One-step immobilization of cationic polymer onto a poly(methyl methacrylate) microchip for high-performance electrophoretic analysis of proteins

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Abstract

One-step covalent immobilization of poly(ethyleneimine) (PEI) onto poly(methyl methacrylate) (PMMA) substrates was investigated to achieve an efficient separation of basic proteins in microchip electrophoresis (MCE). The PEI-treated PMMA chip showed the anodic electroosmotic flow and its rate was almost kept stable during 32 days with over 50 runs. This longer stability of the prepared microchip indicated that the loss of PEI was successfully suppressed by the immobilization through the covalent bond. Furthermore, the PEI modification onto the PMMA chip could apparently reduce the surface adsorption of cationic proteins. In the MCE analysis on the PEI-modified microchip, two proteins were successfully separated within 30 s only utilizing a separation length of 5 mm. While the migration time of the protein gradually increased during only four consecutive runs on an untreated PMMA chip, reproducible analyses were attained by using the PEI immobilized microchip. These results demonstrated that Coulombic repulsion force generated between cationic PEI and basic proteins could avoid the irreversible adsorption of the analytes onto the PMMA surface, which provided a high-performance analysis medium for biogenic compounds.

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Keywords: Microchip electrophoresis; Protein analysis; Surface modification; Poly(methyl methacrylate)

1. Introduction

In recent years, on-chip analysis systems for biogenic compounds have undergone a steady progress and applied to the biological, medical, clinical, and pharmaceutical fields due to a potential for high-throughput parallel analysis of different samples, e.g., DNA, proteins, metabolites, sugar chains, and so on [1–4]. Among various on-chip analysis techniques, microchip electrophoresis (MCE), based on the separation principle of capillary electrophoresis (CE), has been widely used in the analysis of chemical and biogenic compounds because of its high separation efficiency, high-speed analysis and low consumption of samples in addition of the suitability for the parallel analysis on chips [5–8]. In the MCE analysis of biogenic compounds, especially proteins, however, it is well known that sample adsorption onto the surface of a separation microchannel due to electrostatic and/or hydrophobic interactions [9,10] often causes the reduction of the separation efficiency and the analytical reproducibility. Thus, various microchannel coating techniques have been developed to prevent the adsorption of biogenic compounds in MCE [11,12].

In the early stage of the development of MCE, inorganic materials like quartz, Pyrex glass and silicon were mainly employed as substrates. Since ionizable silanol groups (Si-OH) on these inorganic surfaces strongly attract with biogenic samples, considerable efforts have been devoted to develop the wall coating techniques for appropriate analytes. In the glass microchip, the wall modification is conducted by mainly two different methods: (i) physically adsorbed polymer coating (dynamic polymer coating) and (ii) covalently immobilized polymer coating. Dynamic coating which is conducted by adding surface active
polymers to a background solution (BGS) is the easiest technique for the surface modification. For this purpose, cellulose derivatives, e.g., hydroxyethylcellulose (HEC) [13], hydroxypropylmethylcellulose (HPMC) [14], and hydroxypropylcellulose [15], have been employed as buffer additives to suppress the adsorption of proteins. On the other hand, covalent attachment of polymers onto the glass surface based on silanization chemistry has been also applied. After the treatment with a silanization agent on the glass surface, polymers such as poly(acrylamide) [16,17], poly(vinylalcohol) [18] and poly(vinylpyrrolidone) [19] are covalently immobilized to reduce protein adsorption. Although stable coatings are obtained, siloxane bonds can be hydrolyzed in higher pH solution, which limits the use of the coating to acidic and neutral BGS.

On the other hand, polymer microchips have several advantages, e.g., suitability for mass production, less expensive, multiple methods of microchannel fabrication, and so on, which enables to be used as disposable analytical devices. Among various polymers, poly(methyl methacrylate) (PMMA) is one of the most popular substrates for the electrophoretic microdevices and several PMMA microchips are commercially available for the use in the MCE analysis. As well as glass microchips, however, the adsorption of proteins onto the surface of PMMA microchannel is often problematic for the electrophoretic separation, so that several coating techniques have been also introduced in PMMA chips. Dynamic coating of neutral polymers including HEC, HPMC, and poly(ethyleneimine) (PEI) which has been employed to coat fused silica capillaries for the efficient separation of basic proteins in CE was chosen as a surface modifier. High-molecular-mass PEI has a large number of secondary amino groups in PEI dissolved in neutral aqueous solution can proceed as shown in Scheme 1 of PMMA and secondary amino groups in PEI dissolved in basic aqueous solution can proceed as shown in Scheme 1 and the PEI-treated PMMA chip was applied to the MCE analysis of proteins. Effects of the immobilization of PEI onto the PMMA surface on the adsorption and the separation efficiency compared to untreated PMMA. Stable coatings were obtained by covalent modifications, whereas these techniques required to use organic solvents as reaction media and/or rinsing liquids. It is well known that the resistance of PMMA to many organic chemicals, e.g., acetone, chloroform, N,N’-dimethylformamide (DMF), hexane, methanol, tetrahydrofuran, toluene, and so on, is generally low, so that the use of these materials would damage PMMA chips. Therefore, a modification method with no organic solvents should be introduced to the PMMA microchip.

In this study, to obtain a stable coating with easy manipulations, the covalent immobilization of cationic polymer with amino groups onto the surface of PMMA microchip by nucleophilic addition–elimination reaction was investigated. This modification approach has been recently reported by Fixe et al. [26,27] to yield DNA chips based on PMMA substrates. However, the technique has been applied only to the immobilization of small oligonucleotide and hexamethylene-diamine, so that the application to larger polymers has not been reported. To achieve high-performance analysis of proteins, high-molecular-mass poly(ethyleneimine) (PEI) which has been employed to coat fused silica capillaries for the efficient separation of basic proteins in CE was chosen as a surface modifier. High-molecular-mass PEI has a large number of amino groups and a positive net charge over a wide pH range, and thus the adsorption of cationic proteins can be reduced due to a strong electrostatic repulsion force generated between PEI and analytes. We anticipated that the one-step immobilization reaction between acylcarbon of PMMA and secondary amino groups in PEI dissolved in basic aqueous solution can proceed as shown in Scheme 1 and the PEI-treated PMMA chip was applied to the MCE analysis of proteins. Effects of the immobilization of PEI onto the PMMA surface on the adsorption and the separation efficiency of basic proteins were investigated.

2. Experimental

2.1. Chemicals

PEI ($M_W$ 750 000) and rhodamine B isothiocyanate (RBITC) were obtained from SIGMA-ALDRICH (Tokyo,
Japan), methanol and DMF from Nacalai Tesque (Kyoto, Japan), avidin (AVI) and ribonuclease A (RIB) from Wako (Osaka, Japan). Rhodamine B (RB, Tokyo Chemical Industry, Tokyo, Japan) was used as an electroosmotic flow (EOF) marker. All reagents were of analytical or HPLC grade. The BGS used throughout the experiments was 10 mM phosphate buffer (pH 4.0). All 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.

Proteins were labeled with RBITC for laser-induced fluorescence (LIF) detection. An RBITC solution dissolved in DMF was added to a protein solution in 50 mM borate buffer (pH 8.5) at a 10:1 RBITC/protein molar ratio. The derivatization was allowed to proceed in the dark at room temperature for 1 h. The reaction mixture was dialyzed to remove unreacted fluorescent dyes by using a cellulose membrane (Slide-A-Lyser Dialysis Cassettes, MWCO 3500, Pierce Biotechnology, Rockford, IL) for 3 h with a 10 mM phosphate buffer (pH 4.0) as a dialysis buffer. Before MCE separation, a RBITC derivatized protein solution was diluted with an appropriate amount of the BGS.

2.2. Microchip

A PMMA microchip was kindly supplied from Hitachi Chemical (Tokyo, Japan). Fig. 1 shows the schematic of the microchannel chip. The substrate was 85 × 50 × 1 mm. The microchip has a simple cross-type channel (100 μm width × 30 μm depth) with a total separation channel length of 38 mm.

2.3. Immobilization of cationic polymers on the PMMA surface

Immobilization of PEI onto the PMMA surface was performed according to the procedure reported by Fixe et al. [26,27]. Before the immobilization, the PMMA microchip was cleaned by soaking in methanol for 10 min, and rinsed thoroughly with deionized water. A solution of 10–20% (w/w) PEI in 100 mM borate buffer (pH 11.5) was pumped through the microchannel for 2 h at a flow rate of 3.3 μL/min using a syringe pump (KDS100, kd Scientific, Holliston, MA), followed by rinsing with deionized water for 20 min. The PMMA microchip was then dried at 30 ºC overnight. The channel was conditioned with the BGS prior to the MCE measurement.

2.4. Apparatus

MCE analysis of fluorescent samples was performed by using a home-made LIF detection scheme as shown in Fig. 2. A 532-nm laser beam (10 mW diode-pumped solid-state (DPSS) laser, 58GCS411, Melles Griot, Tokyo, Japan) for excitation was introduced to an inverted optical microscope (IX71, Olympus, Tokyo, Japan) and, irradiated to the microchannel (spot size for excitation, ca. 10 μm) through an objective lens ( × 20, NA = 0.40, LCPlanFL20X, Olympus). Fluorescence from analytes collected by the same objective lens was passed through a dichroic filter (U-MWIG2, Olympus) and led to a multichannel photodetector (PMA-11, Hamamatsu Photonics, Hamamatsu, Japan). The sampling rate for data acquisition was 53 Hz. For the fluorescence imaging measurement, a 100-W mercury lamp (HBO103W/2, OSRAM, Augsburg, Germany) and a CCD camera (1K-TU53 H, Toshiba, Tokyo, Japan) were used as a light source and a detector, respectively, in the similar optical configuration. The microchip was placed on the 3D-stage of the microscope and the MCE separation was performed with a 5 channel-type high-voltage power supply (Shimadzu, Kyoto, Japan), which was controlled by a PC with a GPIB board.
2.5. MCE analysis of proteins

The channels were filled with 10 mM phosphate buffer (pH 4.0). Prior to separation, 7 µL of the protein sample was introduced into the reservoir S and a platinum electrode was inserted into each reservoir to provide electrical contact. To introduce the sample solution into the separation channel, the pinched injection technique [30,31] was employed as shown in Fig. 1. For the MCE analysis of basic proteins on the PEI-modified PMMA chip, the sample introduction was conducted mainly by means of the EOF with the reversal polarity. During sample loading, the reservoirs S and BW were grounded, and the applied voltages at the reservoirs B and SW were 150 and 400 V, respectively. During injection and separation, the reservoirs S and SW were set at 900 V, the reservoir B was grounded, and the reservoir BW was set at 2700 V. As for the untreated PMMA chip, the sample injection was performed by means of the electrophoretic mobility of proteins since the EOF rate was smaller in the bare PMMA channel. The voltage programs for the analysis of cationic proteins are summarized in Table 1. These conditions were also employed for the EOF rate measurements.

3. Results and discussion

3.1. Characterization of PEI-modified microchip

The modification approach in this study has been applied only to the immobilization of small molecules as mentioned in Section 1, so that the characterization of the PMMA chips treated with a high-molecular-mass PEI solution is very important to achieve an efficient MCE separation of proteins. First, the EOF rate measurements on the prepared PMMA chips were performed to evaluate the immobilization of PEI. In this study, RB was used as an EOF marker. RB has a carboxylate with the pK_a value of 3.1 and a protonated diethylammonium groups [32], so that the electrophoretic mobility of RB is nearly zero in the solution at the pH value above 4, which is an essential property for the EOF marker. Table 2 shows the electroosmotic mobility (μ_eo) determined with RB in the PMMA channel modified under several conditions. On the PEI treated chips, the anodic EOF was observed and the μ_eo at pH 4.0 was evaluated to be ca. −1.3 × 10^{-4} cm^2 V^{-1} s^{-1}. On the other hand, the EOF rate on the untreated PMMA chip was too small to attain a reproducible injection and detection of the EOF marker since a net charge on the bare PMMA surface is considered to be nearly zero, leading to the weaker EOF [33]. The pK_a value of aliphatic amino groups in PEI is 10–11, so that the amino groups become protonated at pH 4.0. The positively charged PEI immobilized onto the PMMA surface generates the electric potential, which causes the reversal EOF by applying the electric field. Thus, the reversal EOF observed on the prepared PMMA chips indicates a successful immobilization of PEI under basic conditions.

As shown in Table 2, furthermore, the μ_eo on the modified chips were not affected by the concentration of PEI and the temperature during the immobilization. As a preliminary result, the μ_eo on the PMMA chip modified with PEI solution at pH 12.5 also exhibited a similar value with that at pH 11.5. These results indicated that the immobilized PEI on the PMMA surface would be achieved as the saturated amount under the applied conditions, so that the μ_eo values are independent of the PEI concentration, the temperature, and probably the pH value in the range above 11.5. Thus, the stable immobilization of PEI could to be achieved by the modification procedure as shown in Scheme 1.

The endurance of the PEI-modified PMMA chip was also investigated. Fig. 3 shows a long-term stability of the μ_eo and the relative standard deviation (RSD) values of μ_eo.

Table 1
Voltage programs for MCE analysis of proteins on PMMA chips

| Microchip          | Step                  | Time(s) | S   | SW  | B   | BW  |
|--------------------|-----------------------|---------|-----|-----|-----|-----|
| PEI-modified PMMA  | Sample loading        | 60      | 0   | 500 | 190 | 0   |
|                    | Injection and separation | 100    | 750 | 750 | 0   | 1500|
| Bare PMMA chip     | Sample loading        | 60      | 500 | 0   | 420 | 500 |
|                    | Injection and separation | 100    | 1000| 1000| 1500| 0   |

Table 2
Effects of PEI immobilization conditions on μ_eoa

| Entry | PEI concentration (w/w %) | Temperature (°C) | μ_eo (10^{-4} cm^2 V^{-1} s^{-1})b,c |
|-------|--------------------------|------------------|------------------------------------|
| 1     | 10                       | 25               | −1.25                              |
| 2     | 10                       | 50               | −1.32                              |
| 3     | 10                       | 70               | −1.30                              |
| 4     | 20                       | 25               | −1.16                              |
| 5     | 20                       | 50               | −1.37                              |

aModification solution was prepared by dissolving PEI into 100 mM borate buffer (pH 11.5). Detailed procedures are as in Section 2.
bAverage value of five consecutive runs.
cMinus sign represents an anodic (reversal) EOF.
As can be seen, the \( \mu_{\text{eo}} \) values on the PEI-modified PMMA chips were almost unchanged during 32 days with over 50 runs. The run-to-run RSD values of the \( \mu_{\text{eo}} \) were acceptable, less than 8.6% (\( n = 5 \)). It should be noted that the day-to-day reproducibility of the \( \mu_{\text{eo}} \) was good with the RSD value of 5.2% (\( n = 4 \) within 32 days) in the dry storage condition at room temperature. In the previous reports on the covalently modified PMMA chips for MCE, the stability and/or the endurance of the coating have been scarcely mentioned, so that the comparison of the stability on the present coating with that obtained with other modification techniques is difficult. However, it is well known that a cationic polymer physically immobilized fused silica capillary shows a significant decrease in the EOF rate with only 20–25 runs, which indicates the detachment of the polymer layers [29,34]. Thus, the longer stability of the prepared microchip indicates that the loss of PEI was successfully suppressed by the immobilization through the covalent bond. Since the modification was carried out under basic conditions, furthermore, there is a possibility for slight hydrolysis of PMMA, which might provide a negatively charged surface. The electrostatic interaction between the positively charged amino groups of PEI and the negatively charged PMMA surface should stabilize the coated PEI layer. Therefore, the stable PEI coating on the PMMA surface would be provided by the cooperative immobilization based on the covalent binding and the electrostatic interaction.

### 3.2. MCE analysis of proteins on PEI-modified PMMA chips

Prior to the MCE analysis, the adsorption of proteins on the PEI-modified PMMA chip was evaluated. It is well known that AVI shows a strong adsorption on polymers surfaces, so that RBITC-AVI was selected as a test analyte. In the adsorption test for RBITC-AVI, the experimental procedure reported by Liu et al. [23] was performed on both the untreated and PEI-modified PMMA chips. Briefly, an RBITC-AVI solution was introduced to the microchips. After 30 min, the microchannel was rinsed with deionized water for 2 h at a flow rate of 600 \( \mu \)L/min. The injection cross region was irradiated with a 100-W mercury lump being passed through a band-pass filter (535 ± 15 nm), and fluorescence (> 580 nm) was recorded by a CCD camera. As shown in Fig. 4, RBITC-AVI adsorbed strongly on the bare PMMA microchips and the fluorescence could be observed in whole channel surfaces. On the other hand, fluorescence from proteins was apparently decreased on the PEI-modified microchip. The result clearly demonstrates that the protein adsorption was suppressed by the immobilization of PEI. Since the isoelectric point (\( pI \)) of AVI is 10.0 [35], the electric repulsion force generated between the positively charged AVI and PEI layer would reduce the amount of the adsorbed proteins onto the surface. Therefore, the PEI-modified PMMA chips would be suitable for the MCE analysis of basic proteins.

Fig. 5 shows the MCE analysis of RBITC-AVI on the bare and PEI-modified PMMA microchips at pH 4.0. As shown in the figure, RBITC-AVI was detected within 30 s and the peak tailing was scarcely observed on both
microchips, so that a significant effect of the protein adsorption on the analysis might not appear. However, the result would be contradictory to that observed in the fluorescent imaging for the protein adsorption test. To evaluate the effect of the surface adsorption on the MCE analytical performance precisely, the asymmetry factor ($A_S$) was calculated. The $A_S$ is given by the ratio of $w_1$ and $w_2$, where $w_1$ and $w_2$ are the widths measured at a given fraction at the half-peak height before and after the time of peak maximum ($t_R$), respectively. In an ideal chromatographic and electrophoretic analysis, a peak should be obtained as a Gaussian shape, so that the $A_S$ value results in a unity. Thus, the $A_S$ which reflects the peak shape is informative about the existence of irreversible adsorption or slow retention kinetics of solute to the channel surface. According to Giddings [36], these effects can be distinguished by investigating the dependence of the $A_S$ value on the sample concentration. When the $A_S$ increases with increasing the sample concentration, the peak asymmetry is caused mainly by the irreversible surface adsorption. Fig. 6 shows the dependence of the $A_S$ on the concentration of RBITC-AVI on the bare and PEI-modified PMMA chips. The $A_S$ values obtained on the untreated chip were higher than those on the PEI immobilized microchip at higher concentration region. In addition, the $A_S$ on the bare PMMA increased with increasing the sample concentration, while on the PEI-modified chip it was almost independent of the concentration. Therefore, the irreversible adsorption of RBITC-AVI occurred markedly on the bare PMMA surface, which caused the peak asymmetry, and the adsorption of basic proteins was successfully suppressed by the modification of PEI.

However, the peak of RBITC-AVI on the PEI-modified microchip was apparently broader than that on the untreated PMMA. To compare the analytical performance, the theoretical plate numbers ($N$) of the protein obtained with the bare and PEI-modified PMMA chips were calculated to be 680 and 150, respectively. In the case of the PEI-modified chip, the electrophoretic direction of the cationic protein was opposed to that of the EOF since it was generated from cathode to anode. The $\mu_{ce}$ on the PEI immobilized chip and the electrophoretic mobility ($\mu_{ep}$) of RBITC-AVI was estimated to be $-1.3 \times 10^{-4}$ and

![Fig. 5. Electropherograms of RBITC-AVI on the (a) bare, and (b) PEI-modified PMMA microchips. Modification condition, 20% (w/w) PEI, pH 11.5, 25°C; BGS, 10 mM phosphate buffer (pH 4.0); sample concentration, 1000 ppm; distance of detection point from the injection cross, 5 mm.](#)

![Fig. 6. Dependence of the asymmetry factor on the RBITC-AVI concentration. Closed circles and open squares are the data obtained with bare and PEI-modified PMMA microchannels, respectively. Experimental conditions are as in Fig. 5.](#)
ca. $1 \times 10^{-4} \text{cm}^2/\text{V} \cdot \text{s}$, respectively. Thus, the apparent mobility of the protein on the PEI-modified chip would result in the sum of these values, ca. $3 \times 10^{-5} \text{cm}^2/\text{V} \cdot \text{s}$, whereas on the untreated microchip it was evaluated to be ca. $1 \times 10^{-4} \text{cm}^2/\text{V} \cdot \text{s}$ since the EOF rate was very small. Therefore, the lower apparent mobility of the protein on the PEI-immobilized chip caused a band-broadening and brought the lower $N$. Even though the adsorption of basic proteins can be suppressed in the PEI-immobilized channel, these results indicate that there is a possibility of reducing the separation performance for complex basic sample mixtures due to the relatively lower anodic $\mu_{eo}$ which is close to the $\mu_{eq}$ of the cationic analytes, so that the further analytical performance was evaluated by comparing the separation efficiencies of protein sample mixtures on the bare and PEI-modified PMMA microchips.

Two basic proteins, AVI and RIB, were used as the test samples for the MCE separation. The $pI$ values of the AVI and RIB are 10.0 and 9.5, respectively [35,37]. Fig. 7 shows the electropherograms of the basic proteins at pH 4.0. In the case of the uncoated PMMA microchip, a partial separation of the proteins was obtained. On the other hand, further resolved peaks could be detected only utilizing a separation length of 5 mm when the PEI-modified chip was employed. Consequently, resolution of two proteins was improved from 1.28 to 1.88 by using the PEI-modified PMMA microchip as shown in Table 3. Comparing the PEI-modified chip with the bare PMMA chip, the migration order of the proteins was reversed. In the case of the bare chip, the migration order of the proteins depends mainly on their $\mu_{eq}$ due to the suppressed EOF. The faster migration of AVI was obtained on the bare chip though the molecular weight of AVI is larger than that of RIB. This is caused by the higher charge density and/or the higher $pI$ value of AVI compared to RIB since the $\mu_{eq}$ value is determined by both the charge density and the molecular weight of proteins. Under the anodic EOF condition on the PEI immobilized chip, on the other hand, the direction of electrophoretic migration of the cationic proteins is opposite to that of EOF. Since the EOF rate is higher than the electrophoretic migration rate of the proteins, the migration order was reversed on the PEI-modified chip.

Although it is difficult to compare the separation efficiencies between the bare and PEI-modified chips because of the opposite migration order, the $N$ of RBITC-AVI obtained with the PEI chip was apparently lower than that of the bare PMMA chip as well as the MCE analysis of single component sample as shown in Fig. 5. On the other hand, the $N$ value of RBITC-RIB in the bare PMMA channel was lower than that on the PEI immobilized chip. Thus, the separation efficiency on the PEI-modified chip would be almost comparable with that on the untreated PMMA chip. It should be noted that, however, the migration time of the proteins gradually increased during only four consecutive runs on the bare PMMA chip. As a result, 6–14-fold improvements in the run-to-run repeatability of the migration time were obtained by using the PEI chip. In addition, the asymmetry factors of the protein peaks on the PEI-modified chip were apparently improved in comparison with the bare PMMA chip. Therefore, the Coulombic repulsion between cationic PEI layer and basic proteins in the acidic solution can avoid the irreversible adsorption of the analytes, which
provided a high-performance separation medium for biopolymers.

4. Conclusions

One-step immobilization of cationic PEI with high molecular weight was successfully achieved onto the surface of PMMA substrates using nucleophilic addition–elimination reaction, which reduced irreversible adsorption of proteins. As a result, fast, efficient, and reproducible MCE analyses of cationic proteins were achieved by using the PEI-modified PMMA microchip. We believe that this simple surface modification technique with no organic solvents is also applicable to DNA and protein chips based on the PMMA substrate to avoid the surface adsorption of biogenic analytes. However, the peaks of proteins obtained on the PEI-modified microchip were still broader, so that the immobilization of other polymers containing amino groups onto the surface of PMMA chips by using this modification technique will provide a higher-electrophoretic separation efficiency of proteins, and the work along the line is now progress in this laboratory.

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