On-line concentration of proteins in pressurized capillary electrochromatography coupled with electrospray ionization-mass spectrometry

Pressurized capillary electrochromatography (pCEC) and electrospray ionization-mass spectrometry (ESI-MS) have been hyphenated for protein analysis. Taken cytochrome c, lysozyme, and insulin as samples, the limits of detection (LODs) for absolute concentrations are $10^{-11}$ mol (signal-to-noise ratio S/N = 3) with relative standard deviations (RSDs) of retention time and peak area, respectively, of less than 1.7% and 4.8%. In order to improve the detection sensitivity, on-line concentration by field-enhanced sample-stacking effect and chromatographic zone-sharpening effect has been developed, and parameters affecting separation and detection, such as pH and electrolyte concentration in the mobile phase, separation voltage, as well as enrichment voltage and time, have been studied systematically. Under the optimized conditions, the LODs of the three proteins could be decreased up to 100-fold. In addition, the feasibility of such techniques has been further demonstrated by the analysis of modified insulins at a concentration of 20 µg/mL.

Keywords: On-line concentration / Pressurized capillary electrochromatography-mass spectrometry / Protein

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1 Introduction

Capillary electrochromatography (CEC), combining the advantages of high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [1–3], retains their separation mechanisms and achieves high selectivity and efficiency with general applicability and good sample loading capacity. Therefore, it has the potential to become a powerful separation tool for complex mixtures. However, the development of CEC has been restricted by various difficulties, especially the bubble formation in columns. To solve this problem, buffers with low ionic strength are commonly used to decrease the Joule heat. In addition, supplementary pressure could be applied on the column, which is called pressurized or pressure-assisted CEC (pCEC) [4–10]. Although UV detectors are generally used in CEC, mass spectrometry (MS) is a powerful detection method that could provide high sensitivity and reliable detection for various samples, particularly in biochemical applications [11–13]. Moreover, it is able to offer much information about the structure and identity of analytes. To date, pCEC-MS has been successfully applied in the analysis of peptides [5–7, 14, 15], of a crude extract of ergot fungus [16, 17], and of enantiomers [18]. Nevertheless, the relatively low detection sensitivity is still one of the most serious problems, especially for protein analysis. Therefore, the development of on-line concentration techniques is necessary.

Although some methods have been proposed for the enrichment of CEC [19–24], little work has been carried out in pCEC. To the best of our knowledge, an on-line concentration method with combination of the chromatographic zone-sharpening effect and field-enhanced sample-stacking effect has been developed in pCEC-ESI-MS for the first time. With cytochrome c, lysozyme, and insulin as test samples, an obvious improvement of the detection sensitivity has been achieved compared to that with conventional injection. Furthermore, our proposed method has been successfully applied to the analysis of real protein samples with low concentrations.

2 Materials and methods

2.1 Materials

CEC columns packed with 3 µm octadecyl silica (ODS); 100 µm ID, 375 µm OD, total/effective length, 30/20 cm) were purchased from Unimicro Technologies (Pleasanton, CA, USA).
CA, USA). Methanol (HPLC-reagent grade), acetonitrile (HPLC-reagent grade), formic acid (80%, analytical-grade), and ammonium acetate (analytical-grade) were purchased from Yuwang Chemical Plant (Shandong, China). Cytochrome c, lysozyme, and insulin were obtained from Sigma (St. Louis, MO, USA). High-purity water was prepared by a Milli-Q water purification system (Millipore, Milford, MA, USA).

2.2 Hyphenation of pCEC with ESI-MS

A four-way junction was used to connect the inlet end of the pCEC column, pressure reservoir, high-voltage electrode, and a loading capillary connected to a six-port injection valve (Fig. 1). A Jasco PU-1585 HPLC pump (Jasco, Tokyo, Japan) was used to deliver mobile phase and to pressurize the inlet end of a CEC column. The mobile phase consisting of 75 mM formic acid-ammonium acetate/acetonitrile (30:70 v/v), pH 3.4, was provided at a flow rate of 0.2 mL/min. The voltage and pressure applied at the inlet of the CEC column were 18 kV and 7000 kPa, respectively. ESI-MS was performed on a Finnigan LCQ\textsuperscript{DUO} ion trap mass spectrometer (San Jose, CA, USA) with the spray voltage set at 4.0 kV. The temperature of the heated transfer capillary was 200°C. The sheath-liquid flow consisting of 0.5% formic acid in methanol-water (30:50 v/v) was delivered at a flow rate of 3 μL/min by using a syringe pump (Mode 22; Harvard Apparatus, South Natick, MA, USA). The flow rate of the sheath gas was 12 mL/min. The mass range scanning in the MS spectra was set to 300–2000 Da, and the positive ion scan mode was used for all studies in this work. Mass electropherograms and mass spectra were recorded on an LCQ\textsuperscript{DUO} workstation with Xcalibur software, Version 1.2. In our experiment, pCEC and ESI-MS were coupled by a coaxial sheath flow interface, as described previously [17, 25].

2.3 On-line concentration techniques

The on-line concentration procedure in pCEC-ESI-MS was as follows. First, the sample solution was introduced into the CEC column with the propelling force of the mobile phase at the flow rate of 0.05 mL/min, delivered by an HPLC pump under a pressure of 7000 kPa. By calculation, the maximum sample injection volume was 200 nL. Then the pump was stopped and the concentration voltage was applied for a certain time. It should be pointed out that during this period the residue pressure still existed because a sudden drop of pressure to zone might suck the injected sample back. Finally, the pressure was added again with the flow rate at 0.2 mL/min, and the concentration voltage was changed to 18 kV as separation voltage. Between each run, the column was rinsed by the mobile phase for about 5 min.

2.4 Sample preparation

The standard proteins were dissolved in 5 mM formic acid-ammonium acetate buffer (pH 3.4) at the concentration of 2 mg/mL, and diluted with deionized water to 50–500 μg/mL for analysis. Before usage, the sample solution was stored at 4°C. Modified insulin mixtures were gifts from Prof. Huang (Huazhong University of Science and Technology, Wuhan, China). The mixture was dissolved in 5 mM formic acid-ammonium acetate buffer (pH 3.4) at a concentration of 0.5 mg/mL and diluted with deionized water to 20 μg/mL for analysis. Before usage, the sample solution was stored at 4°C.

3 Results and discussion

3.1 Optimization of operation parameters for the separation and detection of standard proteins

3.1.1 Effects of pH value of the electrolyte in the mobile phase on the ESI-MS signal

Since the pH value of the electrolyte in the mobile phase has a great effect on the ESI-MS signal, the optimization of such a parameter is necessary. Although the samples are fully ionized at low pH values, the residue [H\textsuperscript{+}] may...
compete with sample ions in the conversion process from solution to gas phase, leading to weak signal intensity. However, if the pH value is too high, the sample could not be fully ionized, and the [NH\textsubscript{4}\textsuperscript{+}] may compete with sample ions, decreasing the signal as well. Therefore, there should be an optimal pH value for each sample. After systematic study, the maximum ESI-MS signal intensity for lysozyme and insulin was obtained at pH 3.4, and that for cytochrome c was around pH 3.6. In our following experiments, pH 3.4 was chosen as the optimal condition.

### 3.1.2 Effects of the electrolyte concentration in the mobile phase on the ESI-MS signal

The effects of the electrolyte concentration in the mobile phase on the ESI-MS signal have been also investigated, and the results are shown in Fig. 2. For the three proteins, the maximum signal intensity was achieved with 75 mM formic acid-ammonium acetate (pH 3.4). This might be caused by the fact that with the decrease of the electrolyte concentration, the zeta potential of the capillary surface is increased, which might enhance the adsorption of protein to the capillary surface, leading to band-broadening. When the concentration is changed from 25 mM to 75 mM, the zeta potential decreases and the adsorption of protein to the capillary surface becomes weak (this phenomenon has been described in previous works [26, 27]), therefore the sample signal intensity increases. However, with the further increase of electrolyte concentration from 75 mM to 125 mM, high-concentration NH\textsubscript{4}\textsuperscript{+} might compete with the sample ions in the conversion process leading to the decrease of the signal intensity. Therefore, 75 mM formic acid-ammonium acetate was adopted as the final electrolyte concentration in the mobile phase.

### 3.1.3 Effects of separation voltage on the resolution of lysozyme and insulin

In our experiments, the effects of applied voltage on the separation of lysozyme and insulin were discussed. From Fig. 3, it can be seen that when the separation voltage changed from 6 to 18 kV, the resolution of the two proteins increased from 1.01 to 2.40. Considering the operation safety, the voltage was not increased further. Therefore, 18 kV was chosen as the optimum applied voltage in the following experiments.

### 3.2 Optimization of operation parameters for the enrichment of proteins

In pCEC, when the electric field is applied, field-enhanced sample stacking might happen when the concentration of the electrolyte in the sample was lower than that in the mobile phase, similar to that in CE. At the same time, with the coexistence of mobile and stationary phases in a packed column, the solutes could be retained on the stationary phase because of the distribution between the two phases. Once the analytes were eluted by the mobile phase with stronger elution power compared to the sample solution, the sample zone could be further sharpened, leading to improved detection sensitivity, which can be designated as chromatographic zone-sharpening effect. To obtain the best enrichment results in pCEC-ESI-MS, several parameters should be optimized.

#### 3.2.1 Effects of concentration time

In our work, the effects of the concentration time on the signal-to-noise ratio (S/N) of standard proteins were studied systematically. From Fig. 4a it can be seen that first the S/N increased with the concentration time and reached the maximum at 3 min, indicating that the samples zone was continuously sharpened within this period. However, with the further increase of the concentration time, the S/N decreased, which might be caused by the negative effect of the existing pressure even after the pump was stopped, making the sample zone broaden...
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3.2.2 Effects of concentration voltage

The concentration voltage was another factor that affected the detection sensitivity. With the optimized concentration time, the effects of the concentration voltage on S/N and $W_{1/2}$ of lysozyme and insulin were studied. From Figs. 4c and d it can be seen that S/N increased with the increase of the concentration voltage until 18 kV and then decreased. A possible explanation is similar to the effects of the concentration time. Accordingly 18 kV was taken in our experiments.

3.2.3 On-line concentration of a standard protein mixture

For the analysis of large molecules, such as proteins, the detection sensitivity is generally a problem. From Table 1, it can be seen that although the reproducibility of pCEC-ESI-MS is good, the LODs of cytochrome c, lysozyme, and insulin are not ideal, only in the range of $10^{-10}$ to $10^{-11}$ mol. In order to improve the detection sensitivity, an on-line concentration technique with the combination of the chromatographic zone-sharpening effect and field-enhanced sample-stacking effect was applied. Although the reproducibility was slightly lost, the detection sensitivity could be improved by 20- to 100-fold (Table 1), which is demonstrated in Fig. 5.

3.3 Analysis of modified proteins by pCEC-ESI-MS with an on-line concentration technique

The study on modification of proteins is very important in proteomics. For practical application, the protein concentrations are generally low and could hardly be analyzed directly by pCEC-ESI-MS. Therefore, the application of an on-line concentration technique is necessary. With our method, the analysis of a mixture of modified insulins at a concentration of 20 $\mu$g/mL was carried out by pCEC-ESI-MS. 70% acetonitrile in 75 mM formic acid-ammonium acetate at pH 3.4 was selected as the optimum mobile phase; 3 min and 18 kV were chosen as the optimum concentration time and voltage; 0.5% formic acid in methanol-water (30:70 v/v) was used as the sheath-liquid flow. From Fig. 6 it can be seen that good separation and detection of two peaks were achieved despite the low concentration of 20 $\mu$g/mL. By analysis of the full-scan mass spectrogram, the calculated molecular masses of the two peaks were 5777 Da and 5818 Da, respectively, which could be assigned to insulin and its modifier by cholic acid.
Figure 4. Effect of concentration time and concentration voltage on (a, c) S/N and (b, d) peak width at half-height ($W_{1/2}$). Conditions: concentration time, 1–5 min; concentration voltage, 6–20 kV; other conditions as in Fig. 2. (■) Lysozyme; (●) insulin.

Table 1. LODs and reproducibility of three proteins without and with on-line concentration ($n = 3$)

<table>
<thead>
<tr>
<th></th>
<th>Conventional injection</th>
<th>On-line concentration</th>
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<tr>
<td></td>
<td>Cytochrome c</td>
<td>Lysozyme</td>
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<tr>
<td>Reproducibility (RSD%)</td>
<td>Retention time</td>
<td>1.2 &lt; 1.7 &lt; 0.9</td>
</tr>
<tr>
<td>LOD ($S/N = 3$)</td>
<td>Absolute concentration</td>
<td>2.0 × 10^{-11} mol</td>
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Figure 5. Separation of three standards by pCEC-ESI-MS (a) with and (b) without on-line concentration. Conditions: mobile phase, 75 mM formic acid-ammonium acetate/acetonitrile (30:70 v/v), pH 3.4; other conditions as in Fig. 2. Samples: 1, cytochrome c; 2, lysozyme; 3, insulin.

4 Concluding remarks

An on-line concentration technique of pCEC-ESI-MS has been developed with the combination of the field-enhanced sample-stacking effect and chromatographic zone-sharpening effect. Under the optimized separation and concentration conditions, the detection limit of standard proteins could be improved by 20- to 100-fold. Our method has been successfully applied to the analysis of modified proteins at a concentration of 20 μg/mL. All these results demonstrate that our proposed method might play an important role in proteomic study.

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Figure 6. Electrophorogram and full mass spectrum of insulin mixture by pCEC-ESI-MS with on-line concentration. Conditions: separation voltage, 11 kV; sample mixture, 20 μg/mL; other conditions as in Fig. 2.

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5 References

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