Electronic Supplementary Material (ESI) for Analyst.

Capillary zone electrophoresis-mass spectrometry with microliter-scale loading capacity, 140-min separation window and high peak capacity for bottom-up proteomics

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Supporting material I

M*aterials and reagents*

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. LC/MS grade water, formic acid (FA), methanol, acetonitrile (ACN), HPLC grade acetic acid (AA) and hydrofluoric acid (HF) were purchased from Fisher Scientific (Pittsburgh, PA). Acrylamide was purchased from Acros Organics (NJ, USA). Fused silica capillaries (50 μm i.d./360 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ).

Mammalian Cell-PE LB™ buffer for cell lysis was purchased from G-Biosciences (St. Louis, MO). Complete, mini protease inhibitor cocktail (provided in EASYpacks) was purchased from Roche (Indianapolis, IN).

LPA coating for separation capillary of CZE

Bare fused silica capillary (50 μm i.d./360 μm o.d.) was flushed successively with 1 M hydrochloric acid, water, 1 M sodium hydroxide, water, and methanol. Then the capillary was flushed with nitrogen overnight at room temperature. After that, the capillary was filled with 50% (v/v) 3-(trimethoxysilyl) propyl methacrylate in methanol and was kept at room temperature for at least 24 hours with both ends sealed by silica rubber. (***Tips.*** *Based on our experience, the capillary with 3-(trimethoxysilyl) propyl methacrylate inside can be kept at room temperature for up to one week before next step. The silica rubber will block the ends of the capillary, so before next step at least 5 mm length of capillary should be removed from both ends of the capillary.*) The capillary was then flushed with methanol to remove the unreacted reagents and dried under nitrogen. The capillary now can be stored at room temperature with both ends sealed with silica rubber before further steps.

40 mg of acrylamide was dissolved in 1 mL of water. Then 500 μL of the acrylamide solution was used for following steps. 3.5 μL of 5% (w/v) ammonium persulfate (APS) in water was added to the 500-μL acrylamide solution. After mixed via vortex, the solution was degassed using nitrogen to remove the oxygen in the solution for 5 minutes. The solution finally was introduced into the pretreated capillary by vacuum. The filled capillary was sealed with silica rubber at both ends, and incubated in the water bath at

50 ˚C for 35 min. The capillary was then flushed with water to remove excess reagents, and was stored at room temperature before use. (***Tips.*** *The reaction in the capillary is initiated by APS at high temperature. The reaction time can be varied depending on the temperature in the lab. Therefore, the volume of APS and reaction time need to be slightly adjusted. The degassing step is important and this step can be longer (i.e., 10 min). When you flush the capillary with water after the reaction at 50 ˚C, you should be able to see "agarose gel" like substance being pushed out of the capillary at the beginning. The quantity of the "agarose gel" like substance can be small depending on the total volume of the capillary. If you see the "agarose gel" like substance, it means the quality of the coating is good. If the reaction time is too short or APS concentration is too low or the degassing step is not sufficient, you will not see the "agarose gel" like substance. We suggest using a short capillary to test the conditions at the beginning.)*

Sample Preparation

Four standard proteins including bovine serum albumin, cytochrome c (from bovine), myoglobin (from equine) and beta casein (from bovine) were dissolved in 8 M urea and 100 mM ammonium bicarbonate (NH_4HCO_3), pH 8.0, and the solutions were kept at 37 ˚C for 30 min for protein denaturation, followed by protein reduction with dithiothreitol (DTT) at 37 ˚C for 30 min and alkylation with iodoacetamide (IAA) at room temperature for 20 min. After dilute the samples with 100 mM NH_4HCO_3 (pH 8.0) to make the urea concentration lower than 2 M, tryptic digestion was performed at 37 ˚C for overnight with trypsin/protein ratio as 1/30 (w/w). After digestion, the samples were acidified with formic acid to terminate the reactions and desalted with C18 SPE columns (Waters, Milford, MA), followed by lyophilization with a vacuum concentrator (Thermo Fisher Scientific). Finally, we prepared a standard-protein digest sample via mixing the peptides from the four standard proteins. The standard-protein digest sample contains 1 mg/mL of BSA, 0.07 mg/mL of myoglobin, 0.03 mg/mL of beta-casein and 0.006 mg/mL of cytochrome c. This sample was further diluted 10-times with different buffers to get total peptide concentration around 0.1 mg/mL for CZE-MS experiments.

Two 21-week old female OT-1 mice were sacrificed for collection of brain and liver. The mouse brain and liver samples were kindly provided by Professor Xuefei Huang's group

at Department of Chemistry, Michigan State University. The whole protocol related to the mouse samples were performed following guidelines defined by the Institutional Animal Care and Use Committee of Michigan State University. The mouse brain and liver samples taken from the sacrificed mice were stored at -20 ˚C before use. The mouse brain and liver samples were further prepared with the same protocol described below. The tissue was firstly cut into small pieces and washed with PBS to remove the blood. Then, the sample was suspended in 9 mL of lysis buffer containing mammalian cell-PE LB™ buffer and complete protease inhibitor, followed by homogenization with a Homogenizer 150 (Fisher Scientific, Pittsburgh, PA) on ice and sonication with a Branson Sonifier 250 (VWR Scientific, Batavia, IL) on ice for 10 minutes. The lysates were then aliquoted equally into 1.7 mL Eppendorf tubes, followed by centrifugation at 10,000 g for 5 min. The supernatants were collected and a small portion of the sample was used to measure the protein concentration with BCA assay. The supernatants were then subjected to acetone precipitation to purify the proteins. Briefly, 1 volume of sample was mixed with 4 volumes of cold acetone. Then the mixture was kept at -20 ˚C overnight, followed by centrifugation at 10,000 g for 5 min. After removed the supernatant, we added cold acetone to the Eppendorf tube again to simply wash the protein pellet, followed by centrifugation. After centrifugation, the supernatant was discarded and the protein pellet was air dried in the chemical hood for several minutes. Finally, the protein pellets were stored at -20 ˚C before use.

The protein pellet from mouse brain or liver was dissolved in 8 M urea and 100 mM NH_4HCO_3 (pH 8.0) buffer via vortex and sonication to a final protein concentration approximately 4 mg/mL. The proteins were denatured at 37 ˚C for 30 min, followed by protein reduction with DTT at 37 °C for 30 min and protein alkylation with IAA in dark at room temperature for 20 min. The DTT solution was added into the sample again to react with the left IAA for 10 min at room temperature. The sample was then diluted with 100 mM NH_4HCO_3 (pH 8.0) to reduce the urea concentration to 2 M. After that, proteins were digested into peptides with TPCK-treated trypsin at 37 °C overnight with a trypsin/protein ratio as 1/30 (w/w). Then, the protein digests were acidified with formic acid to terminate the reaction. The protein digests were desalted with C18 SPE columns (Waters, Milford, MA), followed by lyophilization with a vacuum concentrator (Thermo

Fisher Scientific). The protein digests were dissolved in different buffers for CZE-MS experiments.

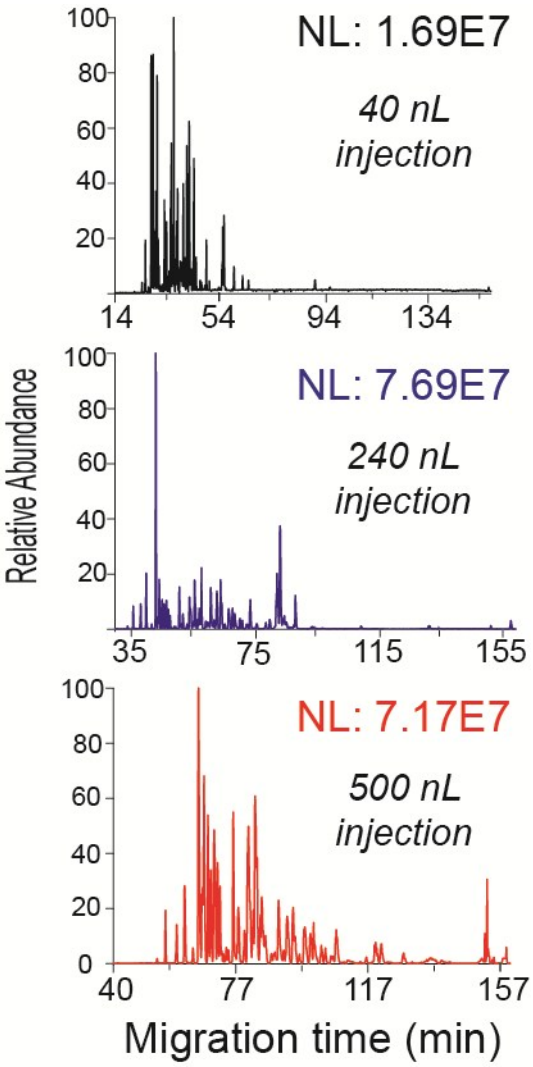
RPLC fractionation of mouse brain proteome digests

500 μg and 50 μg of mouse brain proteome digests were dissolved in 150 μL of 0.1% (v/v) FA for RPLC fractionation. An Agilent Infinity II HPLC system was used. A C18 RP column (Zorbax 300Extend-C18, 2.1 mm i.d. × 150 mm length，3.5 μm particles, Agilent Technologies) was used for peptide separation. Buffer A (water, 0.1% FA) and buffer B (ACN, 0.1%FA) were used as mobile phase to generate gradient for separation. The flow rate was 0.3 mL/min. The peptide samples (500 μg and 50 μg of mouse brain digests) were loaded onto the RPLC column for 4 min at 2% B. Then the peptides were separated by gradient elution: 2 min from 2%B to 6%B, 70 min from 6%B to 40%B, and 1 min from 40%B to 80%B. The mobile phase was kept at 80%B for 4 min, followed by column equilibration with 2%B for 10 min.

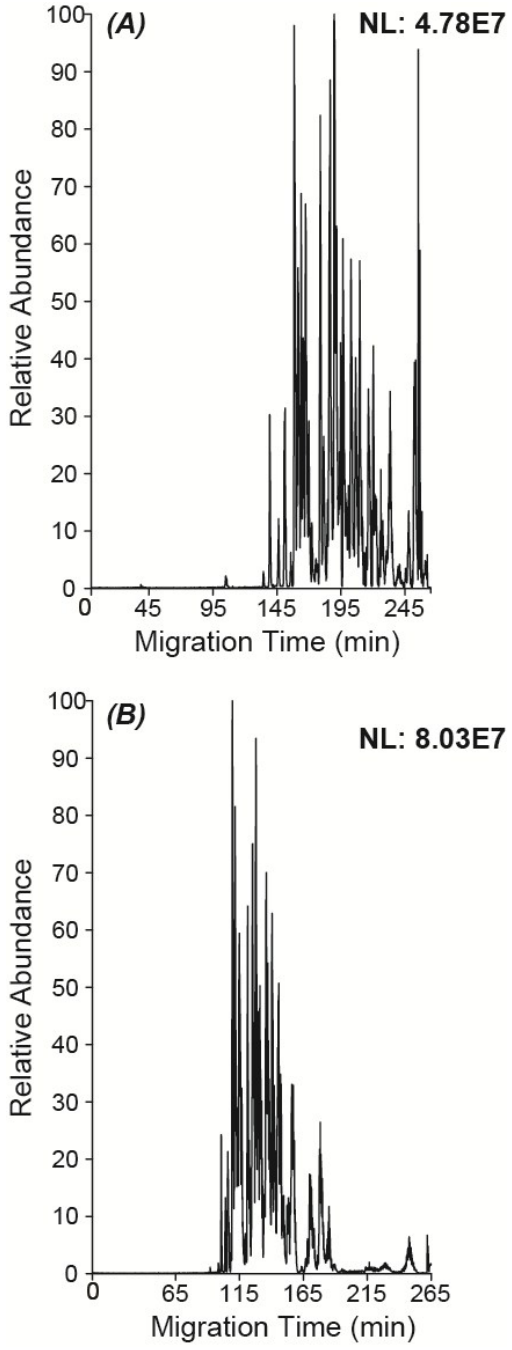
We collected fractions from 11 min to 78 min. Eluate from 11 min to 14 min were collected as one fraction, from 72 min to 78 min was collected as one fraction. From 14 min to 72 min, fractions were collected one fraction/min. In total 60 fractions were collected from each sample.

For the 500 μg of mouse brain digest sample, fraction number “N” and fraction number “N+30” were combined, thus leading to totally 30 fractions. For the 50 μg of protein digest sample, fraction number “N”, fraction number “N+15”, fraction number “N+30” and fraction number “N+45” were combined, thus leading to totally 15 fractions. The fractions were lyophilized and stored at -20 ˚C before use.

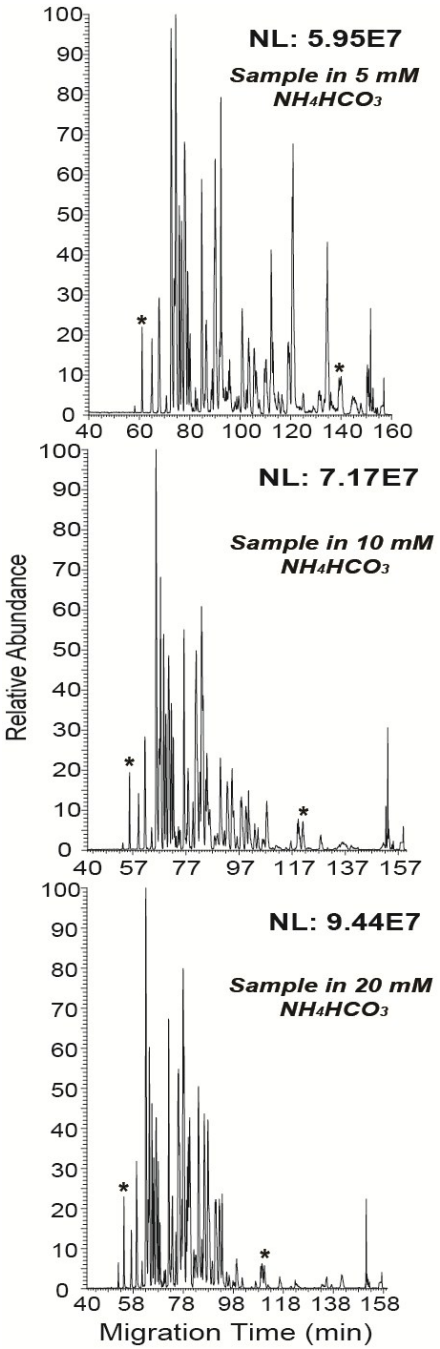
Each RPLC fraction from the 500 μg of protein digest sample was dissolved in 8 μL of 10 mM NH_4HCO_3 (pH 8.0) for CZE-ESI-MS/MS analysis. Each RPLC fraction from the 50 μg of protein digests was dissolved in 4 μL of 10 mM NH_4HCO_3 (pH 8.0) for CZE-ESI-MS/MS analysis.



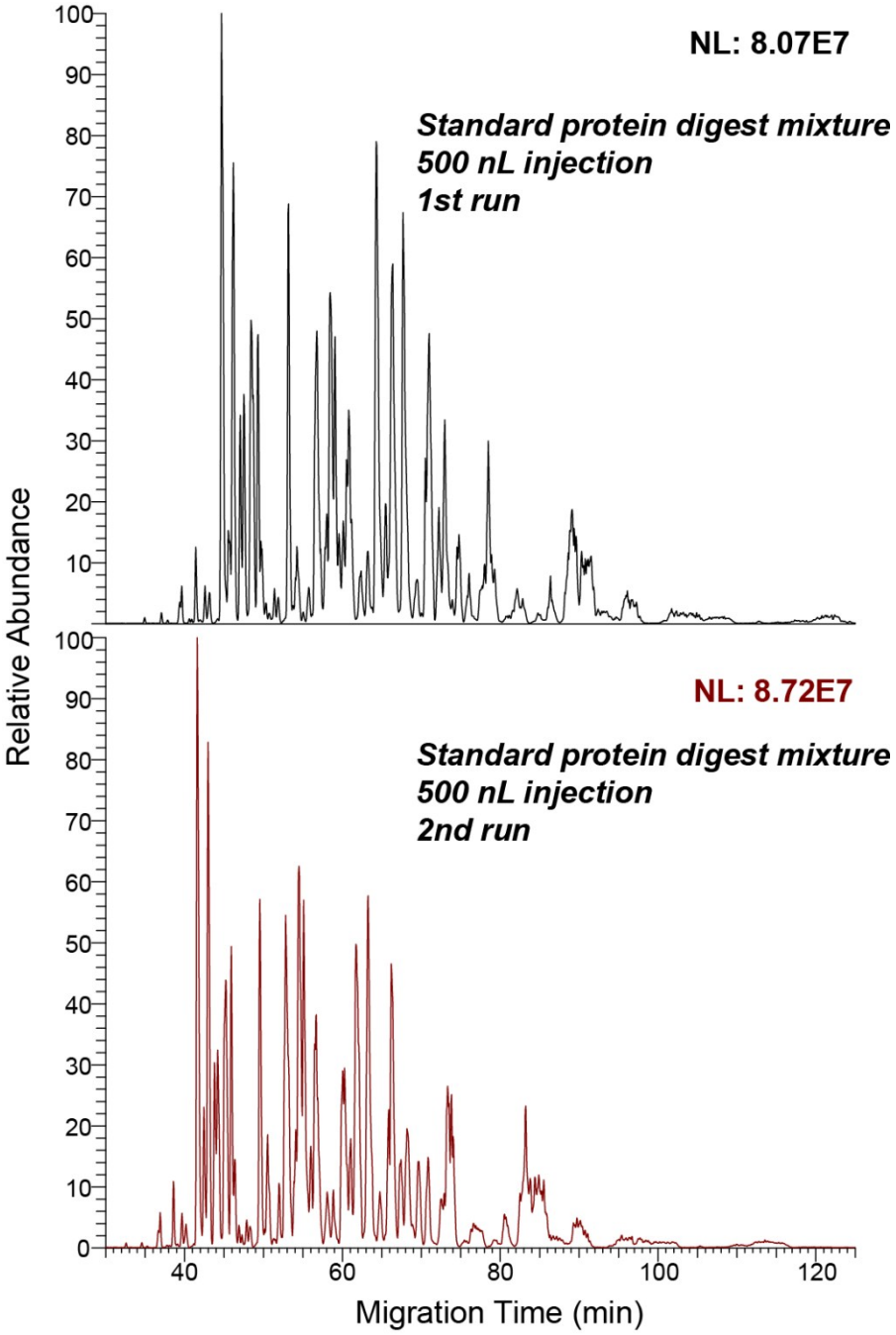
S-Figure 1. Electropherograms of the standard-protein digest sample (0.1 mg/mL in 10 mM NH_4HCO_3, pH 8.0) after CZE-MS analysis with three different sample injection volumes. Top: 40 nL injection; Middle: 240 nL injection; Bottom: 500 nL injection.



S-Figure 2. Electropherograms of the standard-protein digest sample (0.1 mg/mL in 10 mM NH_4HCO_3, pH 8.0) after CZE-MS analysis with two different sample injection volumes. (A): 1 µL injection; (B) 1.5 µL injection.



S-Figure 3. Electropherograms of the standard-protein digest sample (0.1 mg/mL) in 5 mM NH_4HCO_3, pH ~8.0 (top), 10 mM NH_4HCO_3, pH ~8.0 (middle), and 20 mM NH_4HCO_3, pH ~8.0 (bottom) after CZE-MS analysis with 500 nL sample injection volume. Two peptides were marked (*) in the electropherograms to show the different distance between those two peptides.



S-Figure 4. Electropherograms of the standard-protein digest sample (0.1 mg/mL) in 10 mM NH_4HCO_3 (pH ~8.0) after CZE-MS analysis in duplicates with 500 nL sample injection volume per run.