

## Integrated Capillary Zone Electrophoresis—Electrospray Ionization Tandem Mass Spectrometry System with an Immobilized Trypsin Microreactor for Online Digestion and Analysis of Picogram Amounts of RAW 264.7 Cell Lysate

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**Supporting Information** 

**ABSTRACT:** A capillary zone electrophoresis (CZE) electrospray ionization (ESI) tandem mass spectrometry (MS/MS) system was integrated with an immobilized trypsin microreactor. The system was evaluated and then applied for online digestion and analysis of picogram loadings of RAW 264.7 cell lysate. Protein samples were dissolved in a buffer containing 50% (v/v) acetonitrile (ACN), and then directly loaded into the capillary for digestion, followed by CZE separation and MS/MS identification. The organic solvent (50% (v/v) ACN) assisted the immobilized trypsin digestion and simplified the protein sample preparation protocol. Neither protein reduction nor alkylation steps were employed, which minimized sample loss and contamination. The integrated CZE–ESI-MS/MS system generated confident identifications (IDs) when 20 fmol was loaded. When only 1 fmol of BSA was injected, one BSA peptide was consistently detected. For the analysis of a standard protein mixture, the integrated system produced efficient protein



digestion and confident identification for proteins with different molecular weights and isoelectric points when a low-femtomole amount was loaded for each protein. We further applied the system for triplicate analysis of a RAW 264.7 cell lysate;  $2 \pm 1$  and  $7 \pm 2$  protein groups were confidently identified from only 300 pg and 3 ng loadings, respectively. The 300 pg sample loading corresponds to the protein content of three RAW 264.7 cells. In addition to high-sensitivity analysis, the integrated CZE–ESI-MS/MS system produces good reproducibility in terms of peptide and protein IDs, peptide migration time, and peptide intensity.

**B** ottom-up proteomics is routinely used for characterization of complex samples. As an extreme example of the depth of sequencing, the identification of more than 10 000 proteins from mammalian cell lysates has been reported.<sup>1</sup> However, most bottom-up sequencing protocols require microgram to milligram amounts of sample, which limits its applications for material-limited biological samples, such as circulating tumor cells,<sup>2</sup> where the sample size is only a few nanograms or less. In order to improve the performance of bottom-up proteomics for analysis of mass-limited biological samples, improvements in both instrumentation and sample preparation are required for highly efficient enzymatic digestion of trace amounts of proteins, high-capacity peptide separation, and sensitive peptide detection.

Several groups have developed efficient enzymatic digestion of trace amounts of proteins from hundreds of cells,<sup>3-6</sup> where the initial protein sample amounts were on the order of 100 ng. These protocols employ free trypsin to digest proteins. It is likely that the system performance is limited by the combination of low protein and trypsin concentrations. Immobilized trypsin can generate better digestion performance for proteins with very low concentration compared with free trypsin,<sup>7,8</sup> due to the high trypsin concentration and reduced autodigestion of immobilized trypsin.<sup>9,10</sup>

We recently reported a protein sample preparation method based on methanol denaturation and immobilized trypsin digestion; we employed that system for analysis of lownanogram amounts of RAW 264.7 cell lysate.<sup>11</sup> In that protocol, proteins were first denatured with 50% (v/v) methanol. The denatured proteins were reduced in a buffer containing 50% (v/v) methanol and then alkylated. Finally, the treated proteins were digested using trypsin immobilized on magnetic microspheres. The whole process was performed at room temperature in 200 µL Eppendorf tubes. After ultraperformance liquid chromatography (UPLC)-electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis,  $2 \pm 1$  and  $23 \pm 2$  proteins could be identified from only 6 and 30 ng of initial cell lysate, respectively. An additional 16 proteins could be detected from the 6 ng of cell lysate using an accurate mass and time tag method. The results suggest that organic solvent and immobilized trypsin based protocol may be useful for ultratrace-level protein sample preparation.

As an example of high-capacity peptide separation and sensitive peptide detection, Shen et al.<sup>12,13</sup> coupled a highly

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efficient (peak capacities of ~10<sup>3</sup>) 15  $\mu$ m i.d. packed LC capillary column to a mass spectrometer for analysis of lownanogram amounts of proteins from complex samples. Ultrahigh sensitivity (<75 zmol for individual proteins) was obtained. Waanders et al.<sup>14</sup> developed a high-sensitivity LC system based on splitting gradient effluents into a capture capillary and providing an inherent technical replicate for nanogram complex sample analysis with high resolution. More than 2400 proteins could be identified from kidney glomeruli isolated by laser capture microdissection in a single analysis.

Capillary electrophoresis (CE)–ESI-MS/MS has attracted attention as an alternative tool for large-scale, high-sensitivity proteomics analysis.<sup>15–22</sup> This group has focused on the use of an electrokinetically pumped sheath-flow electrospray interface. This system has generated low-attomole peptide detection limit with an LTQ-Orbitrap Velos as detector<sup>19,20</sup> and yielded high-zeptomole level peptide detection limit with triple-quadrupole mass spectrometer employing multiple reaction monitoring.<sup>21</sup> For analysis of low-nanogram and high-picogram amounts of digest, CE–MS/MS generated more peptide and protein identifications than LC–MS/MS produced complementary peptide identifications.<sup>17,22</sup> In addition, CE–MS also produced much higher peptide intensities than LC–MS.<sup>16</sup>

Online integration of an immobilized enzyme microreactor with a CE–ESI-MS/MS system should be a facile method for analysis of trace-level protein samples. Several research groups have developed integrated microreactor–CE–MS systems for online protein digestion, peptide separation, and detection.<sup>23–27</sup> However, the sensitivity of those systems was limited by the immobilized enzyme microreactor, the CE–MS interface, and the mass spectrometer. In those reports, only one or two small standard proteins were analyzed. In addition, denaturation, reduction, and alkylation steps were performed offline, followed by online protein digestion, separation, and detection, which reduced the throughput and increased the potential for protein sample loss and contamination.

As we show in this paper, integration of organic solvent denaturation and immobilized trypsin digestion in a nanoliter volume microreactor further improves sample preparation efficiency. In this work, we integrate an immobilized trypsin microreactor with a CZE–ESI-MS/MS system for online digestion and analysis of picogram amounts of a RAW 264.7 cell lysate.

This system has a number of innovations.

First, the sample was prepared in a 50% acetonitrile (ACN) buffer. Organic solvents are used to unfold the protein structure<sup>28,29</sup> and assist in tryptic protein digestion.<sup>30–32</sup>

Second, no protein reduction and alkylation steps were performed. Eliminating these steps reduced potential sample loss and contamination. Eliminating the steps also improved the throughput.

Third, the immobilized microreactor was based on an acrylamide-based monolithic. This material is hydrophilic and tends to reduce nonspecific adsorption of peptides/proteins.

Fourth, the total volume of the prepared trypsin microreactor was reduced to less than 40 nL. This small volume aids in handling small volume protein samples.

Fifth, the 50% (v/v) ACN sample preparation buffer has lower conductivity than the separation buffer of CZE. This lowconductivity buffer results in sample stacking at the beginning of CZE separation, which improved the CZE separation and MS detection. Finally, an electrokinetically pumped sheath-flow electrospray interface<sup>18</sup> was used to couple the CZE to an LTQ-Orbitrap Velos mass spectrometer. The combination results in highly sensitive peptide detection.

This integrated CZE-ESI-MS/MS system confidently identified  $2 \pm 1$  protein groups from 300 pg of RAW 264.7 cell lysate;  $7 \pm 2$  protein groups were confidently identified from 3 ng of the lysate.

## EXPERIMENTAL SECTION

**Materials and Chemicals.** All chemicals were from Sigma-Aldrich (St. Louis, MO, U.S.A.), unless specified. Poly(ethylene glycol) (PEG, MW 10 000) was ordered from Polysciences, Inc. (Warrington, PA, U.S.A.). Dimethyl sulfoxide (DMSO) was ordered from Electron Microscopy Sciences (Hatfield, PA, U.S.A.). Acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Methanol was purchased from Honeywell Burdick & Jackson (Wicklow, IE). Water was deionized by a Nano Pure system from Thermo scientific (Marietta, OH, U.S.A.). Fused capillaries (50  $\mu$ m i.d. × 150  $\mu$ m o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, U.S.A.).

Dulbecco's modified Eagle's medium (DMEM) with Lglutamine and fetal bovine serum (FBS) was purchased from ATCC (Manassas, VA, U.S.A.). Mammalian cell-PE LB buffer for cell lysis was purchased from G-Biosciences (St. Louis, MO, U.S.A.). Complete, miniprotease inhibitor cocktail (provided in EASYpacks) was purchased from Roche (Indianapolis, IN, U.S.A.).

**Preparation of the Immobilized Trypsin Microreactor.** A diagram outlining the preparation of the immobilized trypsin microreactor is shown in Figure 1A. A fused capillary (50  $\mu$ m i.d. × 150  $\mu$ m o.d., 50 cm) was successively washed with methanol, sodium hydroxide, water, hydrochloric acid, water, and methanol. The capillary was then dried with nitrogen at



**Figure 1.** Procedure for preparation of the immobilized trypsin microreactor (A) and schematic diagram of the integrated CZE–ESI-MS/MS system (B).

room temperature. Subsequently, an ~10 cm portion of the capillary was filled with 50% (v/v) 3-(trimethoxysilyl)-propylmethacrylate in methanol and reacted at room temperature for 24 h. After that, the capillary was washed with methanol to remove the unreacted components and dried again with nitrogen. The treated capillary was stored at room temperature for use.

The details of the polymerization mixture and process were similar to ref 33 with some modifications. A mixture of 20 mg of acrylamide, 30 mg of N,N'-methylenebisacrylamide, and 30 mg of PEG was dissolved in 1 mL of 0.2 M sodium bicarbonate/0.5 M sodium chloride (pH  $\sim 8.0$ ) buffer. The mixture was vortexed for 30 s and then heated at  $\sim$ 50 °C for 15 min to completely dissolve the monomers. Then, 2  $\mu$ L of 20% (v/v) TEMED (N,N,N',N'-tetramethylethylene diamine) was added into 0.5 mL of the prepared mixture, and the mixture was degassed for 15 min using nitrogen. Subsequently, 7  $\mu$ L of N-acryloxysuccinimide (NAS) (140 mg/mL, dissolved in DMSO) was added on top of the solution and degassed for 1 min. After that, 2  $\mu$ L of 20% (w/v) ammonium persulfate (APS) was added into the middle part of the solution to initiate polymerization. After vortexing for several seconds, 190  $\mu$ L of the solution was mixed quickly with 10  $\mu$ L of freshly prepared trypsin (20 mg/mL in a buffer containing 0.5 M benzamidine, pH 8.0). Finally, the vinylized part of the treated capillary ( $\sim 10$ cm) was filled with the mixture through capillary action and reacted at room temperature for 30 min.

After the reaction, ~7 cm of microreactor was discarded. The remaining portion of the capillary including a few centimeters of the microreactor was successively washed with deionized water, glycine (10 mg/mL in phosphate buffer, pH 8.0), and 5 mM NH<sub>4</sub>HCO<sub>3</sub> (pH ~ 8.0) for 30 min. The capillary was stored at 4 °C before use.

**Sample Preparation.** Insulin chain b oxidized was dissolved in 5 mM NH<sub>4</sub>HCO<sub>3</sub> for analysis with the integrated CZE–ESI-MS/MS system. Several standard protein standards were dissolved in 50% (v/v) ACN and 5 mM NH<sub>4</sub>HCO<sub>3</sub> for analysis with the integrated CZE–ESI-MS/MS system. The first sample was bovine serum albumin (BSA). The second was a three-protein mixture of BSA, myoglobin (myo), and cytochrome *c* (cyt *c*). The third was a seven-protein mixture (BSA, myo, cyt *c*,  $\alpha$ -casein,  $\beta$ -casein,  $\beta$ -lactoglobulin, and insulin chain b oxidized).

RAW 264.7 cells were cultured in a T25 flask at 37  $^\circ \text{C}$  and 5% CO<sub>2</sub> in DMEM with L-glutamine and 10% FBS. The cells were lysed with 1 mL of mammalian cell-PE LB buffer (pH 7.5) supplemented with complete protease inhibitor for 30 min on ice. The cell lysate was centrifuged at 18 000g for 15 min, and the supernatant was collected for measurement of protein concentration with the BCA method. After that, a 300  $\mu$ L aliquot of the cell lysate was precipitated with 1.2 mL of cold acetone at -20 °C for 24 h, followed by centrifugation at 18 000g for 15 min. The protein pellet was washed with cold acetone again and dried at room temperature. A 60  $\mu$ g aliquot of the proteins was dissolved in 30  $\mu$ L of 0.5% (w/v) sodium deoxycholate (SDC) and 10 mM NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 8.0). After centrifugation, a 15  $\mu$ L aliquot of the protein solution was mixed with 15  $\mu$ L of ACN, which resulted in an ~1 mg/mL protein solution dissolved in 50% ACN, 0.25% SDC, and 5 mM  $NH_4HCO_3$  buffer (pH 8.0). In addition, 5  $\mu$ L of the 1 mg/mL protein solution was further diluted to 0.1 mg/mL with 50% ACN and 5 mM NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 8.0). The 1 mg/mL

and 0.1 mg/mL protein samples were analyzed by the integrated CZE-ESI-MS/MS system in triplicate.

Online Protein Digestion, Peptide Separation, and Identification with the Integrated CZE–ESI-MS/MS System. The integrated CZE–ESI-MS/MS system included a separation capillary (50  $\mu$ m i.d. × 150  $\mu$ m o.d.) with an immobilized trypsin microreactor at the injection end, an electrokinetically driven sheath-flow electrospray interface,<sup>18</sup> two Spellman CZE 1000R power supplies, and an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific), Figure 1B.

The protocol had three steps. Protein samples were injected into the microreactor end of the separation capillary by air pressure. Proteins were then digested by the microreactor at room temperature. Finally, the digests were separated with CZE and analyzed by the LTQ-Orbitrap Velos.

For oxidized insulin chain b analysis, a 34 cm long separation capillary with a 1.5 cm long microreactor was used, and the digestion time was 1 min. For analysis of BSA and the standard protein mixtures, a 34 cm long separation capillary with a 2 cm microreactor was used, and the digestion time was 2 min. For analysis of the RAW 264.7 cell lysate, a 34 cm separation capillary with 2 cm microreactor and 3 min digestion time was used. In all cases, the separation buffer was 5 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0), and the sheath-flow buffer was 50% (v/v) methanol and 0.05% (v/v) FA. For standard proteins analysis, 19.5 kV was applied at the injection end of the capillary for separation and 1.5 kV was applied for electrospray. For RAW 264.7 cell lysate analysis, 15 kV was applied at the injection end of the capillary for separation, and 1 kV was applied for electrospray. The size of the tip for electrospray was ~8  $\mu$ m.

For LTQ-Orbitrap Velos analysis, the ion transfer tube temperature was held at 300 °C. The mass spectrometer was programmed in data-dependent mode. Full MS scans were acquired in the Orbitrap mass analyzer over m/z 395–1900 range with resolution 60 000 (m/z 400). Ten most intense peaks (for standard protein analysis) and 20 most intense peaks (for cell lysate analysis) with charge state  $\geq 2$  were selected for sequencing and fragmented in the ion trap with a normalized collision energy of 35%, activation q = 0.25, activation time of 20 ms, and one microscan. For standard protein analysis, peaks selected for fragmentation more than twice within 60 s were excluded from selection for 60 s. For the standard protein mixtures and cell lysate analysis, peaks selected for fragmentation more than once within 30 s were excluded from selection for 30 s.

**Data Analysis.** Database searching of raw files was performed in Proteome Discoverer 1.3 with MASCOT 2.2.4. For standard proteins from bovine, the insulin chain b sequence was added into ipi.bovin.v3.68.fasta to form the database. For myoglobin, equine.fasta was used for database searching. For RAW 264.7 cell lysate, Swiss-Prot database with taxonomy as Mus. (16 252 sequences) was used. The database searching parameters included up to four missed cleavages allowed for full tryptic digestion, precursor mass tolerance 20 ppm, and fragment mass tolerance 1.0 Da. For analysis of standard proteins from bovine, oxidation of cysteine (+47.9847 Da) was set as dynamic modification. Database searching against the corresponding decoy database was also performed to evaluate the false discovery rate (FDR) of peptide identification.

For standard protein analysis, MASCOT significance threshold 0.05 (95% confidence) was used to filter the peptide identification. For RAW 264.7 cell lysate data, MASCOT significance threshold of 0.01 (99% confidence) was applied to filter the peptide identification. In addition, manual evaluation was also used for the cell lysate peptide identifications. At the protein level, protein grouping was enabled, and each protein group had at least one unique peptide. Leucine and isoleucine were considered as equal, and the strict maximum parsimony principle was applied.

### RESULTS AND DISCUSSION

**Integrated CZE–ESI-MS/MS System.** The system was composed of a separation capillary with an immobilized trypsin microreactor, an electrokinetically driven sheath-flow CE–MS interface,<sup>18</sup> two high-voltage (HV) supplies, and an LTQ-Orbitrap Velos mass spectrometer, Figure 1B.

The microreactor was synthesized in situ at the injection end of the separation capillary. The integrated system does not require an additional interface to connect the microreactor and separation capillary. The trypsin microreactor was prepared from an acrylamide monolithic due to its good permeability, fast mass transfer, high stability, and easy modification.<sup>34</sup> The hydrophilic nature of the acrylamide microreactor reduced nonspecific adsorption of proteins/peptides. The total volume of the trypsin microreactor used in this work was less than 40 nL, which was efficient for trace protein sample digestion. In order to keep high electroosmotic flow (EOF) in the separation capillary, only a short portion of the separation capillary was vinylized for preparation of the microreactor, Figure 1A. Excess succinimide groups on the monolithic material were blocked with glycine. Glycine's amine group reacted with the succinimide groups, leaving the carboxyl groups of glycine exposed to the separation buffer, which was useful for improving the EOF in the separation capillary.

An electrokinetically driven sheath-flow interface, which was developed by our group,<sup>18</sup> was used due to its very high sensitivity for peptide detection.<sup>19,20</sup> Two power supplies were used in the experiment. HV supply I was applied at the injection end of the capillary. HV supply II was applied to the interface to produce electrospray. The voltage difference between the power supplies drove the capillary electrophoretic separation, while the potential provided by the second power supply drove the electrospray interface. An LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) was used for peptide identification.

In conventional studies of microgram or larger sample amounts, proteins are denatured, reduced, and alkylated, followed by trypsin digestion. However, these sample pretreatment steps can lead to sample losses that are significant when dealing with low-nanogram or picogram protein homogenates. We applied a simplified sample preparation protocol when analyzing samples smaller than a few nanograms. We used a buffer containing 50% (v/v) ACN and 5 mM NH<sub>4</sub>HCO<sub>3</sub> to dissolve the proteins, which was then directly loaded into the integrated CZE–ESI-MS/MS system for protein digestion in the trypsin microreactor, peptide separation in the capillary, and identification by tandem mass spectrometry.

This protocol has at least two advantages. First, the one-step sample preparation reduces the sample loss and also avoids the contamination from the reducing and alkylating reagents. Second, the sample buffer (50% (v/v) ACN and 5 mM NH<sub>4</sub>HCO<sub>3</sub>) has lower conductivity than the separation buffer (5 mM NH<sub>4</sub>HCO<sub>3</sub>). This difference in buffer conductivity results in sample stacking at the beginning of the separation,<sup>35,36</sup> which improves separation efficiency. The

resulting peaks are sharper and of higher amplitude, which assists detection. This protocol also has potential limitations for analysis of highly structured proteins with many disulfide bonds due to the elimination of reduction and alkylation steps. The 50% (v/v) ACN helps to denature non-cross-linked proteins, but the denaturation performance will be limited for highly structured proteins with many disulfide bonds.

**Evaluation of the Integrated CZE–ESI-MS/MS System.** To evaluate the system for protein analysis, we used two model proteins, insulin chain b oxidized and BSA. Insulin chain b oxidized (30 amine acids with sequence FVNQHLCGSHLVEALYLVCGERGFFYTPKA and 3494 Da molecular weight) was first used to evaluate the system. Because it is a small protein, the protein was directly dissolved in the separation buffer (5 mM NH<sub>4</sub>HCO<sub>3</sub>) for analysis. After online digestion, separation, and identification with the integrated CZE–ESI-MS/MS system, 100% sequence coverage was obtained with a 7 fmol sample. Two identified peptides, GFFYTPKA and FVNQHLcGSHLVEALYLVCGER, were extracted from the raw files of triplicate runs with mass tolerance as 5 ppm, Figure 2. The two peptides were well-



**Figure 2.** Extracted ion electropherogram of two peptides of insulin chain b oxidized after analysis by the integrated CZE–ESI-MS/MS system in triplicate with 7 fmol injection amount per run. Five-point Gaussian smoothing was applied to the spectra, and the peptides were extracted with mass tolerance of 5 ppm.

separated, and their migration time was reasonably reproducible (relative standard derivation (RSD) less than 5%) in triplicate runs. In addition, RSD of the peptide intensity in triplicate runs was less than 30% for 7 fmol loading amount and was about 20% for 70 fmol loading amount. These results indicate that the

integrated CZE-ESI-MS/MS system is efficient and reproducible for analysis of low-femtomole amounts of proteins.

We further determined the relationship between the injection amount (A) of insulin chain b oxidized and the peptide intensity (I), Figure 3A. When the sample injection amount



**Figure 3.** Relationship of the injection amount of insulin chain b oxidized and the peptide intensity (GFFYTPKA and FVNQHLcGSHLVEALYLVcGER) (A) and the relationship between the reciprocal of the injection amount and the reciprocal of the peptide intensity, eq 1 (B), produced by the integrated CZE-ESI-MS/MS system in triplicate. The peptide intensity was obtained by peak extraction with mass tolerance of 5 ppm. Gaussian smoothing with five points was applied.

increased from 7 to 70 fmol, the peptide intensity also increased, but the relationship is not linear. In our experiment, the injection volume was fixed, and the concentration of insulin chain b oxidized ranged from 0.01 to 0.1 mg/mL, resulting in 7–70 fmol injection amounts. The digestion time was fixed, so the generated peptide amount directly related to the trypsin digestion rate. The peptide intensity from the mass spectrometer is a good parameter to estimate the peptide amount. Protease digestion can be described by the Michaelis–Menten kinetics. We linearlized the classical Michaelis–Menten equation, which was expressed in terms of peptide intensity (I), the Michaelis constant ( $K_m$ ), and loaded amount (A) (eq 1):

$$\frac{1}{I} = \frac{K_{\rm m}}{I_{\rm max}A} + \frac{1}{I_{\rm max}} \tag{1}$$

A plot of 1/I versus 1/A followed this linearlized equation (R = 0.99), Figure 3B.

To check the system for the analysis of larger and more complex proteins, BSA with 66 kDa molecular weight and 17 disulfide bonds was also analyzed by the system. BSA was dissolved in 50% (v/v) ACN and 5 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0) and directly injected into the microreactor end of the separation capillary and digested at room temperature for 2 min without reduction and alkylation, followed by CZE-ESI-MS/MS analysis in duplicate. When about 4 fmol BSA was injected and analyzed by the system, two peptides and 4.6% sequence coverage were consistently produced, which suggest that the integrated system is also efficient for the analysis of proteins with large molecular weight and many disulfide bonds without reduction and alkylation. When the injection amount was increased to 20 fmol, the peptide number and sequence coverage dramatically increased to an average of 14 and 19%, respectively.

No peptides were detected from the tandem spectra when 1 fmol of BSA was injected. However, we generated an extracted ion electropherogram corresponding to a BSA peptide (KVPQVSTPTLVEVSR, m/z 820.4744, +2) with mass tolerance of 2 ppm. This extracted ion electropherogram had an average intensity of  $1.6 \times 10^4$ , which is consistent with the average intensity produced by 4 fmol sample ( $7.55 \times 10^4$ ). In addition, the charge state of the peptide from 1 fmol was the same as that from 4 fmol, and the migration times of the peptide between the 1 and 4 fmol results were reasonably reproducible. These results indicate that BSA could be detected from injection of only 1 fmol.

Application for Analysis of Standard Protein Mixtures. A three-protein mixture (BSA, 0.12 mg/mL, cytochrome c, 0.01 mg/mL, and myoglobin, 0.01 mg/mL) dissolved in 50% (v/v) ACN and 5 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0) was analyzed by the integrated CZE–ESI-MS/MS system in triplicate. Injection amounts were 7 fmol (BSA), 3 fmol (cytochrome c), and 2 fmol (myoglobin) for each run. The three proteins could be confidently and reproducibly identified by the system with the number of peptide identifications (IDs) as  $10 \pm 2$  (cytochrome c),  $11 \pm 1$  (myoglobin), and  $1 \pm 1$  (BSA), Table 1. Besides the three proteins, transthyretin was also identified with the

Table 1. Results of the Triplicate Analysis of the Three-Protein Mixture

	MW (kDa)	pI	injection amount (pg)	injection amount (fmol)	peptide IDs	sequence coverage (%)
cytochrome c	11.7	9.5	40	3	$10 \pm 2$	69 ± 2
myoglobin	16.9	7.8	40	2	$11 \pm 1$	$74 \pm 17$
BSA	69.5	6.2	480	7	$1 \pm 1$	$2 \pm 2$
transthyretin	15.7	6.3	Un <sup>a</sup>	Un	$1 \pm 1$	6 ± 5

<sup>a</sup>The injection amount of transthyretin was unknown (Un).

number of peptide IDs as  $1 \pm 1$ , Table 1. Transthyretin might be an impurity from BSA, because transthyretin was also identified from the 20 fmol BSA data with six peptides. Interestingly, we also identified transthyretin as an impurity in BSA using top-down analysis of intact proteins (data not shown).

We further analyzed a seven-protein mixture (BSA, 0.11 mg/ mL, cytochrome *c*, 0.009 mg/mL, myoglobin, 0.009 mg/mL,  $\alpha$ casein, 0.015 mg/mL,  $\beta$ -casein, 0.015 mg/mL,  $\beta$ -lactoglobulin, 0.015 mg/mL, and insulin chain b oxidized, 0.01 mg/mL) dissolved in 50% (v/v) ACN and 5 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0) in triplicate. The injection amount was 6 fmol (BSA), 3 fmol (cytochrome *c*), 2 fmol (myoglobin), 2 fmol ( $\alpha$ -casein), 2 fmol ( $\beta$ -casein), 3 fmol ( $\beta$ -lactoglobulin), and 12 fmol (insulin chain b oxidized) for each run. Six proteins (all but  $\beta$ -lactoglobulin) were identified from triplicate runs, and their sequence coverage ranged from 6% to 100% and peptide IDs ranged from 2 to 16, Table 2. Besides the identification of the six

# Table 2. Results of the Triplicate Analysis of the Seven-Protein Mixture

	MW (kDa)	injection amount (pg)	injection amount (fmol)	peptide IDs <sup>a</sup>	sequence coverage (%) <sup>a</sup>
BSA	69.5	440	6	3	6
cytochrome c	11.7	36	3	16	73
myoglobin	16.9	36	2	13	96
$\alpha$ -casein	24.5	60	2	2	20
$\beta$ -casein	25.1	60	2	4	19
insulin chain b oxidized	3.4	40	12	4	100
$\beta$ -lactoglobulin	18.4	60	3	0	0
transthyretin	15.7	$Un^b$	Un	1	7
uncharacterized protein	18.3	Un	Un	4	27
BARX2 protein	31.0	Un	Un	1	2
<sup><i>a</i></sup> Peptide IDs au triplicate runs.	nd seque <sup>b</sup> The in	ence cover iection am	age were	from the	combined teins were

proteins, three additional proteins (transthyretin, uncharacterized protein, and BARX2 protein) were also identified with

unknown (Un).

the number of peptide IDs as 1, 4, and 1, respectively, Table 2. These proteins might be impurities present in the seven standard proteins.

The protein mixtures analyzed in the experiments contained basic and acidic proteins, large and small proteins, and phosphorylated and unphosphorylated proteins, which suggest that the integrated CZE–ESI-MS/MS system is of high sensitivity and efficiency for analysis of complex mixtures of proteins.

Application for Analysis of Picogram Range of RAW 264.7 Cell Lysate. The integrated system was used to analyze RAW 264.7 cell lysate in triplicate. Two buffers were used to prepare the protein samples. In one case, a sample containing 0.1 mg/mL proteins was dissolved in 50% (v/v) ACN, 0.025% (w/v) SDC, and 5 mM NH<sub>4</sub>HCO<sub>3</sub>. In a second case, a 1 mg/mL protein sample was dissolved in 50% (v/v) ACN, 0.25% (w/v) SDC, and 5 mM NH<sub>4</sub>HCO<sub>3</sub>. The injection amounts per run for the 0.1 and 1 mg/mL samples were ~300 pg and ~3 ng, respectively.

After triplicate analysis,  $2 \pm 1$  and  $7 \pm 2$  protein groups were identified from the 300 pg and 3 ng RAW 264.7 cell lysates,

respectively. Information of the identified protein groups is listed in the Supporting Information. The protein groups (except one protein group, glial fibrillary acidic protein) obtained from the 300 pg sample were also identified from the 3 ng sample. For glial fibrillary acidic protein, one unique peptide was identified, and its Exp value and mass error were  $1.4 \times 10^{-6}$  and 1 ppm, respectively, which confirmed the protein group identification. Most of the proteins identified from the 300 pg and 3 ng samples were relatively highly abundant in RAW 264.7 cell lysate according to the protein spectral count information from the large-scale proteome data generated previously in our group. In addition, the peptide identifications from the 300 pg and 3 ng samples were also manually evaluated, and the annotated tandem spectra of those peptides are listed in the Supporting Information. We also analyzed the molecular weight and pI of proteins generated by triplicate analysis of the 3 ng RAW 264.7 cell lysate. The molecular weight ranged from 5.7 to 58.8 kDa, and pI ranged from 4.5 to 11.0, which suggest that the integrated CZE-ESI-MS/MS system is efficient for small and large proteins, and also acidic and basic proteins. We further checked the disulfide bonds of the identified proteins from 3 ng RAW 264.7 cell lysate, and the identified proteins tend to have few or no disulfide bonds, which is expected due to the much easier denaturation of these proteins with 50% (v/v) ACN. The result indicates that the integrated CZE-ESI-MS/MS system might have potential limitations for identification of highly structured proteins with many disulfide bonds. On the other hand, the system might be useful for selective identification of the proteins with few disulfide bonds in complex samples.

We further extracted the electropherograms from a peptide from macrophage migration inhibitory factor, which was identified from both 300 pg and 3 ng samples, Figure 4. The integrated CZE-ESI-MS/MS system yielded reasonably reproducible migration time for the peptide for analysis of both 300 pg and 3 ng samples. In addition, the peptide intensity from triplicate analysis of the 3 ng cell lysate was quite consistent (RSD = 8%), and the peptide intensity from the 300 pg cell lysate was reasonably reproducible (RSD = 30%). In addition, when the injection amount was increased from 300 pg to 3 ng, the average peptide intensity generated by the system increased from 7.5  $\times$  10<sup>4</sup> to 7.6  $\times$  10<sup>5</sup>. The reproducible peptide migration time and peptide intensity indicate that the integrated CZE-ESI-MS/MS system is reproducible for trace complex cell lysate analysis. The peptide intensities generated for 300 pg and 3 ng cell lysates reasonably reflect the difference in sample amounts, which suggests that the system might be useful for further quantitative protein analysis.

We also extracted two peptides of 10 kDa heat shock protein. One peptide (VLQATVVAVGSGGK) was only identified from 0.3 ng cell lysate, S-Figure 1 in the Supporting Information, and another peptide (VLQATVVAVGSGGKGK) was only identified from 3 ng cell lysates, S-Figure 2 in the Supporting Information. These two peptides, extracted with mass tolerance as 2 ppm, generated reasonable signal intensities from all the 300 pg and 3 ng cell lysate data. The migration time and peptide intensity of these two peptides from 300 pg and 3 ng cell lysates were also reproducible, which further confirmed the reproducibility of the integrated CZE–ESI-MS/MS system. The intensity of these two peptides also increased with loading amounts from 300 pg to 3 ng.

One obvious limitation of our analysis is that the protein homogenate was prepared from a large volume and only a small

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**Figure 4.** Triplicate extracted ion electropherograms of one peptide (PMFIVNTNVPR) from the macrophage migration inhibitory factor that was identified from both 300 pg and 3 ng of RAW 264.7 cell lysate. The electropherogram was extracted with a mass tolerance of 5 ppm, and Gaussian smoothing with five points was applied before plotting the results.

aliquot was used for analysis. However, we note that the protein content in a single RAW 264.7 cell is about 0.1 ng,<sup>37</sup> and the protein amounts analyzed in this work correspond to the protein content in three cells (0.3 ng) and 30 cells (3 ng). The simplified sample preparation protocol used in this analysis, when coupled with the high-sensitivity electrokinetically pumped sheath-flow cuvette nanospray interface, suggests that the system could be coupled to a single-cell injection and lysis system for single-cell analysis.<sup>38,39</sup> However, there are still challenges for analysis of small number of cells or of single cell with the system, including the cell injection, efficient cell lysis, removal of interfering compounds in the cell lysate, efficient and accurate control of the transfer of cell lysate to the trypsin microreactor, and the higher capacity peptide separation and deeper protein profiling. We also note that this experiment employed an uncoated capillary for separation. We have recently described the use of a coated capillary for analysis of the Escherichia coli proteome.<sup>40</sup> That approach produced over 1250 peptide IDs in a single run. Use of a coated capillary may also produce enhanced peptide detection in the analysis of minute amounts of the products generated by the on-column digestion system.

#### ASSOCIATED CONTENT

#### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

#### AUTHOR INFORMATION

## Notes

The authors declare no competing financial interest.

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