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Research Article

Stacking and separation of protein derivatives of naphthalene-2,3-dicarboxaldehyde by CE with light-emitting diode induced fluorescence detection

We describe the stacking and separation of proteins by CE under discontinuous conditions in conjunction with light-emitting diode induced fluorescence (LEDIF) detection using a violet LED at 405 nm. The proteins were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) to form NDA–protein derivatives prior to CE-LEDIF analysis. During the separation, poly(ethylene oxide) (PEO) solution containing CTAB enters from the cathodic inlet to the capillary *via* electroosomotic flow (EOF). The optimum conditions are: the capillary was filled with 50 mM glycine buffer (pH 9.0) containing 1.0 mM CTAB, NDA–protein derivatives were prepared in deionized water containing 1.0 mM CTAB, and 0.6% PEO was prepared in 50 mM glycine (pH 9.0) containing 2.0 mM CTAB. The analysis of four NDA–protein derivatives is fast ($<$3 min), with RSD $<$1.5% in terms of migration time. In order to improve the sensitivity of NDA–protein derivatives, a stacking approach based on increases in viscosity and electric field, as well as sieving was applied. The efficient stacking approach provides LODs (S/N = 3) of 2.41, 0.59, 0.61, and 4.22 nM for trypsin inhibitor, HSA, β-lactoglobulin, and lysozyme, respectively. In addition, we also applied the stacking approach to determination of the concentration of HSA in one urine sample, which was determined to be 0.31 $\pm$ 0.05 μM (n = 3).

Keywords:
CE / Cetyltrimethylammonium bromide / Light-emitting diode induced fluorescence detection / Poly(ethylene oxide) / Protein DOI 10.1002/elps.200700315

1 Introduction

With its high sensitivity and efficiency, CE with LIF (CE-LIF) detection has become an important technique for the analysis of trace amounts of proteins such as HSA in biological samples [1–3]. Analysis of HSA is important since it has been suggested that its contents in urine is associated with diseases such as nephropathy and Bence-Jones proteinuria [4]. More importantly, HSA in urine is an essential biological marker for early diagnosis of renal disease of diabetic patients [5]. The concentrations of HSA in urine samples are about 20 μg/mL (~0.30 μM) from healthy people, while those are possibly up to 200 μg/mL (~3.03 μM) from heavy renal sick patients [5]. Owing to complex sample matrixes and low concentrations of HSA, determination of HSA in urine samples remains a challenge.

One of the main concerns of applying CE to protein separation is analyte adsorption on the capillary wall, which leads to loss in resolving power and efficiency, as well as poor repeatability and short capillary lifetime [6–8]. To overcome these disadvantages, several strategies have been developed, including operation of protein separation at extremely high or low pH values, and use of deactivated capillaries [9–11]. Polymers such as linear polyacrylamide [10] and poly(ethylene oxide) (PEO) [6] are commonly used for protein separation in CE because they provide self-coating and sieving capabilities. In order to increase the separation resolution and speed of proteins, surfactants such as SDS [11] and CTAB [12, 13] are usually added to BGEs (polymer solutions). When surfactants are used, a great consideration must be paid to minimizing their effect on protein conformation and thus fluorescence quenching [14]. By applying a short plug of SDS prior to sample injection, the microheterogeneities of eight proteins with p*I* values ranging from 4.5 to 11.1 were separated and detected by CE-LIF using PEO solution [14]. The approach provides high efficiency and sensitivity for protein analysis.

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Abbreviations: LAC, β-lactoglobulin; **LEDIF**, light-emitting diode induced fluorescence; **LYS**, lysozyme; **NDA**, naphthalene-2,3-dicarboxaldehyde; **PEO**, poly(ethylene oxide); **TI**, trypsin inhibitor

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In previous studies, we demonstrated protein separation by CE-LIF in the presence of EOF using PEO solutions [2, 3, 14, 15]. Although these approaches allow sensitive and efficient separations of proteins, a tedious process is needed for the regeneration of high and repeatable EOF between runs. To obtain repeatability, high pH values and high concentrations of Tris-borate (TB) solutions are required for filling capillaries and for preparing PEO solution. At high TB concentration and high pH values, PEO adsorption is minimized, mainly because of salt screening and shielding of SiOH groups by small compounds such as Tris molecules [16]. Unfortunately, this process is time costing.

Another concern on the analysis of proteins in biological sample by CE-LIF is the sensitivity. Although CE-LIF using an UV laser is sensitive, fluorescence quenching caused by salt and Joule heating is common, which reduces the sensitivity greater than one order of the magnitude. Such a detrimental effect is relatively higher than that for the proteins derivatized with fluorophores like naphthalene-2,3-dicarboxaldehyde (NDA). NDA reacts with primary amines in the presence of cyanide to produce cyano[*f*]benzoisoindole products that fluoresce strongly at 490 nm after excitation at 420 nm [17]. For detection of NDA–protein derivatives, a violet light-emitting diode (LED) that emits at 405 nm is a useful light source in a CE system. The advantages of LEDs over lasers include long lifetimes (>10 000 h), high intensities in a variety of wavelengths (ranging from blue to red), stability, low costs, and small sizes [18–21]. However, LED-induced fluorescence (LEDIF) detection is about ten-fold less sensitive than that of LIF detection for proteins [20] and a more complicated optical configuration is required to focus the divergent LED light onto a small capillary [19].

This study aimed at the development of a sensitive and cost-effective method for the analysis of proteins by CE-LEDIF using PEO solution containing CTAB. Representative proteins in this study are HSA, β-lactoglobulin (LAC), lysozyme (LYS), and trypsin inhibitor (TI). Some physical and chemical properties of the four proteins are listed in Table 1. We carefully investigated the effect of CTAB concentration on minimizing PEO adsorption by conducting EOF measurements. The roles that CTAB and PEO concentrations have on sensitivity, resolution, speed, and repeatability of proteins in CE-LEDIF were also explored. In order to detect HSA in urine samples, we applied a stacking technique based on increases in viscosity and electric field, as well as sieving [2, 3].

2 Materials and methods

2.1 Chemicals and sample handling

HSA, LAC, and sodium cyanide (NaCN) were obtained from Sigma (St Louis, MO, USA). Glycine, LYS, and TI are of analytical grade and were obtained from ICN Biomedicals (Aurora, OH, USA). ACN and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium tetraborate was obtained from Acros Organic (Geel, Belgium). PEO (M_r 8.0×10^6) was obtained from Aldrich (Milwaukee, WI, USA). NDA was obtained from Tokyo Chemical Industry (Tokyo, Japan) and dissolved in methanol. Glycine solution (500 mM) was adjusted with 0.5 M NaOH to pH 9.0. For preparation of PEO solutions (0.03–1.0%), the glycine solution was further diluted with deionized water to 50 mM. During the addition of PEO, a magnetic stirring rod was used to produce a well-homogeneous suspension. After the addition was completed, the suspensions were stirred for at least 12 h. Prior to use for CE separation, the solutions were degassed with a vacuum system in an ultrasonic tank for 10 min [23].

2.2 Apparatus

The CE-LEDIF system (CE/LIF, Model: 2100) was purchased from Pebio Scientific Company (Taipei, Taiwan). A fused-silica capillary with 75 μm id and 365 μm od was purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillary length is 40 cm (30 cm to the detector). A DV-E viscometer (Brookfield Engineering Laboratories, Middleboro, MA, USA) was employed to measure the viscosity of PEO solutions in a constant-temperature bath at 25.0 ± 0.2°C. All measurements were performed in triplicate. A fluorometer (Aminco-Bowman Series 2, ThermoSpectronic, Pitsford, NY, USA) was used to measure the fluorescence intensities of the proteins and NDA–protein derivatives that were prepared in 50 mM glycine buffer (pH 9.0) containing different amounts of CTAB (0–10.0 mM).

2.3 Derivatization procedure

The derivatization of proteins with NDA in the presence of cyanide was conducted in 1.5 mL centrifuge tubes according to a report with slight modification [19]. Aliquots of 1.0 mL reaction mixtures (pH 9.3) consisting of individual or the four proteins (each 10 μM), NaCN (0.1 mM), NDA (0.1 mM), and sodium tetraborate (1.0 mM) were prepared for derivatization. Urine samples were taken from a healthy man. The urine samples were mixed with derivatization solution containing 0.1 mM NDA, 1.0 mM sodium tetraborate, and 0.1 mM NaCN in 1.5 mL centrifuge tubes, with a dilution factor of 10. The mixtures reacted in the dark for 20 min at room temperature prior to CE-LEDIF analysis.

2.4 On-line concentration and separation

Prior to analysis, 40 cm capillaries (30 cm in effective length) were filled with glycine solutions (50 mM, pH 9.0) containing CTAB (0.5–7.0 mM). After each run, the capillaries were flushed with the corresponding glycine solutions for 2 min. Hydrodynamic injection of the mixtures of NDA–protein derivatives (5–200 nM) and CTAB (0–10.0 mM) from the ca-

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Table 1. Chemical and physical properties of four studied proteins

Protein (source)	M_r	p*I*	Conformation	Hydrophobicity[a)]
HSA (human serum)	66 000	4.6	Globular	960
LAC (bovine milk)	35 000	5.31	Globular	1070
LYS (chicken egg white)	14 300	11.0	Globular	890
TI (soybean)	24 500	4.58	Globular	1150

a) Data are taken from ref. [22].

thodic end was applied at 15 cm height (the height difference between anodic and cathodic ends) for 10 s. During separation, PEO solutions that were prepared in glycine solution (50 mM, pH 9.0) containing CTAB (0.5–10.0 mM) were introduced to the capillaries *via* EOF from the cathodic side. The separation was conducted at −15 kV. The shift of the baseline (trough) due to detection of ACN was used to calculate the EOF mobility. The sample volume was estimated according to our previous method [19] In order to improve the sensitivity of the NDA–protein derivatives, a stacking approach based on increases in viscosity and electric field, as well as sieving was applied. NDA–protein derivatives were hydrodynamically injected to the capillary for periods of 60–180 s. The NDA–protein derivatives were then subjected to stacking and separation under the similar CE-LEDIF conditions described above. Stacking enhancement factor was used to evaluate quantitatively the stacking efficiency [19]. In this study, stacking enhancement factors are the ratios of the LOD of the analytes that were injected for 10 s to those for certain injection times. The LODs of the NDA–protein derivatives were estimated at the concentrations that the peak heights generated are three times the noise.

3 Results and discussion

3.1 Generation of a reversed EOF in the presence of PEO

In the presence of EOF, the resolving power of CE using PEO is dependent on the electrophoretic mobility (μ_{ep}) values of analytes and EOF mobility [16]. To prevent the tedious process of generating high and repeatable EOF using 0.5 M NaOH, a reversed EOF was generated by treating the capillary with BGEs containing CTAB (no PEO). A reversed EOF was generated as a result of CTAB absorbed on the capillary wall with the ammonium ions toward the bulk solution [24]. Along with our previous finding of suppression of PEO adsorption on the capillary wall at high pH and high ionic strength [16], we tested the protein separation under alkaline conditions in the presence of EOF using a PEO solution containing CTAB.

In order to generate a high reversed EOF, a capillary is treated with 50 mM glycine solution (pH 9.0) containing 0.5–7.0 mM CTAB for 2 min and then is filled with the same glycine solution (Fig. 1A). A mixture of NDA–protein derivatives and CTAB (0–10.0 mM) is then hydrodynamically injected to the capillary (Fig. 1B). We note that CTAB bound to NDA–protein derivatives to form NDA–protein–CTAB complexes (discuss later). Once PEO enters the capillary *via* EOF, the NDA–protein derivatives that possess positive charges migrate against EOF and entered PEO zone (Fig. 1C). Finally, the NDA–protein derivatives are separated and detected by CE-LEDIF (Fig. 1D).

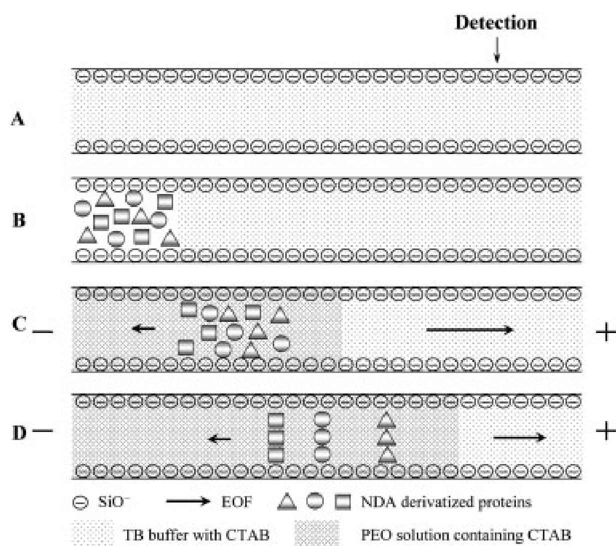

Figure 1. Evolution of protein separation using PEO solution containing CTAB in the presence of reversed EOF. (A) Prior to sample injection; (B) sample injection; (C) introduction of PEO *via* EOF; and (D) separation. Representative conditions are: a capillary is filled with 50 mM glycine solution (pH 9.0) containing 1.0 mM CTAB. 0.6% PEO is prepared in 50 mM glycine (pH 9.0) containing 2.0 mM CTAB. NDA–protein derivatives are diluted in deionized water containing 1.0 mM CTAB.

3.2 Impact of CTAB on protein separation

The magnitude of EOF toward the anode and the electrophoretic mobilities of NDA–protein derivatives toward the cathode are both dependent on CTAB concentration. In addition, adsorptions of protein complexes and PEO are expected to be related to CTAB concentration. With increas-

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ing CTAB concentrations, the EOF mobility become greater, while proteins and PEO adsorption is suppressed to a greater extent. However, denaturation of proteins in the presence of high concentrations of CTAB might occur [25], possibly leading to loss in resolution, sensitivity, and repeatability. Thus, a careful consideration of the CTAB concentration on the analysis of proteins by the present CE-LEDIF approach must be taken. In order to generate high and repeatable EOF, while to minimize the detrimental effects that CTAB and PEO adsorption have on protein separation and sensitivity, we conducted the separation under discontinuous conditions. In other words, the CTAB concentrations in the glycine solutions that were used to fill the capillary, in the sample solutions, and in the PEO solutions are different.

To investigate the effect that CTAB concentration in the glycine solutions (filled in the capillary) has on EOF, we calculated the EOF mobility under different conditions. In this study, ACN was used as EOF marker. Because of a differential refractive index value between ACN and glycine solution, a negative peak for ACN was detected. In this study, the migration time for ACN was used to calculate the EOF value under different conditions. Figure 2 displays that the EOF increased with increasing CTAB concentration over the range of 0.5–7.0 mM. The increases in EOF due to Joule heat are negligible because the separation currents were all less than 15 μA. Table 2 summarizes the impacts of CTAB on the migration times and their repeatability, peak widths, and peak heights of the NDA–protein derivatives. The results suggested that 50 mM glycine solutions (pH 9.0) containing 1.0–2.0 mM CTAB are suitable for filling the capillary in terms of repeatability. At 1.0, 2.0, and 5.0 mM CTAB, the EOF mobilities were $-7.4, -8.1$, and -9.1×10^{-4} cm^2 V^{-1} s^{-1}, respectively, when using 0.6% PEO containing 2.0 mM CTAB. The results also suggest that PEO adsorption on the capillary wall was suppressed in the presence of CTAB.

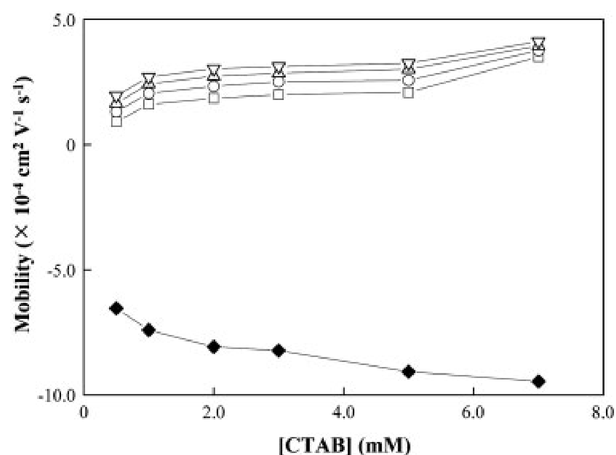

Figure 2. Effect of CTAB in 50 mM glycine solution (pH 9.0) that was filled in the capillary on the EOF mobility and electrophoretic mobilities of the four NDA–protein derivatives. Capillary: 40 cm of total length, 30 cm of effective length. 0.6% PEO solution was prepared in 50 mM glycine solution (pH 9.0) containing 2.0 mM CTAB. The mixture of four proteins (each at 200 nM) was prepared in deionized water containing 1.0 mM CTAB. Sample injections were conducted hydrodynamically by raising the capillary inlet 30 cm height for 10 s and the separations were conducted at −15 kV. Curves: (◆), EOF; (□), NDA-TI; (○), NDA-HSA; (△), NDA-LAC; and (▽), NDA-LYS.

The RSD of the EOF mobility was 1.2% ($n = 3$) at 1.0 mM CTAB when using 0.6% PEO containing 2.0 mM CTAB. At 1.0 mM CTAB, the EOF mobility was -8.3×10^{-4} cm^2 V^{-1} s^{-1} in the absence of PEO, which is higher than that (-7.4×10^{-4} cm^2 V^{-1} s^{-1}) in the presence of 0.6% PEO. Therefore, we further suggest that an increase in viscosity is the main reason for the smaller EOF mobility in the case of using 0.6% PEO (viscosity 105 mPa·s). To obtain repeat-

Table 2. Effect of CTAB concentrations in glycine solution on the migration time, peak width, and peak height of four NDA–protein derivatives[a)]

	Migration time (min)					Peak width (min)[b)]					Peak height (a.u.)				
CTAB (mM)	0.5	1.0	2.0	3.0	5.0	0.5	1.0	2.0	3.0	5.0	0.5	1.0	2.0	3.0	5.0
NDA-TI	2.37 (4.1%)[c)]	2.30 (1.6%)	2.14 (1.5%)	2.14 (2.8%)	1.91 (3.2%)	0.09	0.09	0.10	0.12	0.07	1.4	1.3	1.3	1.1	1.4
NDA-HSA	2.55 (3.7%)	2.49 (1.5%)	2.32 (1.4%)	2.33 (2.5%)	2.05 (3.5%)	0.08	0.06	0.07	0.07	0.05	3.7	4.1	4.2	3.5	4.9
NDA-LAC	2.73 (3.8%)	2.68 (1.4%)	2.49 (1.4%)	2.48 (2.9%)	2.20 (3.3%)	0.06	0.06	0.06	0.05	0.04	3.8	4.0	4.0	4.2	5.3
NDA-LYS	2.91 (4.8%)	2.83 (1.6%)	2.64 (1.5%)	2.61 (3.0%)	2.29 (3.8%)	0.07	0.05	0.06	0.03	0.04	2.3	2.6	2.6	3.0	3.4

a) Capillary was filled with 50 mM glycine solution (pH 9.0) containing different amount of CTAB; 0.6% PEO solution was prepared in 50 mM glycine solution (pH 9.0) containing 2.0 mM CTAB; sample (100 nM) was prepared in deionized water containing 1.0 mM CTAB and was hydrodynamically injected at 30 cm height for 10 s; separation was conducted at −15 kV.
b) Peak width was calculated at half height of the peak.
c) RSD, $n = 3$.

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ability, it is required to flush the capillary with about 20 μL of 50 mM glycine solution containing 1.0 mM CTAB after each run. When compared to our previous studies [14, 15], this process is simple, effective, and time-saving for regenerating repeatable and high EOF. We point out that the EOF directions of the two methods are different; EOF toward cathode in our previous study and toward anode in the present study.

As shown in Fig. 2, the μ_{ep} values of the four NDA–protein derivatives (NDA–protein–CTAB complexes) are positive (toward the cathode). The result shows the formation of the positively charged NDA–protein–CTAB complexes. In the absence of CTAB, HSA, LAC, and TI and their corresponding NDA–protein derivatives possess negative charges at pH 9.0. Further evidence to support the formation of NDA–protein–CTAB complexes is the fact that their positive μ_{ep} values slightly increases with increasing CTAB concentration. Because the 50 mM glycine solution inside the capillary contain greater concentrations of CTAB than that (1.0 mM) in the sample solution, stronger interactions of NDA–protein derivatives with CTAB ions occurred when they migrated inside the capillary. By conducting fluorescence measurements (using a fluorometer), we found that the fluorescence intensities of the NDA–protein derivatives at 460 nm (excitation wavelength at 405 nm) in the presence of CTAB ($<$3.0 mM) was about five-fold higher than those of NDA–protein derivatives. At the CTAB concentration above 0.9 mM [26], CTAB molecules formed micelles. Thus, we suggested that the increases in fluorescence are mainly due to binding of CTAB monomers and micelles to the NDA–protein derivatives [27]. We also observed that the fluorescence intensities of the NDA–protein derivatives are smaller ($\sim$30%) at 7.0 mM CTAB than those at 2.0 mM CTAB, mainly due to conformational changes in the proteins.

The electropherograms depicted in Fig. 3 display the impact of CTAB concentration in sample solutions on the separations of the four NDA–protein derivatives under discontinuous conditions. During the separation, 0.6% PEO solution prepared in 50 mM glycine containing 2.0 mM CTAB entered the capillary that was filled with 50 mM glycine (pH 9.0) containing 1.0 mM CTAB. When the sample solutions did not contain CTAB, the peak corresponding to NDA-TI derivative (relatively hydrophobic to the other three proteins) is very broad, mainly because of its stronger interactions with the capillary wall. On the other hand, at the CTAB concentration greater than 5.0 mM the losses in the intensity, resolution, and repeatability of the four NDA–protein derivatives were found. As suggested above, partial denaturation of the proteins is the main contributor for the detrimental effects at high concentrations of CTAB. The electropherograms depicted in Fig. 3 suggest that the optimum CTAB concentration in the sample solution is 1.0–2.0 mM. The peaks for the NDA–protein derivatives are greater in the presence of 1.0 and 2.0 mM CTAB, mainly because of minimum adsorption and greater fluorescence intensities of the derivatives. It is important to note that the separation order is related to their increased order in p*I* values of the four proteins. The migration time for NDA-LYS is the longest because it has the greatest negative μ_{ep} value against EOF among the four derivatives. We note that LYS has the highest p*I* value among the four proteins.

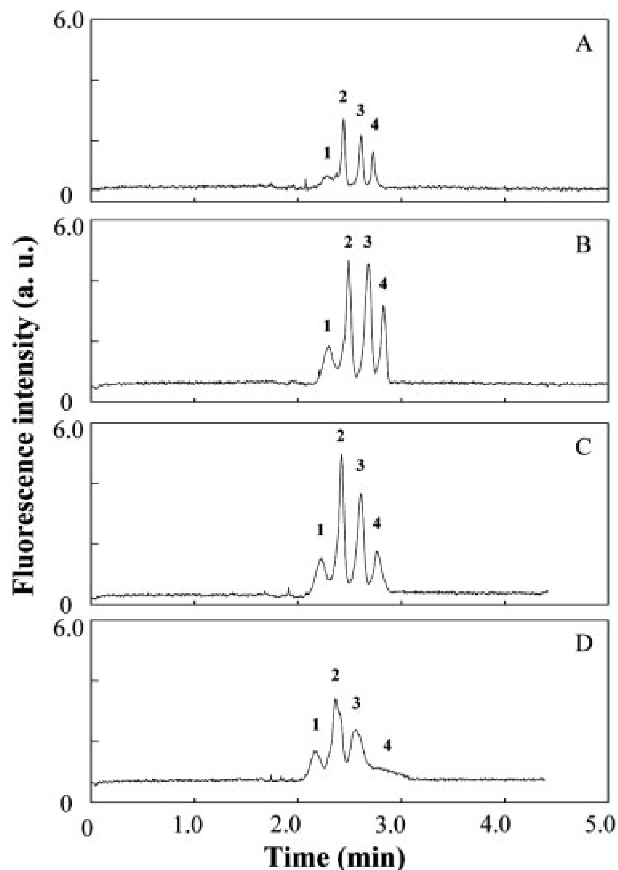

Figure 3. Electropherograms of the mixtures of four NDA–protein derivatives containing (A) 0 mM, (B) 1.0 mM, (C) 2.0 mM, and (D) 5.0 mM CTAB. Capillary was filled with 50 mM glycine solution (pH 9.0) containing 1.0 mM CTAB. Peak identities: 1, NDA-TI; 2, NDA-HSA; 3, NDA-LAC; and 4, NDA-LYS. The fluorescence intensities are plotted in arbitrary units (au). Other conditions are the same as in Fig. 2.

Next we investigated the impact of CTAB concentration (0.75–10.0 mM) in 0.6% PEO solution on the separation of an NDA–protein mixture (Fig. 4). The glycine and sample solutions both contained 1.0 mM CTAB. When CTAB concentration was lower than 0.5 mM, we did not detect NDA–protein derivatives. This is mainly because of PEO adsorption on the capillary wall, leading to small EOF. With increasing CTAB concentration over the range of 0.75–2.0 mM, the EOF mobility increased, mainly because of decreased PEO adsorption and increased adsorption amounts of CTAB bilayers on the capillary wall. Over the CTAB concentration range of 2.0–10.0 mM, the EOF mobility slightly decreased as a result of increases in ionic strength. By calculating the μ_{ep} values for the NDA–protein derivatives, we also found that their values toward the cathode increased

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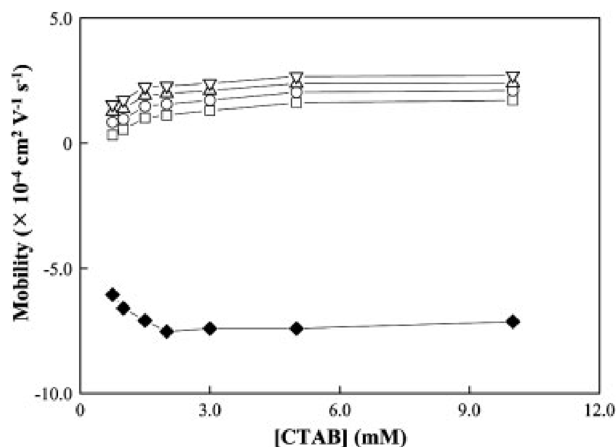

Figure 4. Effect of CTAB amounts in PEO solutions on protein analyses by CE-LEDIF. Capillary was filled with 50 mM glycine solution (pH 9.0) containing 1.0 mM CTAB. Curves: (◆), EOF; (□), NDA-TI; (○), NDA-HSA; (△), NDA-LAC; and (▽), NDA-LYS. Other conditions are the same as in Fig. 2.

with increasing CTAB concentration in PEO solutions, again supporting that stronger interactions of NDA–protein derivatives with CTAB occurred during separation [28]. The change in the morphologies of PEO matrixes due to their interaction with CTAB molecules might be another contributor for varying protein mobilities [29]. In terms of resolution, speed, sensitivity, and repeatability, we suggested that the optimum CTAB concentration was 2.0 mM.

3.3 Impact of PEO solution

To further verify the role that PEO play on the separation of NDA–protein derivatives, different concentrations (0–1.0%) of PEO solutions were tested (Fig. 5). In the absence of PEO, the separation was unsuccessful. With increasing PEO concentration, the resolving power for the proteins increased due to minimum protein adsorption on capillary wall, smaller EOF values, and sieving. For example, when the PEO concentration was increased from 0 to 0.03%, the resolution between NDA-HSA and NDA-LAC derivatives improved from 0 to 1.0. In the presence of PEO solution, the peaks of NDA–protein derivatives were sharper than those in the absence of PEO solution. The entanglement threshold concentration of the PEO solution is 0.07% [30]. When adding CTAB to PEO solution, the viscosity of PEO solution changed due to the interactions between PEO coil and CATB micelles and monomers, thus the entanglement threshold concentration of PEO solution also changed [29]. The viscosity of 0, 0.03, 0.1, 0.3, 0.6, and 1.0% PEO solutions containing 2.0 mM CTAB are 1.2, 1.5, 3.1, 25, 105, and 5100 mPa·s, respectively. With increases in the viscosity of the solution inside the capillary, the EOF mobility became smaller. In addition, interactions between PEO and CTAB are stronger at higher PEO concentrations, leading to decreases in the

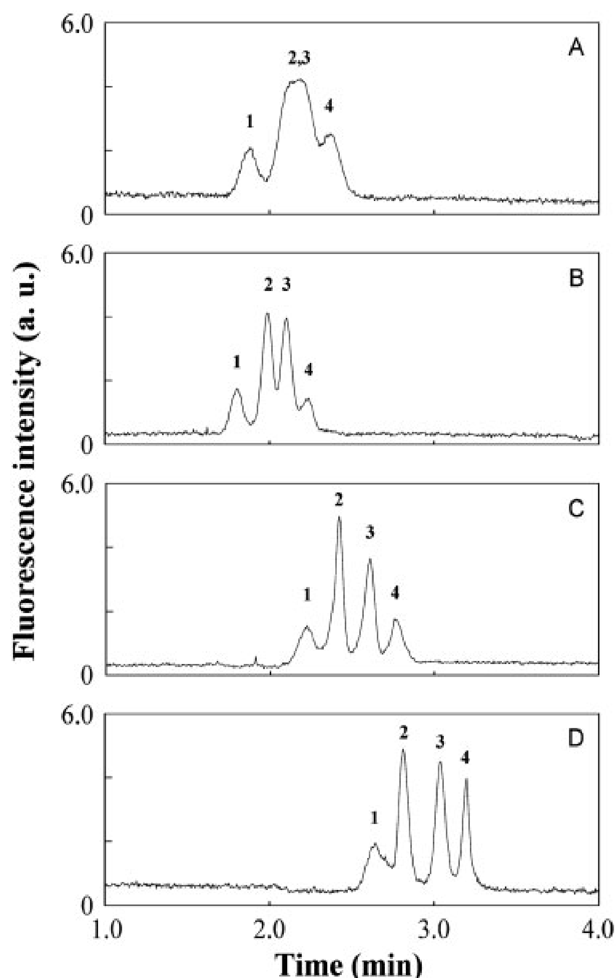

Figure 5. Electropherograms of protein analyses by CE-LEDIF using (A) 0%, (B) 0.03%, (C) 0.6%, and (D) 1.0% PEO solutions. The PEO solutions were prepared in 50 mM glycine solutions (pH 9.0) containing 2.0 mM CTAB. Capillary was filled with 50 mM glycine solution (pH 9.0) containing 1.0 mM CTAB. Peak identities: 1, NDA-TI; 2, NDA-HSA; 3, NDA-LAC; and 4, NDA-LYS. Other conditions are the same as in Fig. 2.

surface charge of the capillary and thus EOF. The EOF mobility values were −9.1, −8.5, −8.3, −7.5, −7.4, and -5.9×10^{-4} cm^2 V^{-1} s^{-1} in 0, 0.03, 0.1, 0.3, 0.6, and 1.0% PEO solutions containing 2.0 mM CTAB, respectively. The separations were quite successful when using PEO solutions at the concentrations greater than 0.3%. Although the peak profiles for NDA-LAC and NDA-LYS derivatives are sharper at 1.0% PEO, we experienced in difficulty of preparing and handling high-viscosity PEO solutions. When using 0.6% PEO, the separation was completed within 3 min, with the RSD values of the migration times for the analytes less than 1.5%. Again, the results clearly suggested that PEO and CTAB are both required for minimum protein adsorption and improving sensitivity, resolving power, and repeatability of this approach.

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3.4 On-line concentration of proteins

Based on the results listed above, we suggested that the optimal separation conditions are: the capillary is filled with 50 mM glycine buffer (pH 9.0) containing 1.0 mM CTAB, NDA–protein derivatives are prepared in deionized water containing 1.0 mM CTAB, and 0.6% PEO is prepared in 50 mM glycine (pH 9.0) containing 2.0 mM CTAB. Under this condition, we separated a standard sample containing four NDA–protein derivatives. The LODs at S/N = 3 for the NDA derivatives of TI, HSA, LAC, and LYS were 26.2, 13.5, 15.1, and 29.0 nM, respectively. The RSD values ($n = 3$) of the migration times for the four proteins were 1.4, 1.2, 1.3, and 1.5%, respectively. The capillary-to-capillary and day-to-day repeatability are both good; the RSD values of the migration times for the four proteins were all less than 1.5%.

The result shows that this method is not sensitive enough to determine HSA in urine samples. In order to further improve the sensitivity, a stacking approach was applied. After hydrodynamic injection of large volumes of NDA–protein derivatives that were prepared in deionized water, they migrated with greater electrophoretic mobility values in the sample zone than those in PEO solution. As a result, the NDA–protein derivatives were stacked in the interface when migrated to the PEO zone as a result of increases in viscosity and electric field, as well as sieving. Figure 6 displays the electropherogram of on-line concentration of 0.3 μL NDA–protein derivatives. Based on the electropherogram, the LODs for NDA derivatives of TI, HSA, LAC, and LYS were 2.41, 0.59, 0.61, and 4.22 nM, respectively. The stacking enhancement factor values were 7–25-folds. The separation efficiencies for the NDA derivatives of TI, HSA, LAC, and LYS were 6.6×10^4, 2.1×10^5, 2.4×10^5, and 1.1×10^5 plates/m, respectively. The linearity ranges for the four NDA–protein derivatives were all in the range from 50 nM to 10 μM ($R^2 > 0.98$). The results show effective stacking of the analytes by applying this simple approach. The quantitative repeatability of this method was good, with the RSD values (peak height) for the four derivatives were less than 1.5%. Run-to-run (same capillary) repeatability is excellent; the RSD of the migration times for the four NDA–protein derivatives ($n = 3$) were less than 1.5%. By testing three different capillaries (each for three runs), we calculated the RSD values for the four NDA–protein derivatives were less than 1.8%, suggesting that the capillary-to-capillary repeatability of the present approach is great. We point out that the capillary can be used for more than 100 runs, with the RSD values less than 1.3%.

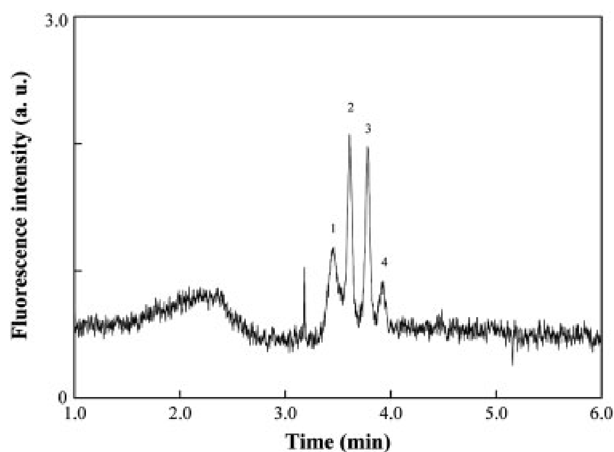

Figure 6. On-line concentration of four proteins by CE-LEDIF. The concentrations of TI, HSA, LAC, and LYS are 10, 5, 5, and 10 nM, respectively. Hydrodynamic injection time is 180 s. Peak identities: 1, NDA-TI; 2, NDA-HSA; 3, NDA-LAC; and 4, NDA-LYS. Other conditions are the same as in Fig. 5C.

3.5 Determination of HSA in urine

The above result suggests that our method is sensitive enough for the determination of HSA in urine. One representative electropherogram for the analysis of 0.1 μL urine sample by our CE-LEDIF is depicted in Fig. 7A. In order to identify the peak for HSA, we analyzed a urine sample spiked with 0.1 μM HSA. The electropherogram depicted in Fig. 7B displays that the intensity for the peak at 2.7 min (first peak) is greater than that in Fig. 7A. The peak height increases upon increasing HSA concentrations over the range of 5.0 nM to 1.0 μM, confirming that the first peak is corresponding to HSA. By applying a standard addition method (peak height) ($R^2 = 0.97$), we determined the con-

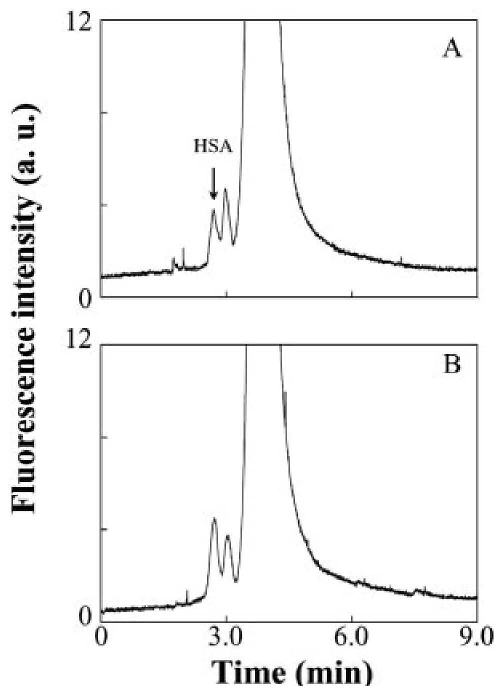

Figure 7. Electropherograms of a diluted urine sample without and with spiking with 0.1 μM HSA (final concentration). Hydrodynamic injection time is 60 s. Other conditions are the same as in Fig. 6.

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centration of HSA in a urine sample, with a result of 0.31 (± 0.05) μM ($n = 3$). The broad peak at the migration time around 4 min are likely due to small molecules such as catecholamines containing amino groups.

4 Concluding remarks

In this study, we have demonstrated that CTAB plays several important roles in determining the protein analysis in the presence of EOF by CE-LEDIF using PEO solution. CTAB is effective for minimizing PEO adsorption on the capillary wall, which allows fast and repeatable regeneration of EOF by treating the capillary with the glycine solution containing 1.0 mM CTAB after each run. We further demonstrated stacking of proteins due to increases in viscosity and electric field, as well as sieving. Under the optimum conditions, the separation of NDA–protein derivatives at the nM level was achieved within 4 min by CE-LEDIF. The successful example of the determination of HSA in urine samples shows great potential of this method for diagnosis of diabetes, nephropathy, and Bence-Jones proteinuria.

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The authors have declared no conflict of interest.

5 References

[1] Tseng, W.-L., Chiu, T.-C., Weng, J.-M., Chang, H.-T., *J. Liq. Chromatogr. Rel. Technol.* 2001, *24*, 2971–2982.

[2] Tseng, W.-L., Chang, H.-T., *Anal. Chem.* 2000, *72*, 4805–4811.

[3] Huang, Y.-F., Hsieh, M.-M., Tseng, W.-L., Chang, H.-T., *J. Proteome Res.* 2006, *5*, 429–436.

[4] Friedberg, M. A., Shihabi, Z. K., *Electrophoresis* 1997, *18*, 1836–1841.

[5] Sakti, S. P., Hauptmann, P., Zimmermann, B., Bühling, F., Ansorge, S., *Sens. Actuators B* 2001, *78*, 257–262.

[6] Hsieh, M.-M., Chiu, T.-C., Tseng, W.-L., Chang, H.-T., *Curr. Anal. Chem.* 2006, *2*, 17–35.

[7] Dolník, V., *Electrophoresis* 2006, *27*, 126–141.

[8] García-Campaña, A. M., Taverna, M., Fabre, H., *Electrophoresis* 2007, *28*, 208–232.

[9] McCormick, R. M., *Anal. Chem.* 1988, *60*, 2322–2328.

[10] Manabe, T., Oota, H., Mukai, J., *Electrophoresis* 1998, *19*, 2308–2316.

[11] Mikšík, I., Deyl, Z., *J. Chromatogr. A* 1999, *852*, 325–336.

[12] Ye, M., Hu, S., Quigley, W. W. C., Dovichi, N. J., *J. Chromatogr. A* 2004, *1022*, 201–206.

[13] Baryla, N. E., Melanson, J. E., McDermott, M. T., Lucy, C. A., *Anal. Chem.* 2001, *73*, 4558–4565.

[14] Tseng, W.-L., Lin, Y.-W., Chang, H.-T., *Anal. Chem.* 2002, *74*, 4828–4834.

[15] Chiu, T.-C., Lin, Y.-W., Huang, C.-C., Chrambach, A., Chang, H.-T., *Electrophoresis* 2003, *24*, 1730–1736.

[16] Tseng, W.-L., Hsieh, M.-M., Wang, S.-J., Chang, H.-T., *J. Chromatogr. A* 2000, *894*, 219–230.

[17] Matuszewski, B. K., Givens, R. S., Srinivasachar, K., Carlson, R. G., Higuchi, T., *Anal. Chem.* 1987, *59*, 1102–1105.

[18] Hillebrand, S., Schoffen, J. R., Mandaji, M., Termignoni, C., Grieneisen, H. P. H., Kist, T. B. L., *Electrophoresis* 2002, *23*, 2445–2448.

[19] Chiu, T.-C., Chang, H.-T., *J. Chromatogr. A* 2007, *1146*, 118–124.

[20] Sluszny, C., He, Y., Yeung, E. S., *Electrophoresis* 2005, *26*, 4197–4203.

[21] Xiao, D., Zhao, S., Yuan, H., Yang, X., *Electrophoresis* 2007, *28*, 233–242.

[22] Fasman, G. D., in: Sober H. A. (Ed.), *Handbook of Biochemistry and Molecular Biology*, CRC Press, Boca Raton, FL 1976, pp. 210.

[23] Chiou, S.-H., Huang, M.-F., Chang, H.-T., *Electrophoresis* 2004, *25*, 2186–2192.

[24] Chang, H.-T., Yeung, E. S., *Anal. Chem.* 1993, *65*, 650–652.

[25] Li, Y., Wang, X., Wang, Y., *J. Phys. Chem. B* 2006, *110*, 8499–8505.

[26] Liu, J.-F., Ducker, W. A., *J. Phys. Chem.* B 1999, *103*, 8558–8567.

[27] Arun, K. T., Ramaiah, D., *J. Phys. Chem. A* 2005, *109*, 5571–5578.

[28] Brackman, J. C., Engberts, J. B. F. N., *Langmuir* 1991, *7*, 2097–2102.

[29] Mya, K. Y., Jamieson, A. M., Sirivat, A., *Langnuir* 2000, *16*, 6131–6135.

[30] Radko, S. P., Chrambach, A., *J. Phys. Chem.* 1996, *100*, 19461–19465.

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