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On-Line Concentration and Separation of Proteins by Capillary Electrophoresis Using Polymer Solutions

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Proteins were separated in 0.6% poly(ethylene oxide) (PEO) solutions using a capillary filled with buffers prior to analysis and were detected by laser-induced native fluorescence using a pulsed Nd:YAG laser. PEO solutions entered the capillary by electroosmotic flow (EOF) during the separation. The composition and concentration of the buffer affected the adsorption of PEO molecules on the capillary surface and, consequently caused changes in the EOF. Short separation times (<7 min) were achieved on a sample solution of five proteins in a 0.6% PEO solution containing 5 μg/mL ethidium bromide using a capillary pre-filled with 100 mM TRIS-borate (TB) buffers (pH 10,0). We also extended this method for on-line concentration and separation of proteins. Proteins dissolved in low-conductivity media stacked in both TB buffers and in PEO solutions. The peak height was proportional to the injection volume up to 2.1 μL using an 80-cm capillary filled with 400 mM TB buffers. Using large injection volumes (2.1 μL), we achieved a limit of detection (S/N = 3) of 31 pM for carbonic anhydrase, which was a 1696-fold sensitivity enhancement compared to a conventional injection method (1 kV for 10 s). In high-conductivity media (urine matrix), stacking occurred at the boundary between the sample zone and PEO solutions. A urine sample without any pretreatment was analyzed, and after stacking, several peaks were detected. Spiking the urine sample with human serum albumin (HSA) affected the fluorescent intensity of some analytes as a result of interaction with HSA.

The need for high-resolution and sensitive protein separation has become increasing important due to the recent advances in molecular biology. One example is the use of capillary electrophoresis (CE) to verify the purity of recombinant proteins to be used as therapeutic agents.[1,2] CE is also of potential value in the study of the proteome.[3] Proteomics is the study of protein properties (posttranslational modification, interaction, etc.) to obtain a global and integrated view of disease processes, cellular processes, and networks at the protein level.[4,5] CE has also been successfully applied to the analysis of proteins in single cells.[6,7]

Protein analysis by CE is complicated by the heterogeneity and stability of proteins and by interactions with the silica surface. The adsorption of proteins causes tailing and broadening of the analyte peak, which leads to loss of resolution and sensitivity. The change of ζ potential of the capillary wall due to the adsorption of proteins also affects the electroosmotic flow (EOF), giving improper migration times and poor reproducibility. To overcome the protein adsorption, a variety of techniques have been developed. Separations have been accomplished at either low or high pH to increase the Coulombic repulsion between proteins and the capillary wall.[8−10] Reducing the protein adsorption has also been achieved by modifying the charge density of proteins with suitable chemicals or surfactants.[11] However, these methods are limited by variations of protein structure and activity and the results may not reflect the behavior of proteins under physiological conditions. Chemically bonded capillaries are stable and commonly used in protein separation, but they are expensive and have a limited lifetime.[12−17] Capillaries dynamically coated with polymers through hydrogen bonding, hydrophobic interaction, Coulombic interaction, and/or van der Waals interaction have also been used for protein analysis.[18−22]

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To separate proteins of different sizes, deactivated capillaries filled with gel matrixes or polymer solutions have been employed.[23−25] Compared to gels, polymer solutions offer the advantages of having a dynamic pore structure, flexibility, low viscosity, and ease of preparation and, thus, are more suitable for protein analysis in CE. Common polymer solutions used in CE are prepared from linear polyacrylamide,[26] dextran,[27] poly(vinyl alcohol),[28] and poly(ethylene oxide) (PEO).[29−33] Filling deactivated capillaries with polymer solutions is generally accomplished by pressure, necessitating the use of large capillaries and polymer solutions at low viscosity. In addition, the separation of proteins at a pH near the p*I* value (in order to achieve better resolution) is limited by low electrophoretic mobility (EPM). Although surfactants such as sodium dodecyl sulfate (SDS) are commonly used to alter the charge/mass ratio of proteins, the protein conformation and activity may be changed. A high background fluorescence from surfactants and decreases in fluorescent intensity of proteins may reduce the sensitivity in CE with laser-induced fluorescence (LIF).[34]

The other problem in protein analysis is poor concentration sensitivity, generally limited to micromolar levels, using absorbance measurement in CE. Techniques, such as isotachophoresis,[35,36] field amplification,[37] and the use of hollow fibers[38] and membranes,[39] have been developed to enhance the sensitivity of protein detection by absorbance measurement in the UV range. However, the problems of concentration efficiency, resolution, and migration time due to the effects of the matrix and protein adsorption must be overcome. For analysis of trace proteins in biological sample such as plasma, techniques of on-line concentration combined with LIF in CE may be useful. To perform CE-LIF, proteins must be labeled with suitable fluorephores or contain tryptophan, tyrosine, and phenylalanine residues.[40−44] Many inexpensive lasers, such as an argon ion laser at 488 nm and a He−Cd laser at 440 nm, have been commonly used to excite labeled proteins. However, labeling is tedious and problems of contaminants and byproducts must be overcome. For further identification, such as mapping and determination of structures by mass spectrometry, it is more convenient and accurate if proteins are detected without labeling using UV lasers. Although it is more sensitive for the protein analysis using continuous UV lasers than pulsed lasers, the high cost of the continuous lasers makes them unpopular in many labs.[43,44]

In an attempt to overcome problems associated with the matrix, protein adsorption, and labeling, we have developed a novel on-line concentration and separation method in CE for protein analysis using a relatively inexpensive Nd:YAG pulsed laser. After injecting samples into capillaries filled with TB buffers, PEO solutions entered the capillaries by EOF.[45−47] The effects of sample matrix composition and buffer concentration on the adsorption of PEO molecules to the capillary surface and, in turn, on migration time, resolution, and concentration were carefully elucidated.

EXPERIMENTAL SECTION

Apparatus. The basic design of the separation system has been previously described.[45] Briefly, a high-voltage (HV) power supply (Gamma High Voltage Research Inc., Ormond Beach, FL) was used to drive electrophoresis. The entire detection system was enclosed in a black box with an HV interlock. The high-voltage end of the separation system was housed in a plexiglass box for safety. A Nd:YAG laser with 266-nm, 3000-HZ output from Continuum (model EPO-5000, Santa Clara, CA) was used for excitation. One UG 5 filter from Melles Griot (Irvine, CA) was used to block 532-nm light from the laser. The emission light was collected with a 10× objective (NA = 0.25). One UG 1 filter from Edmund (Barrington, NJ) was used to block scattered light before the emitted light reached the phototube (Hamamatsu R928). The amplified current was transferred directly through a 10-kΩ resistor to a 24-bit A/D interface at 5 Hz (Borwin, JMBS Developments, Le Fontanil, France) and stored in a PC. Fused-silica capillaries (BIOTAQ.COM INC, Potomac, MD) with 75-μm i.d. and 365-μm o.d. were used for protein separations without any coating process.

Chemicals. All chemicals for preparing buffers and ethidium bromide (EB) were obtained from Sigma (St Louis, MO), except that PEO (MW 8 000 000) was from Aldrich (Milwaukee, WI). Buffers prepared from *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), sodium phosphate dibasic heptahydrate, sodium tetraborate, and *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), respectively, were adjusted by NaOH to pH 10.0. TRIS-borate (TB) buffers prepared from tris(hydroxymethyl)-aminomethane (TRIS) were adjusted with boric acid to pH 10.0 and 9.0, respectively. PEO (0.6 g) was added to 100 mL of these buffers in the presence and absence of EB. Please note that EB is a highly carcinogenic compound and gloves must be worn when it is handled. Carbonic anhydrase (CA), conalbumin, α-lactalbumin, trypsin inhibitor, bovine serum albumin (BSA), human serum albumin (HSA), trypsinogen, and catalase were dissolved in water and diluted to suitable concentrations with buffers prior to analysis.

Polymer Solutions. Increasing amounts of PEO were gradually added to the above buffers, respectively, in a water bath at 85−90 °C. During the addition of PEO, a magnetic stirring rod

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was used at high speed to produce a homogeneous suspension. After adding the PEO, the solution was stirred for a further hour. PEO solutions were degassed with a vacuum system in an ultrasonic tank. Polymer solutions stored in a refrigerator at 4 °C were usable for 4 days.

Treatment of Capillaries. Capillaries were treated with 0.5 M NaOH overnight prior to use. Before runs, capillaries were washed with 0.5 M NaOH at 1.0 kV for 10 min to remove polymer solutions and refresh the capillary wall.

On-Line Concentration and Separation. Protein samples were injected into capillaries filled with buffers at different applied voltages for certain times. The end of the capillary was then immersed in PEO solutions. During the analysis, PEO solutions entered capillaries by EOF.

Urine Analysis. A urine sample was collected from a normal male prior to analysis and was separated without any pretreatment process. Either 10 μL of water or 0.1 mM HSA was added to 990 μL of the urine sample. The sample was injected into a capillary filled with 400 mM TB buffers, pH 10.0, at 5 kV for 30 s and separated in PEO solutions prepared in 800 mM TB buffers at 20 kV.

RESULTS AND DISCUSSION

Adsorption. Protein separation using entangled polymer solutions in the presence of EOF has been challenging because of the adsorption of polymer molecules on the capillary wall, leading to poor reproducibility and even loss in detecting proteins. The adsorption is dependent on the surface area, hydrophobic properties, and charge density of the polymer molecules and the capillary surface. Thus, factors affecting the properties of the capillary surface and polymer molecules, including electrolyte species and concentration, pH, ionic strength, and additives, must be carefully considered in order to obtain a reproducible EOF. We have demonstrated that the reproducibility (RSD of migration times <3%) of CE using PEO solutions was obtained after washing the capillary with 0.1 M NaOH at 1.0 kV for 10 min.[45] Thus, effects of the variation of the capillary surface on EOF prior to analysis was not addressed in this study. PEO molecules are neutral, and the PEO adsorption on the capillary wall has been suggested to occur through hydrogen bonding between hydroxyl groups of the capillary surface and the ether oxygen of PEO molecules and through hydrophobic interactions between the capillary surface and the ethylene groups of PEO molecules.[32,48,49] To evaluate the PEO adsorption, EOF coefficients at different concentrations of PEO solution using a capillary filled with different buffers were calculated. The EOF coefficient was calculated from the time when the baseline shifted, and Figure 1A shows the EOF decreased with increasing PEO concentration. There was a steeper reduction at lower PEO concentrations which kept fairly constant at concentrations above 0.7%. The relationship between PEO concentration and EOF and viscosity and EOF was poorly correlated (entangled threshold of PEO solutions is 0.05%).[33] In addition, the reduction in EOF was much greater than the reduction in EPM of CA when PEO solutions were changed from 0.5 to 1.0% (results not shown). This is much higher than our prediction based on the known effects of viscosity changes and retardation.[50,51], Thus,

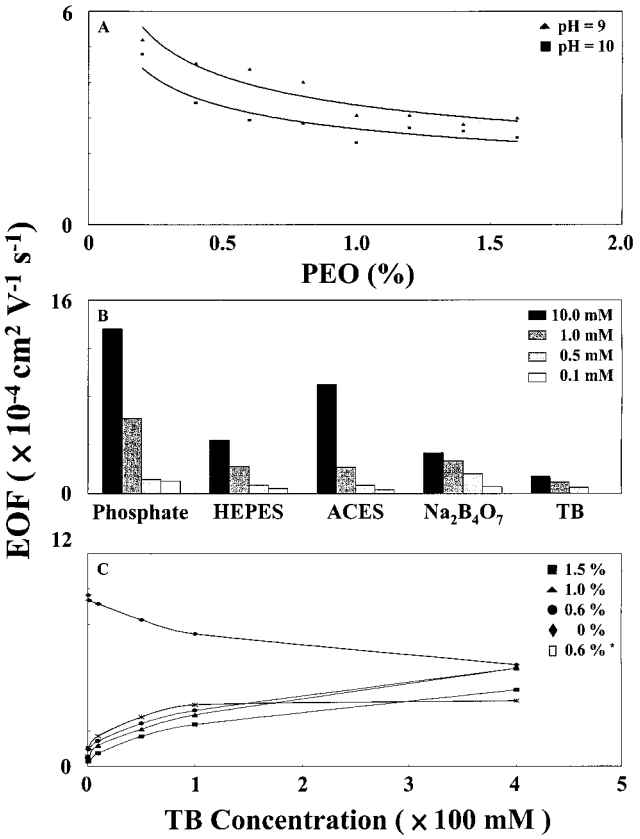

Figure 1. EOF coefficient obtained under different conditions at 15 kV. (A) Effects of PEO concentration using a capillary filled with 100 mM TB, pH 10.0; (B) effects of buffer composition and concentration using a capillary filled with different buffers, pH 10.0; and (C) effects of PEO concentration and concentration of TB buffers, pH 10.0, used to fill the capillary. Capillary: 40 cm in total length and 30 cm in effective length. Buffer vials contained different PEO solutions. PEO solutions contained 5 μg/mL EB.

PEO adsorption also played major roles in reducing the EOF. The fact that EOF coefficients obtained at pH 9.0 were higher than that at pH 10.0 was likely due to less PEO adsorption because of a larger amount of borate.

In an attempt to gain more insight into the effects of buffer composition on the PEO adsorption, we performed separations of four proteins using a capillary filled with phosphate, HEPES, ACES, borate, and TB buffers, respectively, prior to analysis. PEO solutions (prepared in 100 mM TB buffers, pH 10.0) entered the capillary from the injection side by EOF, resulting in at least two different zones in the capillary. Consequently, the bulk EOF coefficient ($\mu_{eof,b}$) was decreased and was related to the local EOF coefficients ($\mu_{eof,l}$) in the different zones.[52,53] For simplicity, eq 1 can be used to express the relationship:

$$\mu_{eof,b} = x_i\mu_{eof,b} + (1 - x_i)\mu_{eof,p} \quad (1)$$

where x_i is the fraction of length filled with buffers and $\mu_{eof,b}$ and $\mu_{eof,p}$ are the local EOF coefficients when buffers or PEO solutions

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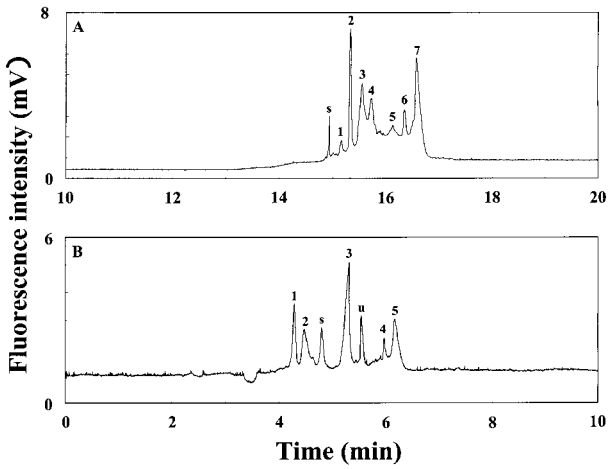

Figure 2. Separation of proteins at 15 kV using a capillary filled with TB buffer, pH 10.0. Sample injection, 1 kV for 10 s. (A) The capillary was filled with a 10 mM TB buffer, pH 10.0, and buffer vials contained 0.6% PEO prepared in 100 mM TB, pH 10.0; protein concentration, 2.5 μM. Peak identities: s, system peak; 1 and 2, CA; 3, conalbumin; 4−6, catalase; and 7, BSA. (B) The capillary was filled with a 100 mM TB buffer containing 5 μg/mL EB, pH 10.0, and buffer vials contained 0.6% PEO, 5 μg/mL EB, and 100 mM TB, pH 10.0; protein concentration, 1 μM, except that the concentration of α-lactalbumin was 10 μM. Peak identities: 1, CA; 2, conalbumin; *, system peak; 3, α-lactalbumin; 4, trypsin inhibitor; and 5, BSA. Other conditions were as in Figure 1.

was used alone. Due to the PEO adsorption and higher viscosity, $\mu_{eof,b}$ is higher than $\mu_{eof,p}$ at the same ionic strength. Figure 1B shows EOF coefficients obtained in the presence of PEO decreased with decreasing ionic strength (<10 mM) of buffers, which is in contrast to the result using buffers only. To minimize any contribution from Joule heating, we compared EOF coefficients obtained at buffer concentrations less than 1 mM (current <6 μA). The EOF coefficients obtained at the same buffer concentration diminished in the following order: phosphate (6.21×10^{-4} cm^2 V^{-1} s^{-1}) > borate (2.67×10^{-4} cm^2 V^{-1} s^{-1}) > HEPES (2.21×10^{-4} cm^2 V^{-1} s^{-1}) > ACES (2.16×10^{-4} cm^2 V^{-1} s^{-1}) > TB (0.51×10^{-4} cm^2 V^{-1} s^{-1}), indicating that the interaction between the capillary wall and PEO molecules was weaker using buffers prepared from chemicals with highly negative charges (higher ionic strength at the same concentration). These values were smaller than that (9.38×10^{-4} cm^2 V^{-1} s^{-1}) in 1 mM TB (in the absence of PEO). The higher the charge density of the capillary surface due to adsorption of charged species, the more hydrophilic the capillary surface was, weakening van der Waals interactions between PEO molecule and the capillary surface. On the other side, the strength of hydrogen bonding between PEO molecules and the capillary surface was probably reduced by the adsorption of the cations on the capillary surface. Thus, our data suggest that the PEO adsorption occurred as a result of hydrogen-bonding and hydrophobic interactions. Note that the interactions between buffer components and the capillary wall also affected the effective surface charge of the capillary, leading to changes in EOF. Figure 1C further shows that in the presence of PEO solutions the EOF coefficients increased with increasing TB concentration, unlike with TB buffers only. These results again suggest that the reduction of EOF is mainly due to PEO adsorption. It is interesting to note that the EOF coefficient was

Table 1. Limits of Detection for Proteins in the Presence and Absence of EB

	LOD (S/N = 3) (×10 nM)[a]	
protein	EB[b]	without EB[c]
CA	1.28 ± 0.02	2.55 ± 0.03
conalbumin	2.09 ± 0.04	4.11 ± 0.05
α-lactalbumin	7.86 ± 0.06	nd[d]
trypsin inhibitor	2.64 ± 0.03	nd[d]
BSA	1.66 ± 0.02	2.45 ± 0.03

[a] Mean for $n = 3$. [b] Conditions as shown in Figure 2A. [c] Conditions as shown in Figure 2B. [d] Not determined.

higher in the presence of EB when the TB concentration was less than 100 mM, presumably resulting from the adsorption of ethidium cations on the capillary surface or the interaction between EB and PEO molecules.[54] The effects of EB and TB concentration on the separation of proteins using 0.6% PEO solutions (100 mM TB, pH 10.0) are further shown in Figure 2A and B and Table 1. In our study, proteins migrated against the EOF. As a result of higher EOF compared to the EPM of proteins, proteins with lower EPM values were detected earlier at the cathode end. The migration order was not correlated with the charge/mass ratio of proteins, indicating that the shape of proteins, solvent-accessible charges of proteins, and possible interactions between PEO molecules and proteins may play important roles in determining the migration order. Several peaks corresponding to CA and catalase were separated, presumably because of proteolytic degradation of the two proteins. Compared to Figure 2A, the separation of five proteins was faster (<7 min) in the presence of EB using the capillary filled with higher concentrations of TB buffers (100 mM). It is interesting to note that the five proteins were not well separated in the absence of EB. These results may be due to the effect of EB on protein conformations and/or protein adsorption on the capillary wall. Since the capillary wall is not completely covered by polymer molecules,[22] the adsorption of EB on the capillary wall may further reduce protein adsorption. Table 1 shows the effect of EB on the sensitivity of protein detection using a relatively low-cost Nd:YAG pulsed laser at 266 nm with 3000 Hz as a light source. Although we have found that the fluorescent intensity of BSA reaches a plateau at the pulse rate ~3500 Hz, a pulse rate at 3000 Hz was chosen to minimize damage to the capillary by laser light while optimal sensitivity was obtained. The LOD values of proteins shown in Table 1 are near nanomolar levels and ~10 times higher than those obtained using a continuous UV laser in buffers. Although EB caused a slightly higher fluorescent background, the LOD obtained in the presence of EB was lower. In addition to the possible effect of bromide anions or ethidium cations on changing protein conformations, the reduced adsorption of proteins on the capillary surface in the presence of EB must be taken into account. The fact that more symmetrical and sharper peaks were obtained in the presence of EB supports our reasoning. The peaks corresponding to 2.5 μM catalase were not detectable in the presence of EB, which indicated that interactions between EB and proteins might affect fluorescence. It should be noted that the system peaks occurred at different times in the absence and

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Table 2. Effect of the Sample Matrix on Resolution and Separation Window of Separating Four Proteins

TB (mM)	resolution (peak pair)[a] 1/2	2/3	3/4	4/5	5/6	6/7	separation window[b](min)
100	2.2	1.2	0.0	1.4	2.4	0.0	3.53−5.50
50	1.6	1.1	0.0	4.3	4.4	0.0	5.01−6.92
10	1.5	2.8	1.4	3.0	2.3	2.4	8.05−9.45
1	3.9	2.2	1.1	3.7	2.6	2.2	15.17−16.57
0.5	3.7	2.4	1.2	0.0	0.0	3.2	19.05−20.63

[a] Separation conditions and peak numbers are as in Figure 2A. [b] Migration times between the first peak and last peak.

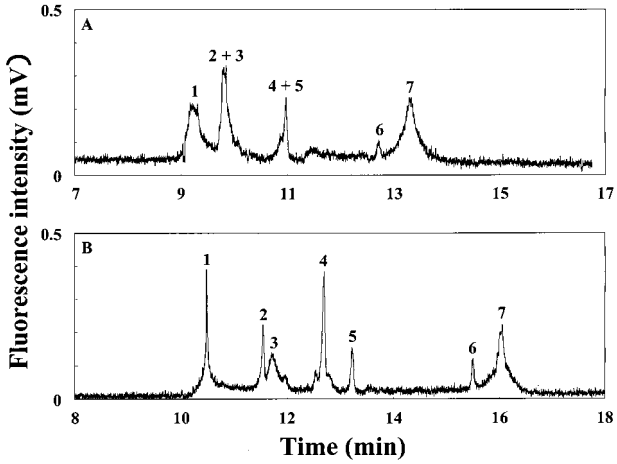

Figure 3. Separation of proteins at 15 kV using a 60-cm capillary filled with 1.5 M TB buffer, pH 10.0. Effective capillary length, 50 cm. Sample injection, 2 kV for 10 s. Buffer vials contained 200 mM TB buffers, pH 9.0 in (A) and 0.6% PEO prepared in 200 mM TB, pH 9.0, in (B). Peak identities: 1, trypsinogen (2.5 μM); 2, CA (0.5 μM); 3, conalbumin (2 μM); 4, β-casein (50 μM); 5, α-lactalbumin (3 μM); 6, trypsin inhibitor (1 μM); and 7, BSA (2 μM). Other conditions were as in Figure 1.

presence of EB. In the absence of EB, the system peak may be due to the change of index of refraction at the interface between the TB buffers and the PEO solution. On the other hand, in the presence of EB, the system peak may be due to more ethidium cations traversing the zone containing more negatively charged species to balance the charge.

In the presence of EOF, resolution depends not only on the difference in migration mobilities between analytes but also on the EOF coefficient. Table 2 shows resolution and separation windows (the difference of the migration times between the first and last peaks) when separating CA, conalbumin, catalase, and BSA in 0.6% PEO solutions (100 mM TB, pH 10.0) using a capillary filled with TB buffers at different concentrations. Resolution increased with decreasing EOF coefficients because the differential migration of proteins became larger. The separation window was wider using higher concentrations of TB buffers, indicating that the EPM of proteins became larger probably due to changes in the conformation and the charge density of proteins. It has been shown, for example, that borate interacts with glycoproteins through their carbohydrate residues, which leads to an increase in the EPM of proteins.[55] In addition, changes in EPM may be due to different interactions between PEO molecules and proteins at different ionic strengths, as suggested by Lee at al.[56] and Iki and Yeung.[32]

To further explore the role of PEO and buffer composition in minimizing protein adsorption, we performed separations of sample proteins, including one basic protein (trypsinogen, pI 9.3), in the absence and presence of PEO using a capillary filled with 1.5 M TB buffers. It is impossible to achieve a symmetric peak corresponding to trypsinogen at a pH near its p*I* using low concentrations of TB buffers (<1 M) because it tends to adsorb on the capillary wall. Figure 3A shows that the separation of the sample proteins was not successful in the absence of PEO, while they can be separated in the presence of 0.6% PEO. In the presence of PEO, the peaks became sharper, presumably because the interaction between proteins and the capillary wall was weaker. It is worth noting that the peak corresponding to trypsinogen was comparatively sharp. This is likely because it carried very few negative charges and stacked at the boundary between PEO and TB buffer.

Stacking. In the absence of EOF using polymer solutions, stacking efficiency can be optimized by using samples in water. However, in terms of speed, it is not recommended in the presence of PEO solutions because of PEO adsorption. Similarly, low concentrations of TB buffers are not suitable for preparing PEO solutions and samples. In the presence of PEO solutions, stacking occurs both in buffers and in PEO solutions when using proteins dissolved in low-conductivity TB buffers (<100 mM). Stacking at the boundary between the sample zone and buffers is due to the decrease in the mobility of proteins migrating from high electric fields to lower ones when a large volume of proteins is injected into a capillary filled with buffers. After PEO enters the capillary by the EOF, proteins with negative charges are subjected to the second stacking when they traverse the boundary between the sample zone and the PEO solution. Stacking occurs probably because of decreasing electric field strengths and retardation of proteins by PEO matrixes.

A model sample containing 50 nM CA, and 200 nM α-lactalbumin and BSA was not detected as shown in Figure 4A when the injection was performed at 1 kV for 10 s using PEO solutions prepared in 100 mM, TB, pH 9.0. Note that the LOD of proteins obtained at pH 10.0 shown in Table 1 is ∼10-fold lower than at pH 9.0. However, the three major peaks shown in Figure 4B were sharp and well resolved when the sample was injected at 10 kV for 300 s (1.4 μL). Table 3 shows good linearity between the peak height and the injection time up to 300 s at 10 kV. The LOD values of three proteins were in the subnanomolar to nanomolar levels when 1.4 μL of sample was injected. Sensitivity improvements for CA, α-lactalbumin, and BSA were 148-, 35-, and 48-fold, respectively, when injecting at 10 kV for 300 s compared to that achieved at 1 kV for 10 s. The stacking efficiency for CA was comparatively better, presumably because of a good retardation by PEO matrixes and/or the sample self-stacking.[57] The separation was slower when a long plug of the sample at a low ionic strength was injected due to the PEO adsorption.

As demonstrated above, the PEO adsorption is dependent on buffer composition and concentration. The choice of sample matrix

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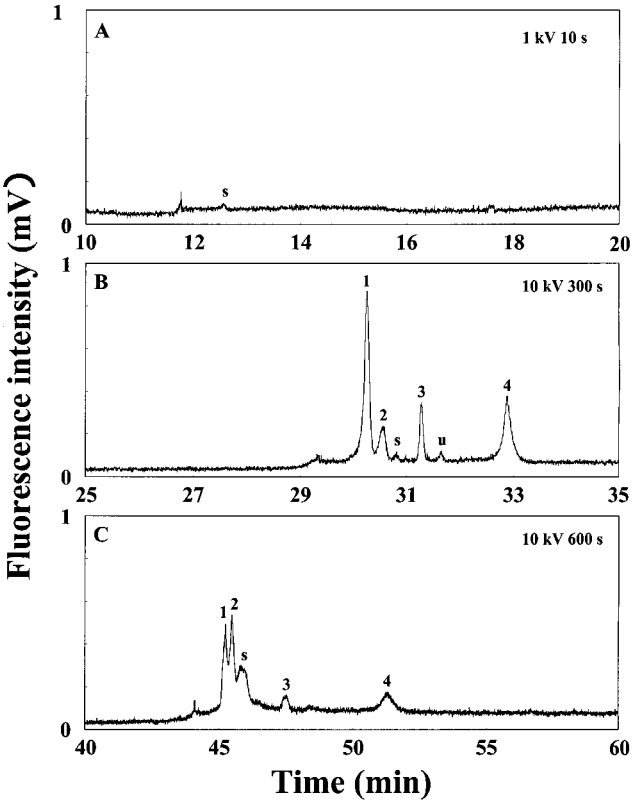

Figure 4. On-line concentration and separation of sample proteins dissolved in TB buffers in 0.6% PEO solutions using capillaries filled with 400 mM TB buffers, pH 10.0. Sample injection: (A) 1 kV for 10 s; (B) 10 kV for 300 s; (C) 10 kV for 600 s. The capillary length is 60 cm in (A) and (B) and 80 cm in (C). Peak identity in (B): 1 and 2, CA (50 nM); 3, α-lactalbumin (200 nM); 4, BSA (100 nM). Peak identity in (C): 1, CA (1 nM); 2, conalbumin (25 nM); 3, α-lactalbumin (50 nM); 4, BSA (25 nM). The symbol s in (B) and (C) represents the system peaks and u represents unknown in (B).

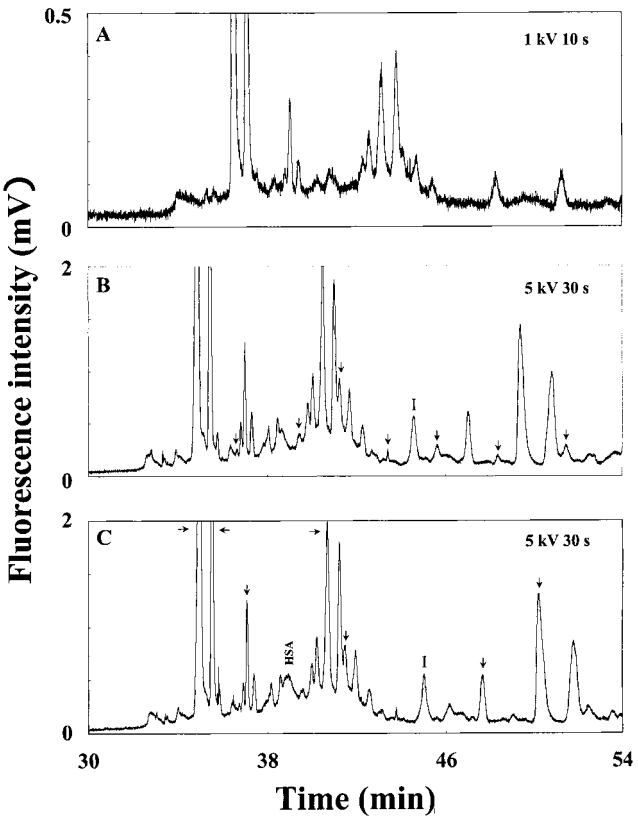

Figure 5. On-line concentration and separation of a urine sample in 0.6% PEO solutions using a capillary filled with 400 mM TB buffers, pH 10.0. Sample injection: (A) 1 kV for 10 s; (B) and (C) 5 kV for 30 s. Urine samples, 990 μL , were spiked with 10 μL of water in (A) and (B), and 10 μM HSA in (C), respectively.

must be carefully considered for optimal separation and concentration. To evaluate the effect of the ionic strength of the model sample on migration time and concentration, proteins dissolved in 25−75 mM TB were injected, respectively, at 10 kV for 600 s using an 80-cm capillary. Table 4 clearly shows that the migration time became longer using proteins prepared in low-conductivity media. The separation of proteins dissolved in 75 mM TB was not complete due to poor stacking efficiency. The optimal matrix concentration of TB buffers in terms of concentration factor and speed was ∼50 mM under these separation conditions. Figure 4C shows the electropherogram from the separation of 2.1 μL of the sample containing four model proteins at concentrations from 1 to 25 nM using an 80-cm capillary. By using this long capillary, the LOD for CA fell to 31 pM and the sensitivity was enhanced 1696-fold. The use of a long capillary allows loading a large sample and better resolution but results in a long separation time and broader peaks due to diffusion.

Proteins in Urine. Figure 5A shows a great number of peaks detected in a urine sample from a normal male injected at 1 kV for 10 s. It should be noted that these peaks most likely correspond to proteins, peptides, cathecholamines and their metabolites, small heterocyclic aromatic compounds, and so on. The first peak of the two big peaks around 36 min corresponds mainly to tryptophan, which is present at less than 40 mg/L in a normal urine sample.[58] Figure 5B shows not only that peak intensity was reasonably enhanced and peaks were well resolved but also that there were many new peaks marked by arrow bars detected when the urine sample was injected at 5 kV for 30 s. Although it is not our goal to identify all these peaks in this study, the result clearly shows the potential of this method for analysis of biological samples. Figure 5C shows the separation of the urine sample spiked with HSA, a good carrier of small molecules including tryptophan in the circulatory system. The density of an unidentified peak marked as I in Figure 5B did not change after spiking with 1 μM HSA and made itself a good candidate for an internal standard. Comparing peak intensities with and without HSA, the intensity of some peaks (marked as arrow bars including the two big peaks in Figure 5C) decreased, indicating that they might interact with HSA. The fact that the intensity of the peak corresponding to HSA was considerably smaller than anticipated supports our suggestion. These results suggest that simply adding suitable reagents to biological samples may provide additional information when analytes are being screened. Note that migration times of analytes moving more slowly than that of HSA became longer in Figure 5C compared to that in Figure 5B, probably due to the effect of HSA on the system such as adsorption of HSA on the capillary wall**.** The reproducibility of this method is still good. The RSD values for the migration times of peak I in both electropherograms were 0.43 and 0.72%, respectively.

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Table 3. On-Line Concentration and Separation of Three Model Proteins in 0.6% PEO Solutions Using a 60-cm Capillary Filled with 400 mM TB Buffer, pH 10.0

	CA	α-lactalbumin	BSA
linear regression[a]	$y = 0.18x - 0.14$	$y = 0.62x - 0.39$	$y = 0.99x - 0.18$
linear regression coefficient	$r^2 = 0.997$	$r^2 = 0.994$	$r^2 = 0.985$
LOD (nM) (S/N = 3)	0.79	8.8	3.7
enhancement	148	35	48
migration time (min) (RSD)[b]	30.25 (0.68%)	31.27 (0.88%)	32.87 (1.12%)
plate number	5.8×10^5	1.0×10^6	3.7×10^5

[a] y is the peak height (mV) and x is the injection time (min) at 10 kV. [b] $n = 3$.

Table 4. Effect of the Sample Matrix on Migration Time, LOD, and Concentration Factor (CF) When Samples Were Injected into an 80-cm Capillary at 10 kV for 600 s

	TB (mM)											
	25			50			75			50[a]		
protein	migration time (min)	LOD (nM)[b]	CF	migration time (min)	LOD (nM)[b]	CF	migration time (min)	LOD (nM)[b]	CF	migration time (min)	LOD (nM)[b]	CF
CA	69.79	0.22	246	45.25	0.031	1696	41.16	nd[c]	nd[c]	48.25	0.22	238
conalbumin	70.20	3.44	75	45.49	0.72	360	41.16	nd[c]	nd[c]	48.82	0.62	426
α-lactalbumin	71.85	5.04	61	47.53	9.09	33	43.26	10.90	28	49.92	8.30	37
BSA	75.45	1.80	100	51.22	3.00	60	46.87	3.53	51	52.77	4.16	43
system peak (min)		68.26			44.08			40.13			47.18	

[a] TB buffers, pH 11.0. [b] S/N = 3. [c] Two peaks corresponding to CA and conalbumin were not resolved.

CONCLUSION

We have demonstrated a new method for on-line concentration and separation of proteins in PEO solutions in the presence of the EOF using a capillary filled with buffers prior to analysis. We showed that the buffer composition and ionic strength play important roles in determining the EOF, which in turn affects resolution and concentration efficiency. The LOD of proteins is in the subnanomolar level, indicating that this method is useful for analysis of biological samples. More importantly, we demonstrated on-line concentration and separation of urine samples without any pretreatment. To enhance sensitivity when a longer plug of biological samples is injected at high salt concentrations, a method for reducing Joule heating is needed. To achieve the goal of faster analysis, use of other polymer solutions to minimize the adsorption of polymer molecules on the capillary wall is currently being studied. To separate more basic proteins, we are also focusing on searching for suitable polymer solutions, which should be stable above pH 11.

Changes in the intensity of several peaks after adding HSA to urine samples may simplify the identification of analytes. It may also be possible for us to develop techniques to screen important peaks by adding selected proteins or chemicals with high affinity for analytes. Since a great number of peaks were well resolved, this method may become popular in clinical chemistry if these peaks are further identified. It is also our future goal to extend this method to proteomics.

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