1000 kDa. The code of protein peaks: 1, LZ (14.4 kDa); 2, TI (20.5 kDa); 3, CA (29.0 kDa) and each protein was labeled with the fluorescent dye, Cy5. Other experimental conditions were the same as those in Fig. 1.

Modification of channel walls of PMMA

Pristine channel walls of PMMA adsorb SDS-PC *via* a hydrophobic interaction.[13] To prevent the adsorption, we modified the walls with hydrophilic polymer, methylcellulose (MC). The method of the modification was very simple. MC was dissolved in water to the 0.15% (w/v) concentration by stirring slowly until the solution appeared to be homogeneous and optically transparent. Prior to electrophoresis, the MC-containing aqueous solution was introduced through the reservoir, SW, by means of a 1-ml syringe. After completely filled with the solution, the channels were dried for 1 day at room temperature (typically 25˚C) in order to immobilize the MC coating. Next, the coated channel walls were filled with running buffers and electrophoresis was then carried out.

Microchip electrophoresis

Microchip electrophoresis was done using the μ-CE system, SV1210. Two channels (injection and separation channels) and 3 reservoirs (SW, BR, BW) were filled with running buffers by syringe and then SDS-PC were introduced at SR by a pipette. For the process of sample injection, 250 V was applied at SW for 40 s, while the other reservoirs were grounded and then, for that of sample separation, 950 V was applied at BW during separation, whereas 250 V was applied at both SR and SW, and BR was grounded. Injected SDS-PC were separated during electrophoresis and were detected at a point of 30 mm downstream from the intersection of the separation and injection channel by an LED confocal fluorescence detection method. The electrophoretic data were obtained by 10 different measurements using 10 different channels.

Measurement of electro-osmotic flow (EOF)

The current monitoring method was used to measure the mobility of EOF.[14] All of the channels and 2 reservoirs (BR and BW) were filled with 5.0 mM Tris–HCl (containing 4% of L-PA and 3.5 mM SDS) and SW was filled with 25 mM Tris–HCl (containing 4% of L-PA and 3.5 mM SDS). In addition, the sample buffer was introduced to SR so as to correspond with the experimental conditions of protein separation. Then a positive voltage (250 V) was applied to SW and the other reservoirs were grounded. The mobility of EOF was calculated based on the time of the current variation.

Results and Discussion

Protein separation after modification of channel walls

Pristine channel walls of PMMA microchip induced hydrophobic adsorption between the walls and SDS-PC as well as SDS *via* hydrophobic interaction.[13] Such adsorption induced cathodic electroosmotic flow (EOF) extensively interfering with introduction of the SDS-PC to detection point (Fig. 1A), whereas the introduction was achieved well in MC-coated channel walls (Fig. 1B). Tanaka *et al.*[15] reported that MC worked as EOF suppressor in the same channel walls of PMMA (also i-chip 3, Hitachi High-Technologies). Their coating method was to precondition the channel walls with MC-containing buffer which was never evaporated; then MC was further added to running buffer, which resulted in successfully separating saliva samples. We also tried their coating method, but no remarkable improvement was obtained. We thought that evaporation of MC aqueous solution contributed to formation of hydrophilic MC layers on the channel walls and such layers repelled the hydrophobic interaction inducing the strongly cathodic EOF. Actually, cathodic EOF in MC-coated channels was decreased to 1.97×10^{-5} cm^2/V s^{-1}, whereas the value is 5.86×10^{-3} cm^2/V s^{-1} in pristine channels.

In this MC-coated PMMA chips, we separated 3 proteins using an entangled polymer, L-PA, at various concentrations (2.0 – 4.0%). The high speed separation was performed within 130 s and quite good reproducibility was obtained with a relative standard deviation (RSD) within 2.2% at any concentration of L-PA (Fig. 2). These RSD values were comparable to those in other reports.[9-13] In this connection, L-PA with the concentrations above 4.0% could not be filled in channels due to its high viscosity. We selected the 3 proteins since we usually analyze 2 – 4 protein markers with molecular weight range from 10 to 30 kDa in cancer diagnosis.[16] In addition, proteins with larger M_W than CA (29 kDa) could not be detected in the MC-coated chips. We thought it was caused by the relatively higher EOF (1.97×10^{-5} cm^2/V s^{-1}, above mentioned). The mobility of CA in 4.0% L-PA solution was 7.71×10^{-5} cm^2/V s^{-1}. Generally, SBPS in CE was performed under the condition that the mobility difference between SDS-PC and EOF was 10 or more.[17] Therefore, the larger proteins than CA could be hardly introduced to the detection point only with difficulty since they should have lower mobility than CA. To separate more SDS-PC, further improvement for decreasing EOF was needed.

Noncross-linked polymers such as L-PA begin interacting with the surrounding polymers and creating mesh networks which can sieve SDS-PC above the concentration threshold of L-PA.[18] SDS-PC migrated through the mesh network of L-PA chains and was apparently sieved by the network since the threshold of L-PA was regarded to be 0.40 – 0.65% (w/v)[19] which was much lower than the concentrations adopted in this work. Therefore, as shown in Fig. 2, when the concentration of L-PA was increased, the resolutions were clearly improved; at the same time, the migration time of sample peaks became longer. It could be seen that the peaks of LZ at any concentration of L-PA had shoulder shape, which was caused by the different numbers of Cy5 attached to LZ. We used the impurity-free proteins, so that the 2 proteins (TI and CA) had a single peak. In the Cy5 staining step, the different number of Cy5 attached to LZ, which made a wide molecular range of LZ, and then the shoulder peaks appeared.

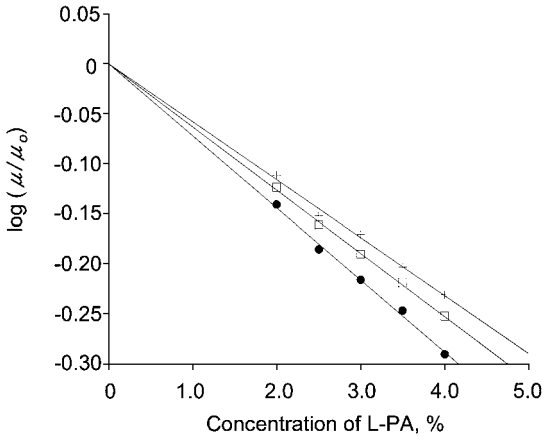

Fig. 3 Ferguson plots: (+) LZ, (□) TI, and (●) CA. Calibrate equation: the line +, $y = -0.058002x + 1.4901 \times 10^{-9}$ ($R^2 = 0.99017$); the line □, $y = -0.063171x - 8.9407 \times 10^{-9}$ ($R^2 = 0.99783$); the line ●, $y = -0.07205x - 8.9407 \times 10^{-9}$ ($R^2 = 0.99415$).

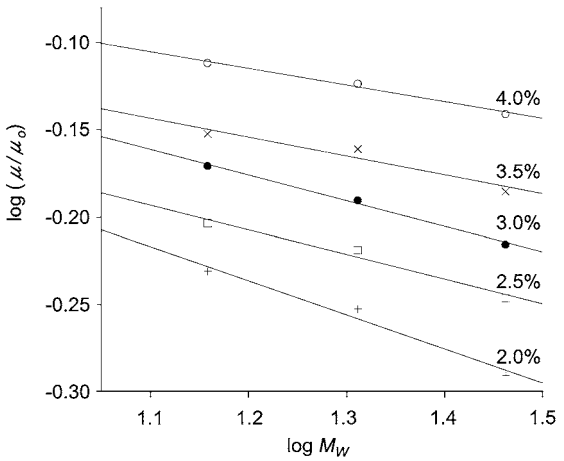

Fig. 4 Relationship between logarithm of relative electrophoretic mobility (μ/μ_o) of each SDS-PC and logarithm of M_W of proteins. The concentration (%) of L-PA was inserted in the figure.

Coating stability

We checked whether our coating method ensured the long-term reproducibility of electrophoretic performances by demonstrating consecutive runs. Unfortunately, the performances could not be repeated after the first electrophoresis (data not shown). This result meant that MC layers immobilized on channel walls were supposedly dissolved in the running buffer during first electrophoresis, which led to no reelectrophoresis. In addition, this coating unstability seemed to affect the band broadening (electropherogram of 4.0% L-PA in Fig. 2).

We challenged to regenerate the layer by repeating our coating method after the first electrophoresis. However, the channels were clogged during the evaporation step and reelectrophoresis could not be conducted. We also checked another challenge that the MC was added to the running buffer containing L-PA, so that MC would dynamically modify channel walls during the first electrophoresis. Unfortunately, the MC inserted into running buffer began to be clouded due to the interaction between the polymers, MC and L-PA, resulting in poor electrophoretic performances. PMMA is a disposable material and we therefore thought that these demerits of our coating could be entirely overcome. Further investigation is needed to construct a more stable coating.

Separation property of entangled L-PA solution

The retardation of SDS-PC in entangled polymer solution is a function of the concentration of separation polymer (C) and the retardation coefficient (K_R):[20]

$$\mu = \mu_o \exp(-K_R C) \quad (1)$$

where μ is the actual electrophoretic mobility of SDS-PC and μ_o is the mobility of SDS-PC in the solution without the entangled polymer. The Eq. (1) was converted into logarithm scale:

$$\log(\mu/\mu_o) = -K_R C \quad (2)$$

As given in Eq. (2), plots of the logarithm of the electrophoretic mobility and the concentration of the entangled polymer are known as Ferguson plots.[21] In Fig. 3, Ferguson plots showed quite linear responses (correlation efficient $R_2 > 0.99$) with a slope equal to K_R and the extrapolation of the lines for all of the proteins at 0% L-PA was almost zero (RSD = 0.19%). Generally, the intercepts of all the SDS-PC were independent of their own molecular weight (M_W) since all the complexes had similar charge to weight ratios: 1.4 g of SDS per 1.0 g of protein.[22] According to Ogston model, Ferguson plots offer almost the same y-intercepts ($\log \mu/\mu_o = 0$) with small RSDs (< 0.20%) as well as rather linear behavior.

Additionally, Ferguson plots of SDS-PC (14 to 65 kDa) which separated in cross-linked polyacrylamide synthesized by 15% (w/v) *N*,*N*-methylenebis(acrylamide) exhibited linear behavior when their separation performance obeyed the Ogston model according to some experimental evidence.[23]

Moreover, according to Nakatani *et al.*,[24] the logarithm of relative electrophoretic mobility, $\log(\mu/\mu_o)$, *versus* the logarithm of M_W, $\log M_W$ of protein should also exhibit linear response when the performance was in agreement with Ogston model, *i.e.*,

$$\log(\mu/\mu_o) \sim \log(M_W) \quad (3)$$

Figure 4 shows the plots of $\log(\mu/\mu_o)$ *versus* $\log(M_W)$ of protein. The plots exhibited a linear behavior and were matched to Eq. (3). Above-mentioned results and discussion validated the assertion that the separation mechanism of SDS-PC on a coated PMMA microchip with L-PA separation matrix was performed in accordance with the Ogston model.

We used L-PA with the M_W range (600 – 1000 kDa); other ranges were not tested. According to Guttman's report,[25] the larger M_W of PEG used as an entangled polymer for SBPS in CE would make the separation mechanism of SDS-PC fit to the reptation model rather than the Ogston model. Thus, in the case of larger M_W of L-PA used in separation of SDS-PC, the separation mechanism may be different from the Ogston model, which needs to be further investigated.

Conclusions

Migration of SDS-PC was disarranged by a strong cathodic flow, EOF, which was derived from the adsorption of SDS-PC on channel walls of PMMA microchip *via* hydrophobic interaction. MC-mediated channel wall coating transferred

hydrophobic channel walls into hydrophilic ones by non-covalently modifying channel walls using MC as the coating reagent, which noticeably suppressed the adsorption and therefore EOF. Injection and separation of SDS-PC succeeded in a separation buffer containing L-PA. Ferguson plots and the double logarithm plots of relative electrophoretic mobility *versus* M_Ws of proteins indicated that the electrophoretic performance in MC-coated PMMA microchips fitted to Ogston model, which were comparable to SBPS in CE or other analytical devices. Therefore, our work was applied to protein sizing as well as improved the compatibility between SDS-PC and PMMA microchip. We are convinced that our work will help to construct a high-throughput screening system for proteome analysis on PMMA microchips.

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