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One-step Preparation of Amino-PEG modified Poly(methyl methacrylate) Microchips for Electrophoretic Separation of Biomolecules

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Abstract

A simple method for a chemical surface modification of poly(methyl methacrylate) (PMMA) microchips with amino-poly(ethyleneglycol) (PEG–NH$_2$) by nucleophilic addition–elimination reaction was developed to improve the separation efficiency and analytical reproducibility in a microchip electrophoresis (MCE) analysis of biomolecules such as proteins and enantiomers.   In our procedure, the PEG chains were robustly immobilized only by introducing an aqueous solution of PEG–NH$_2$ into the PMMA microchannel.   The electroosmotic mobilities on the modified chips remained almost constant during 35 days with 37 runs without any recoating.   The PEG–NH$_2$ modified chip provided a fast, reproducible, efficient MCE separation of proteins with a wide variety of isoelectric points within 15 s.   Furthermore, the application of the modified chip to affinity electrophoresis using bovine serum albumin gave a good chiral separation of amino acids.

1. Introduction

The integration of various chemical/analytical operations on a microchip, which is called micro-total analysis systems (μ-TAS), allows the minimal sample consumption, fast sample pretreatment, chemical reaction, analysis, and detection. In μ-TAS, polymeric substrates have attracted much attention due to low cost, easy replication, and disposability. Among several operations in μ-TAS, microchip electrophoresis (MCE) is one of the most important separation techniques, and thus the application of polymer channel chips to MCE has been studied during past 15 years [1–9].

In the MCE analysis of proteins, sample adsorption onto the surface of a separation microchannel should reduce the separation efficiency and the analytical reproducibility [10, 11]. It has been well-known that dynamic coating of several polymers is one of the useful approaches for suppressing the protein adsorption [12–15]. However, desorption of the coated polymers from the surface of the microchannel is sometimes problematic, *e.g.*, in case of the MCE separation combined with mass spectrometric detection. In the covalent bonding methods, on the other hand, stable coating can be obtained but troublesome and time-consuming modification processes are often required [13–22]. Glass/quartz microchips can be chemically modified on the basis of silane chemistry, while for polymeric channel chips chemical modification techniques have not been well established. Thus, a stable modification of polymer chips with a simple procedure is still desired in MCE.

In our previous study, a poly(methyl methacrylate) (PMMA) chip was modified with poly(ethyleneimine) (PEI) by nucleophilic addition–elimination reaction [23–25] for the MCE analysis of cationic proteins [26]. In the nucleophilic addition–elimination, esters in PMMA undergo at their acyl carbon atoms when treated with primary or secondary amines in the presence of electron donators in a basic aqueous solution. The binding of amine to PMMA is most likely an SN2 reaction in which the primary amino groups in coating polymer are involved in a nucleophilic attack on electron-poor carbon on the surface esters of the PMMA, resulting in elimination of a methanol from the ester. In this modification, therefore, an aqueous solution of coating polymer is only introduced into non-activated PMMA microchannel. Although the surface adsorption of cationic proteins could be well suppressed on the PEI modified PMMA chip due to the electrostatic repulsion force, the modified chip could not be applied to the MCE analysis of anionic proteins. In this study, to overcome this limitation, we investigated the possibility of the one-step and covalent immobilization of terminal amino-poly(ethyleneglycol) (PEG–NH$_2$) onto the inner surface of PMMA microchannel. PEG is well-known to be effective for suppressing

the surface adsorption of proteins due to steric repulsive force of the PEG chains [27–29]．　Hence，PEG is often employed for dynamic coating of capillary/microchannel.　In our procedure, the reaction between acylcarbon of PMMA and primary amino group in PEG–NH_2 dissolved in a basic aqueous solution is expected to proceed as shown in Fig. 1.　Effects of the modification of PEG–NH_2 onto the PMMA channel surface on the adsorption and the separation performance of proteins were studied.　The application of the PEG–NH_2 modified PMMA chip to the affinity MCE (AMCE) analysis of tryptophan enantiomers was also investigated.

2.　Experimental

2.1.　Chemicals

Terminal amino-poly(ethyleneglycol) (PEG–NH_2, M_W = 30000) was obtained from NOF corporation (Tokyo, Japan), rhodamine B isothiocyanate (RBITC), fluorescein isothiocyanate (FITC), bovine serum alubumin (BSA) and myoglobin (MYO) were from SIGMA-ALDRICH (Tokyo, Japan), polyethylene glycol (PEG, M_W = 25000), N,N'-dimethylformamide (DMF) and DL-tryptophan (Trp) from Nacalai Tesque (Kyoto, Japan), sulforhodamine B (SRB) from Tokyo Chemical Industry (Tokyo, Japan), and ribonuclease A (RIB) from Wako (Osaka, Japan).　All reagents were of analytical or HPLC grade.　Background solutions (BGSs) used in the MCE analysis of proteins and Trp enantiomers were 10 mM borate buffer (pH 9.0–10.0) and 0.5 mg/mL BSA in 20 mM phosphate buffer (pH 7.2), respectively.　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 12 h with 10 mM borate buffer (pH 9.0–10.0) as a dialysis buffer.　Prior to the MCE separation, RBITC labeled proteins were diluted to be 100 μg/mL with an appropriate amount of the BGS.　Fluorescence labeling of Trp with FITC was also carried out with the same procedure.

2.2.　Microchip

A PMMA microchip was kindly supplied from Hitachi Chemical (Tokyo, Japan). The substrate was $85 \times 50 \times 1$ mm. The microchip has a simple cross-type channel (100 μm width $\times$ 30 μm depth) with a total separation channel length of 38 mm. Immobilization of PEG–NH$_2$ onto the PMMA channel was carried out using the same procedure in the previous report [26]. Briefly, a solution of 10% (w/w) PEG–NH$_2$ in 100 mM borate buffer (pH 12.5) was pumped through the microchannel at a flow rate of 1.7 μL/min for 6 h using a syringe pump (KDS100, kd Scientific, Holliston, MA), followed by rinsing with deionized water for 20 min. The modified PMMA microchip was then dried at 30 °C overnight. The channel was conditioned with the BGS for 10 min prior to the MCE measurement.

2.3. *Apparatus*

MCE analysis of fluorescent samples was performed using a home-made LIF detection scheme as reported previously [26]. A 532-nm laser beam (10 mW diode-pumped solid-state laser, 58GCS411, Melles Griot, Tokyo, Japan) or 488-nm laser beam (20 mW solid-state laser, 85BCD020, Melles Griot) 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, LCPlanFL-20X, Olympus). Fluorescence from analytes collected by the same objective lens was passed through a dichroic filter (U-MWIG2 and U-MNIBA2 (Olympus) for 532- and 488-nm excitation, respectively) and led to a multichannel photodetector (PMA-11, Hamamatsu Photonics, Hamamatsu, Japan). For the fluorescence imaging measurement, an 100-W mercury lamp (HBO103W/2, OSRAM, Augsburg, Germany) and a CCD camera (1K-TU53H, Toshiba, Tokyo, Japan) were used as a light source and a detector, respectively. 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).

2.4. *Procedure*

The channels were filled with the BGS. Prior to separation, the sample solution (7 μL) was introduced into the sample reservoir and a platinum electrode was inserted into each reservoir to provide the electrical contact. To introduce the sample solution into the separation channel, the pinched injection technique [30] was employed. Voltage programs for the injection on bare and PEG–NH$_2$ modified chips are summarized in Table 1. Except for the MCE analysis of proteins on the bare PMMA chip, the sample

introduction was carried out mainly by means of the electrophoretic migration of the anionic analytes with the reversed polarity.   Since the electroosmotic mobility on the bare PMMA chip was larger than the electrophoretic mobility of the sample proteins at pH 10.0, the sample injection was performed with the EOF under the normal polarity condition.   These voltage programs were also employed for the EOF rate measurements.

3.   Results and discussion

3.1.   Characterization of PEG–NH$_2$ Modified Microchip

In the modification of PEG–NH$_2$ onto the inner surface of the PMMA channel, the degree of the modification was evaluated from the electroosmotic mobility (μ_{eo}).   Since the EOF was suppressed in the PEG–NH$_2$ coated channel, neutral EOF marker or current monitoring method [31] could not be employed to determine the EOF rate in MCE.   To evaluate the EOF velocity, hence, the fluorescent SRB was analyzed to calculate the apparent electrophoretic mobility (μ_{app}).   By subtracting the electrophoretic mobility of SRB obtained in the CE experiments (1.3×10^{-4} cm^{2}V^{-1}s in 10 mM phosphate buffer (pH 7.0)), the EOF velocity in the channel was determined. On the bare PMMA chip, an anodic EOF was observed with μ_{eo} of $+1.8 \times 10^{-4}$ cm^{2}V^{-1}s at pH 7.0 [32, 33].   By immobilizing PEG–NH$_2$ on the PMMA channel, μ_{eo} was apparently decreased as shown in Table 2.   To optimize the immobilization condition of PEG–NH$_2$, the reaction time and temperature were varied.   Both μ_{eo} and its relative standard deviation (RSD) were decreased with increasing the modification time from 2 h to 12 h at higher temperature, indicating the increase in the immobilization amount of PEG–NH$_2$.   In this study, the reaction time and temperature were set at 6 h and 70 °C, respectively.   Comparing the immobilization of PEI onto the PMMA channel in our previous study (2 h, 25 °C) [26], the long reaction time and high temperature was required in modifying PEG–NH$_2$.   This is due to small amount of amino groups in PEG–NH$_2$ (only one amino group bounds to PEG with Mw 30000) relative to PEI. The effect of the molecular weight of PEG–NH$_2$ on the modification was also investigated.   As a result, the PMMA chip modified with PEG–NH$_2$ of smaller molecular weight (Mw = 1000, 20000) showed lower ability for suppressing the adsorption of proteins and lower stability of the coating layer than Mw 30000.   Thus, PEG–NH$_2$ with Mw 30000 was employed in the remaining study.   Such high molecular weight PEG would be effective to avoid the adsorption of rather high-molecular weight proteins [27–29].

To investigate the durability of the PEG–NH$_2$ coating onto the PMMA surface, 6~7

replicate measurements were carried out every seven days.   As shown in Fig. 2, the μ_{app} of SRB was almost constant during 37 runs within 35 days.   This indicated that μ_{eo} on the PEG–NH$_2$ coated channel remained almost constant during approximately 1 month.   The day-to-day reproducibility of μ_{eo} was acceptable with the RSD of 9.6% (n = 4 within first 21 days) under the dry storage condition.

To compare the physical coating, the modification polymer was replaced from PEG–NH$_2$ to normal PEG (Mw 25000).   The PMMA chip modified with PEG at the same condition (10% PEG, pH 12.5, 6 h, 70 °C) gave a sharp peak of BSA at first run. After only 5 consecutive runs, however, the peak width became broader and the detection time was increased from 30 s to 85 s, indicating the desorption of PEG from the PMMA surface.   Hence, the longer stability of the PEG–NH$_2$ coated microchip demonstrated that the loss of PEG–NH$_2$ was successfully suppressed by the immobilization via the covalent bonding between amino groups in PEG–NH$_2$ and acyl carbon on the PMMA surface.   Since the coating was carried out with alkaline solutions, furthermore, there is a possibility of slight hydrolysis of PMMA, resulting in a negatively charged surface.   The electrostatic interaction between the positively charged amino groups in PEG–NH$_2$ and the negatively charged PMMA surface should stabilize the PEG–NH$_2$ coating layer.   Therefore, the stable PEG–NH$_2$ coating on the PMMA surface might be obtained by the cooperative immobilization based on the covalent bonding and the electrostatic interaction.

3.2.   *MCE Analysis of Proteins on PEG–NH$_2$ Modified PMMA Chips*

To evaluate the analytical performance of the PEG–NH$_2$ modified chip, the MCE analysis of BSA was carried out.   As shown in Fig. 3, a broad peak was obtained on the bare PMMA microchip, whereas in the PEG–NH$_2$ modified channel a sharper peak was observed at 11.6 s.   The efficiency was increased from 150 to 1450 by immobilizing the PEG–NH$_2$ onto the PMMA channel surface.   On the PEG–NH$_2$ modified chip, furthermore, the RSD of the detection time was better (1.8%) than the bare PMMA channel (9.4%).   Fig. 4 shows the fluorescence images of the bare and PEG–NH$_2$ modified PMMA chips after 10 consecutive runs of fluorescently labeled BSA.   On the bare PMMA chip, a strong fluorescence from the adsorbed BSA was observed from the entire channel surface, while on the PEG–NH$_2$ modified channel the surface adsorption of BSA was apparently reduced.   Thus, the sharp peak observed in Fig. 3b was due to the suppression of the surface adsorption of the analytes by the PEG–NH$_2$ coating.

In the MCE separation of proteins, BSA (pI 4.0), MYO (pI 7.2) and RIB (pI 9.2)

were used as the test mixture.   On the bare PMMA chip, the separation of RIB and MYO was insufficient as shown in Fig. 5a.   On the other hand, a successful separation of three proteins was achieved on the PEG–NH$_2$ modified chip within 15 s only utilizing a separation length of 5 mm (Fig. 5b).   Comparing the PEG–NH$_2$ modified chip with the bare PMMA, the migration order of the proteins was reversed.   This was due to the reversal polarity of the separation voltage as mentioned in the Experimental section.   Since μ_{eo} on the bare chip was larger than the electrophoretic mobilities of the negatively-charged proteins, the analytes migrated toward the cathode.   On the PEG–NH$_2$ modified chip, on the other hand, the EOF was well suppressed and the polarity of the separation voltage was negative, so that the anionic analytes moved toward the anode.   As summarized in Table 3, the efficiencies were improved from 110–190 to 550–3400 by the PEG–NH$_2$ coating.   In addition, the RSDs of the detection time were 4–18% on the bare PMMA chip, whereas on the PEG–NH$_2$ modified chip the RSD values were improved to less than 4%.   These results demonstrated that the PEG–NH$_2$ immobilized on the PMMA surface could avoid the irreversible adsorption of proteins, which provided a high performance analysis medium for proteins with a wide variety of isoelectric points.

3.3.   Application to affinity MCE analysis of enantiomers

In affinity electrophoresis, a BGS containing affinity ligands is employed for the separation of closely-resembled biogenic compounds.   It has been well-known that several proteins such as BSA, avidin, ovomucoid and α$_1$-acid glycoprotein, show chiral recognition ability for racemic amino acids and drug components.   Hence, affinity electrophoresis is one of the most effective separation modes for the chiral analysis in CE [34].   On the other hand, only a few reports on the application of affinity electrophoresis using chiral selective proteins as additives in the BGS to MCE has appeared [35] since the adsorption of affinity ligands onto the channel surface makes the electrokinetic sample injection difficult, resulting in poor reproducibility [36].   To overcome this problem, the PEG–NH$_2$ modified PMMA chips were employed to the affinity MCE (AMCE) analysis of Trp enantiomers by using BSA as the affinity ligand.

On the basis of the results of affinity CE experiments with BSA by Liu *et al.* [37], 0.5 mg/mL BSA in 20 mM phosphate buffer (pH 7.2) was selected as the BGS in the present AMCE analysis.   Even on the bare PMMA chips, Trp enantiomers could be separated by AMCE at the first run as shown in Fig. 6a.   At the second run, however, a single peak was obtained.   Such low repeatabilities on the bare PMMA chips would be caused by poor sample injection.   In each run, the surface condition of the bare PMMA

might be changed by unstable surface adsorption of BSA, resulting in low repeatability of injected sample amount. On the other hand, the chiral separations of Trp were attained within 40 s on the PEG–NH$_2$ modified PMMA chip during 10 consecutive runs. As shown in Fig. 6b, closely-resembled electropherograms were obtained at both the first and fifth runs.   The repeatability of the detection time was better with the RSD of 1.8% ($n = 5$) on the modified chip, while on the bare PMMA chip the RSD was 6.9%. These results showed that, therefore, the PEG–NH$_2$ modification onto the PMMA channel was effective for not only zone electrophoresis separation of proteins but chiral AMCE analysis using proteins as additives to the BGS.

4   Conclusions

To reduce irreversible adsorption of proteins, one-step immobilization of PEG–NH$_2$ onto the surface of the PMMA microchips was developed on the basis of nucleophilic addition-elimination reaction.   By using the modified microchips, efficient and reproducible MCE analyses of proteins and racemic amino acids were attained.   The application of both PEG–NH$_2$ and PEI modified PMMA chips to MCE should be useful for the separation of anionic, cationic and neutral proteins and peptides, which will be valuable to high-throughput proteomic analyses.

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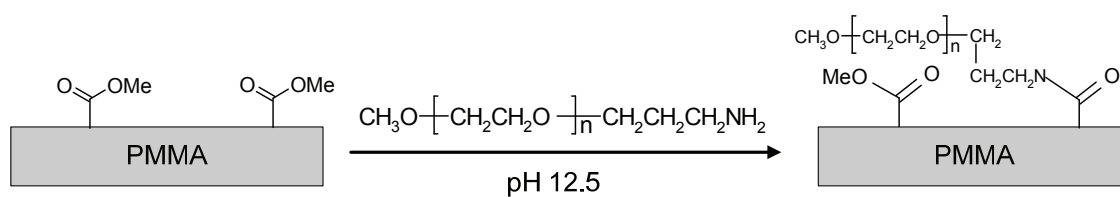
Fig. 1. Covalent immobilization of PEG–NH$_2$ (n = 680, Mw = 30000) onto the PMMA surface.
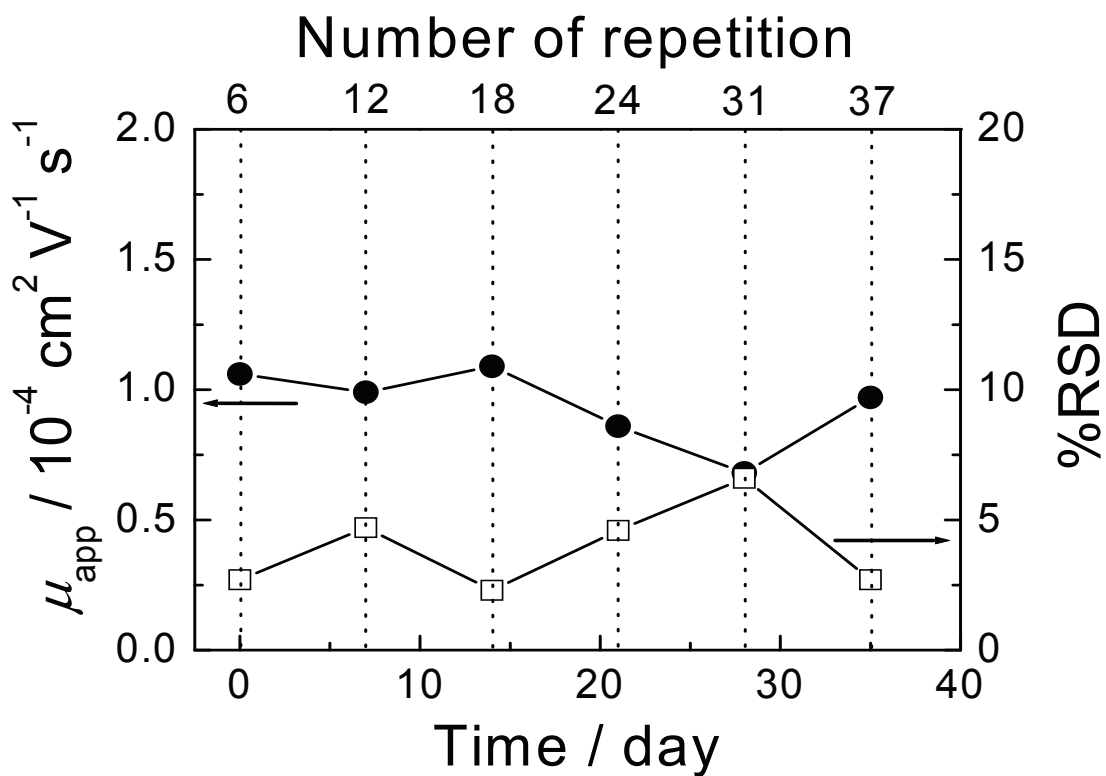
Fig. 2. Long-term stability of the PEG–NH$_2$ modified PMMA microchip. The μ_{app} values were determined from the migration time of SRB in 10 mM phosphate buffer (pH 7.0). %RSD of μ_{app} was calculated from five consecutive runs.
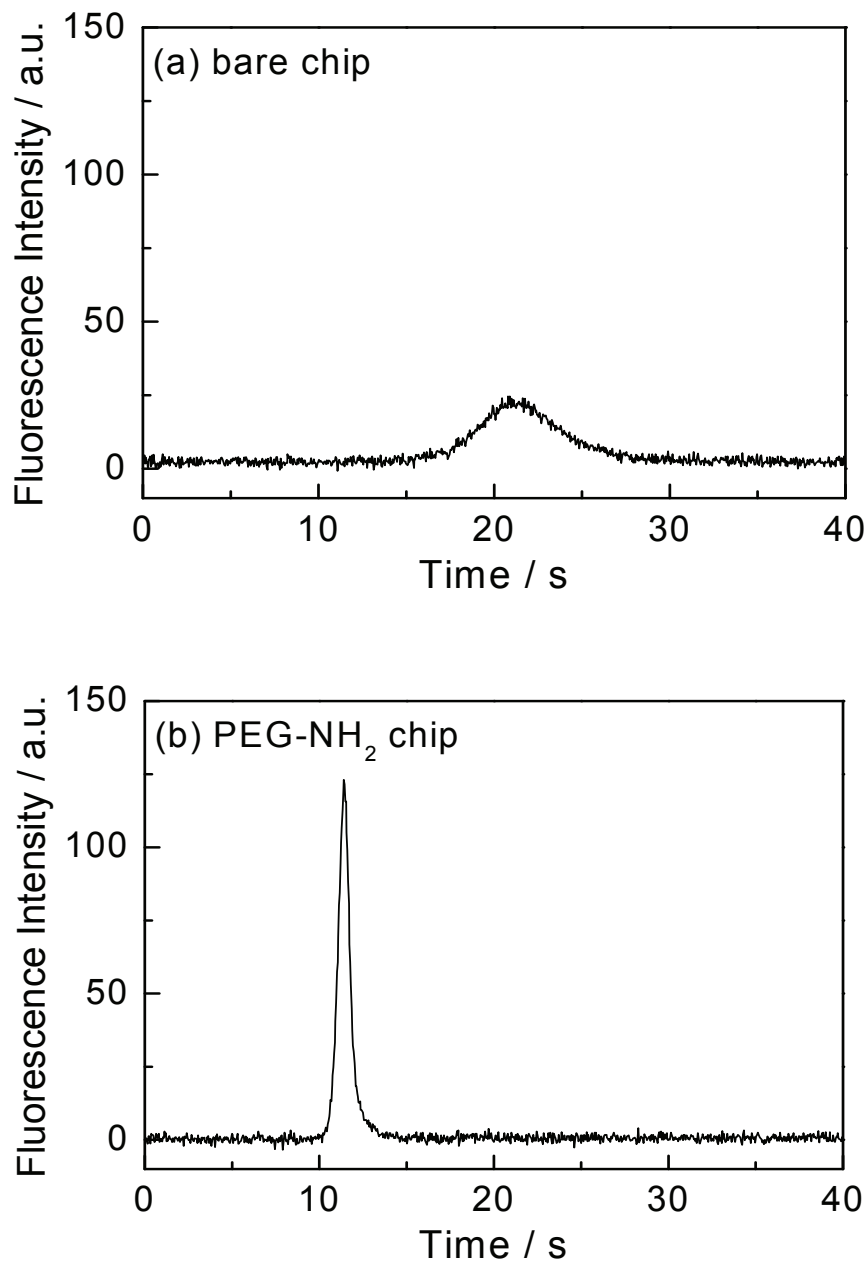
Fig. 3. Electropherograms of 100 ppm BSA on the (a) bare and (b) PEG–NH$_2$ modified PMMA microchips. Modification condition, 10% (w/w) PEG–NH$_2$, pH 12.5, 70 °C, 6 h; BGS, 10 mM borate buffer (pH 9.0); sample concentration, 100 μg/mL; distance of detection point from the injection cross, 5 mm.
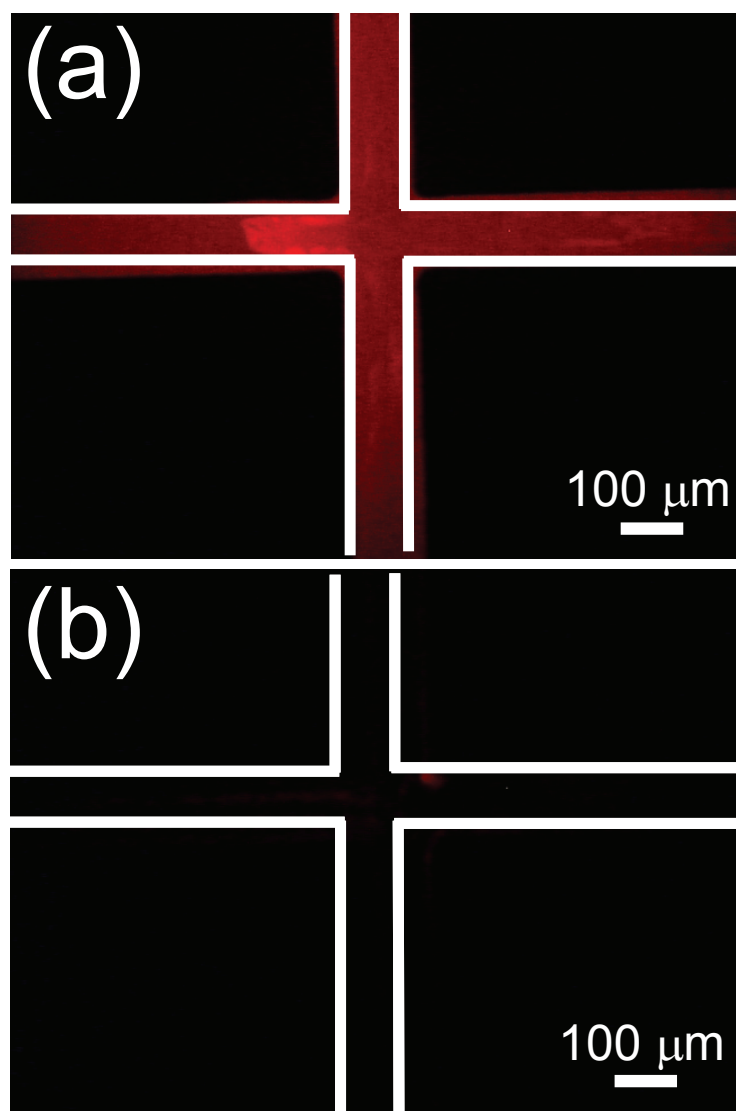
Fig. 4. Fluorescence images of the (a) bare and (b) PEG–NH$_2$ modified PMMA microchips after 10 consecutive runs of fluorescently labeled BSA.
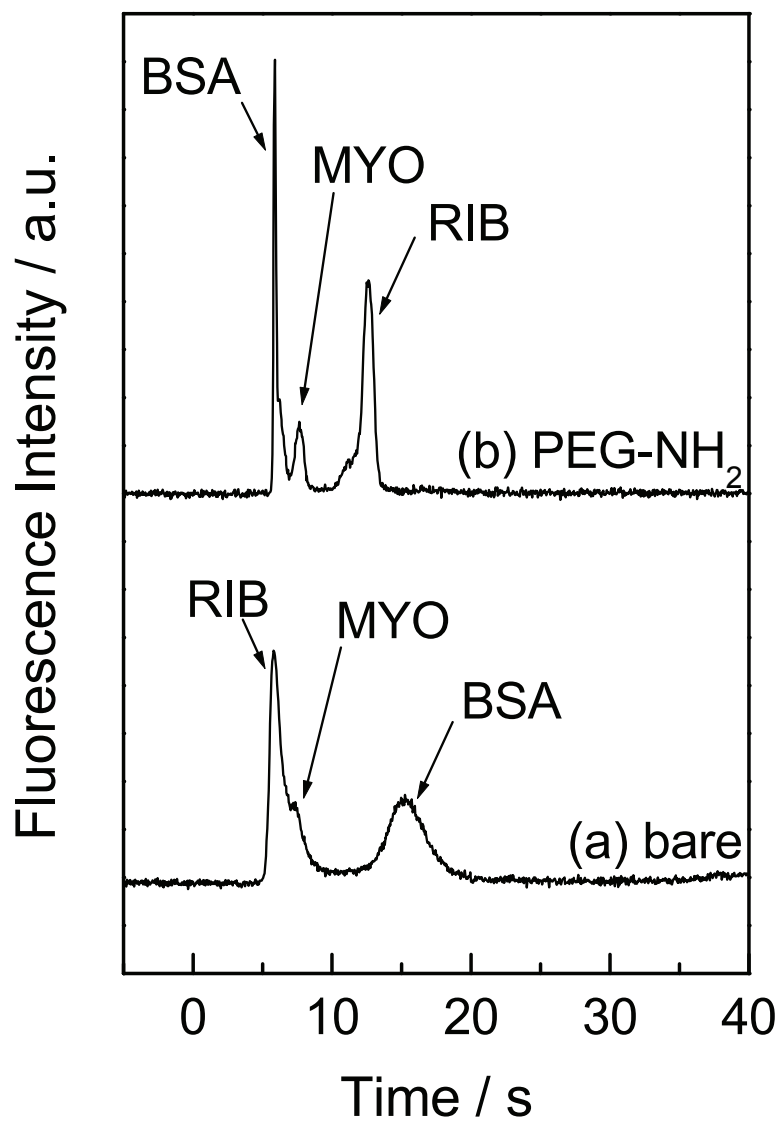
Fig. 5. MCE separation of three proteins on the (a) bare and (b) PEG–NH$_2$ modified PMMA microchips. BGS, 10 mM borate buffer (pH 10.0); sample concentrations, 100 μg/mL each. Other conditions as in Fig. 2.
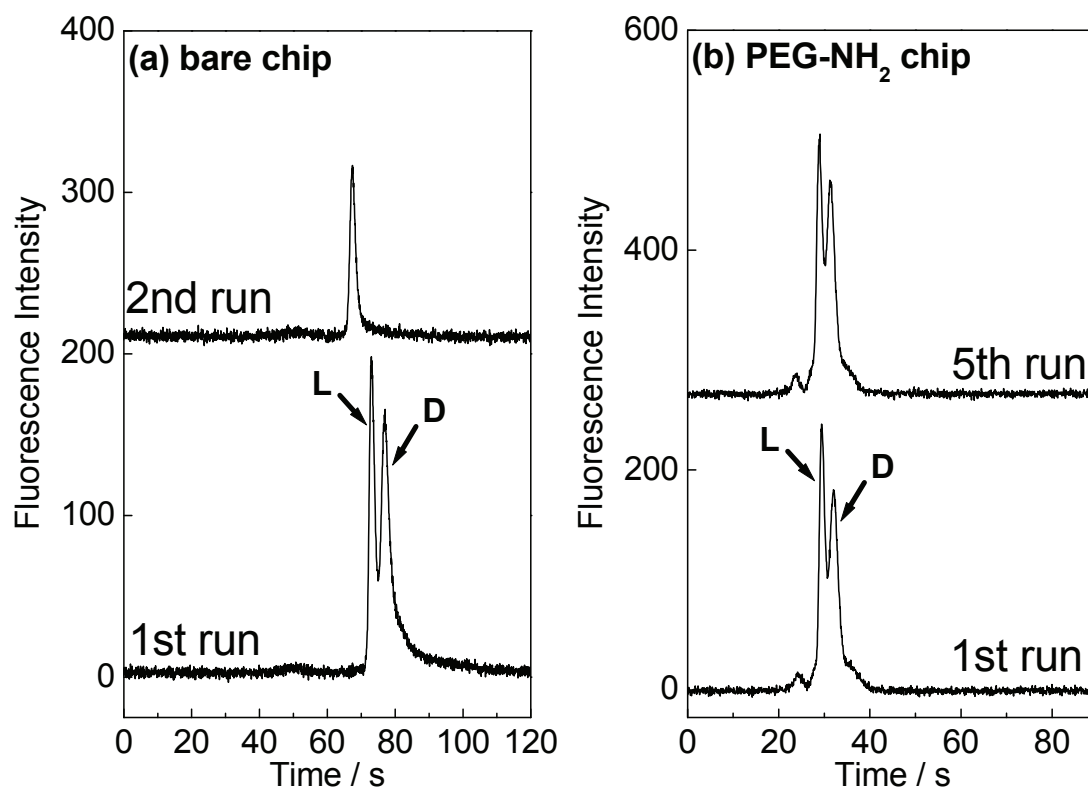
Fig. 6. MCE analysis of FITC labeled-Trp enantiomers on the (a) bare and (b) PEG–NH$_2$ modified PMMA microchips. BGS, 0.5 mg/mL BSA in 20 mM phosphate buffer (pH 7.2); sample concentration, 1 μM; distance of detection point from the injection cross, 10 mm.

Table 1. Voltage programs for the MCE analysis of proteins and enantiomers on the PMMA chips.

microchip	step	time (s)	analytes	applied voltage (V)			
				S	SW	B	BW
PEG–NH$_2$ modified PMMA chip	loading	90	proteins	0	750	0	0
			enantiomers	0	650	0	0
	injection / separation	120	proteins	750	750	0	1800
			enantiomers	1050	1050	0	1800
bare PMMA chip	loading	90	proteins	650	0	550	500
			enantiomers	0	650	0	0
	injection / separation	120	proteins	1050	1050	1800	0
			enantiomers	1050	1050	0	1800

Table 2. Effect of the modification condition of PEG–NH$_2$ on the μ_{eo}.[a]

PEG–NH$_2$ concentration (%)	temperature (°C)	modification time (h)	μ_{eo} (10^{-4} cm^{2}V^{-1}s^{-1})	%RSD of μ_{eo}
0	–	–	~1.3 [b]	–
10	25	2	~1.3 [b]	–
10	70	2	~1.3 [b]	–
10	70	4	0.48	12.4
10	25	6	0.46	4.3
10	70	6	0.13	2.7
10	70	12	0.12	1.5

[a] BGS, 10 mM phosphate buffer (pH 7.0).

[b] SRB could not be detected under the applied fields of both +450 and –450 V/cm, and this result allowed a rough estimation: μ_{app}(SRB) = μ_{eo}.

Table 3. MCE separation of proteins on the PMMA chips.

	PEG–NH$_2$ modified chip				bare PMMA chip			
	t_R / s	%RSD	N	R_S	t_R / s	%RSD	N	R_S
BSA	6.4	3.1	3400		18.3	18.0	110	
				2.01				2.14
MYO	8.3	3.1	550		7.8	6.1	–	
				3.34				–
RIB	14.0	4.0	830		5.8	3.8	190	