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Single Shot Capillary Zone Electrophoresis-Mass Spectrometry Produces Over 27,000 Peptide and nearly 4,400 Protein Identifications by Combining a Very Low-Electroosmosis Coated Capillary, a Third-Generation Electrokinetically-Pumped Sheath-Flow Nanospray Interface, an Orbitrap Fusion Lumos Tribid Mass Spectrometer, and an Advanced Peak Determination Algorithm

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Abstract

We show that capillary zone electrophoresis-electrospray ionization-tandem mass spectrometry (CZE-ESI-MS/MS) generates very large numbers of peptide and protein identifications (IDs) by combining four technologies: a separation capillary coated to generate very low electroosmosis, an electrokinetically-pumped sheath-flow nanoelectrospray interface to produce high-sensitivity ionization, an Orbitrap Fusion Lumos Tribrid platform to provide high-speed analysis, and use of an advanced peak determination (APD) algorithm to take advantage of the mass spectrometer's data acquisition speed. The use of the APD algorithm resulted in two-times more identification than the Standard Peak Algorithm. We also investigated the effect of the isolation window, injection time, and loading amount. Optimization of these parameters produced over 27,000 peptide identifications and nearly 4,400 protein group identifications from 220 ng of K562 cell digest in a single 120 min run, which is 2.7 times the number of IDs in the previous state of the art produced by CZE-ESI-MS/MS.

Graphical Abstract

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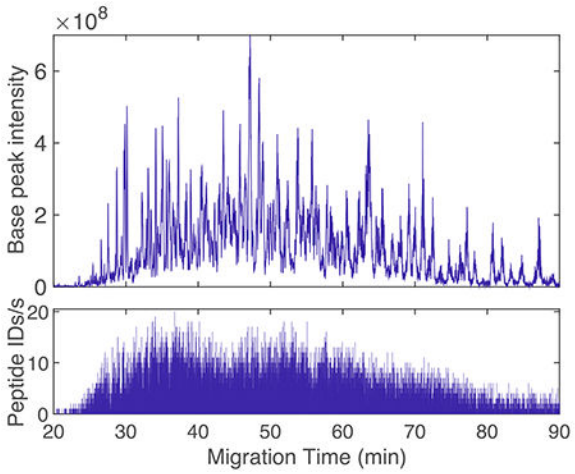
Supporting Information

Summary of CZE-ESI-MS/MS data for bottom-up proteomics and distribution of peak widths. An Excel spreadsheet with information on peptide and protein identifications. The Supporting Information is available free of charge on the ACS Publications website.



In shotgun proteomics, a complex set of proteins is digested to create a much more complex set of peptides.[1–3] These peptides undergo one or more stages of separation before analysis by tandem mass spectrometry. The vast majority of bottom-up proteomic studies use reversed-phase liquid chromatography (RPLC) for peptide separation. However, the performance of mass spectrometry is exceeding the capabilities of RPLC to provide peptides for analysis, and alternative separation technologies will be of value.[4]

Capillary zone electrophoresis (CZE) is such an alternative. CZE separations are complementary to RPLC, and the combination of the technologies provides deeper proteomic coverage than either alone.[5] CZE is particularly useful for detection of hydrophilic peptides that are lost in the void volume of RPLC separations.[6] CZE consistently produces more peptide identifications than RPLC when analyzing submicrogram amounts of a tryptic digest.[5,7] CZE has very low operational costs, particularly if capillaries are coated in-house using straightforward chemistry.[8] Finally, CZE separations are much easier to accurately model than RPLC; such models are very useful in detecting peptides that are misidentified by search engines.[6]

CZE-ESI-MS/MS analysis has undergone remarkably rapidly improvement in the number of peptide and protein identifications IDs. This improvement arises due to the development of a robust CZE-MS interface, the incremental improvement in capillary coatings with reduced electroosmotic flow, and the continual improvement in the performance of mass analyzers.[9–15] To date, the best single shot CZE-ESI-MS/MS bottom-up proteomic analyses of eukaryotic systems have identified approximately 10,000 peptides and 2,000 proteins.[8, 10]

In this paper, we more than double the number of IDs produced by the state-of-the-art in single-shot CZE–MS/MS for analysis of a complex proteome. This improvement results from the combination of a number of technologies. We employ a surface-confined aqueous reversible addition-fragmentation chain transfer polymerization for preparation of a coated capillary with very low electroosmotic flow.[11] We also employ a third-generation electrokinetrically-pumped electrospray interface for high-sensitivity analysis. In addition, we use an advanced peak determination (APD) algorithm with an Orbitrap Fusion Lumos Tribrid platform.[14] This mass spectrometer can generate up to 60 ion trap MS/MS scans per

second. However, such high acquisition rates are significantly underutilized in most experiments because the instrument exhausts the number of detectable peptide determined by the standard peak determination (SPD) algorithm from a survey scan.[14] The SPD algorithm only considers local maxima in a given mass-to-charge (*m/z*) region when annotating the most abundant peptide feature, and lower abundance features are not selected for data-dependent MS/MS. The APD algorithm uses an iterative approach to annotate densely populated *m/z* regions, and enables nearly full utilization of the sampling capacity of the mass spectrometer. This RPLC-MS/MS analysis generated ~53,000 peptide IDs in a 90 min gradient from 1 μg of HeLa sample.[14]

MATERIALS AND METHODS

Chemicals and Materials

Formic acid (FA), acetic acid (HAc), acrylamide, trifluoroacetic acid (TFA), 4,4 -azobis(4-cyanovaleric acid), cyanomethyl [3-(trimethoxysilyl)propyl] trithiocarbonate, bovine pancreas TPCK-treated trypsin, tris(2-carboxyethyl)phosphine (TCEP), and iodoacetamide (IAA) were purchased from Sigma-Aldrich (St. Louis, USA). Methanol was purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). Uncoated fused silica capillary (50 μm i.d. × 350 μm o.d.) was purchased from Polymicro Technologies (Phoenix, AZ). Water was deionized by a Nano Pure system from Thermo Scientific (Marietta, OH).

Sample preparation

Human K562 cells were homogenized in 6 M guanidine HCl by probe sonication, followed by boiling for 5 minutes. Methanol was added to 90% final concentration, samples were centrifuged at 10,000 × g for 5 min, and the supernatant was discarded. The protein pellet was dissolved in 8 M urea, 100 mM tris pH 8, 10 mM TCEP, and 40 mM chloroacetamide. Endoproteinase LysC was added to an estimated 50:1 (protein:enzyme) ratio and the digestion was incubated at ambient temperature for 2 hours. The digest was diluted to 2 M urea in 100 mM tris; trypsin was added at a 50:1 ratio followed by overnight incubation at ambient temperature. Samples were titrated with TFA to approximately pH 2, followed by centrifugation and solid phase extraction of the peptides in the supernatant, and dried down.

Preparation of linear poly(acrylamide) (LPA) coated capillaries by surface-confined aqueous reversible-addition fragmentation transfer polymerization

A published protocol was use for coating the capillary.[8] Briefly, a piece of ~110 cm long, 50-μm ID, 360 μm OD fused silica capillary was pretreated by flushing with 0.1 M NaOH for 2 h, flushing with water until the outflow reached pH ~7.0, flushing with 0.1 M HCl for 12 h, flushing again with water until the outflow reached pH ~7.0, and drying with a nitrogen stream overnight at room temperature. Then, the capillary was filled with 50% cyanomethyl [3-(trimethoxysilyl)propyl] trithiocarbonate solution in MeOH(v/v) and incubated in a water bath at 45 °C for 12 h. The capillary was then rinsed with MeOH to flush out the residual reagent and dried with a nitrogen stream. A polymerization mixture was prepared by mixing acrylamide (0.56 M) and 4,4'-azobis(4-cyanovaleric acid) (5.42×10^{-4} M) in an acetate buffer (pH 5.2, 0.27 M acetic acid, and 0.73 M sodium acetate) at room temperature and stirring for about 10 min under nitrogen to form a homogeneous

solution. The mixture then was introduced into the pretreated capillary and incubated at 60 °C for 7 hours. The coated capillary was flushed with H_2O and MeOH to remove residual reagents.

Capillary etching

The outer portion of a 5-mm length of the LPA coated separation capillary was etched with HF to a ~80 μm diameter using a published protocol.[10] The final length of the etched capillary was 105 cm.

Caution: use appropriate safety procedures while handling HF solutions.

CZE-ESI-MS/MS analysis

The CZE system consisted of two high-voltage power supplies (Spellman CZE 1000R) and an electrokinetically pumped nanospray interface,[9–11] which coupled the CZE separation capillary to the mass spectrometer. The electrospray emitter was made from a borosilicate glass capillary (1.0 mm o.d. × 0.75 mm i.d., 10 cm long) pulled with a Sutter instrument P-1000 flaming/brown micropipette puller; the size of the emitter opening was 15-20 μm.[8] As noted above, the distal tip of the capillary was etched to a ~80-μm outer diameter. The electrospray sheath electrolyte was 10% (v/v) methanol with 0.5% FA. The background electrolyte was 1 M HAc in H_2O. The sample was injected by nitrogen pressure. The separation voltage of 23.8 kV was applied at the injection end of the capillary. 1.8 kV was applied to the sheath flow reservoir for electrospray. Voltage programming was controlled by LabView software. The mass spectrometer's operating parameters are described below.

Mass Spectrometer Operating Parameters

Peptides were analyzed on an Orbitrap Fusion Lumos Tribrid platform with Instrument Control Software version 3.0. Typical analyses used a 240 000 resolving power survey scan with an AGC of 10^6, followed by MS/MS of the most intense precursors for 1 s. The MS/MS analyses were performed by 0.4 m/z isolation with the quadrupole, normalized HCD collision energy of 25%, and analysis of fragment ions in the ion trap using the "Turbo" scan speed scanning from 200 to 1200 m/z. Dynamic exclusion was set to 20 s. Monoisotopic precursor selection (MIPS) was set to Peptide. For MS/MS analyses, the maximum injection time was set to 11 ms with an AGC target of 30 000, and charge states unknown, 1, or >5 were excluded. Advanced peak determination was either off or on for SPD and APD analyses, respectively.

Database Searching

Database searching of the raw files was performed in Proteome Discoverer 2.2 (Thermo) with Sequest HT and MS Amanda 2.0 database search engines.[17, 18] The SwissProt database with taxonomy as *Homo sapiens* was downloaded from Uniprot and for the K562 cell line proteome digest. The database searching parameters included full tryptic digestion and allowed up to two missed cleavages, the precursor mass tolerance was set at 10 ppm, fragment mass tolerance was 0.4 Da. Carbamidomethylation of cysteines (+57.0215 Da) was set as a fixed modification, and variable modifications of methionine oxidation (+15.9949 Da), Acetyl (N-terminus) (+42.011 Da) and deamination (Asparagine or Glutamine) (+0.984

Da) were allowed. The false discovery rate (FDR) was determined by using a target-decoy search strategy. The decoy sequence database contains each sequence in reverse orientations, enabling FDR estimation. On the peptide level, the corresponding FDR on peptide level was less than 1%. On the protein level, protein grouping was enabled, and a Protein FDR Validator was used with the target FDR (strict) set at 1%.

For peak widths analysis, raw files were also analyzed by MaxQuant (version 1.61.0.). MS/MS spectra were searched by the Andromeda search engine against the Uniprot human protein database using the default parameters.[19] The distribution of the peak widths was generated from the “evidence” file.

RESULTS AND DISCUSSION

We investigated the effect of a number of experimental parameters on the numbers of peptide and protein IDs, Table 1. We first compared the APD and the SPD algorithms for single shot CZE-ESI-MS/MS analysis of whole cell K562 lysates. Under the same separation conditions, the APD algorithm generated nearly twice the number of peptide IDs as the SPD algorithm. The fractional increase in the number of peptide IDs produced by the APD algorithm for CZE-ESI-MS/MS is over twice the increase produced by this algorithm for LC-ESI-MS/MS analysis.[14] CZE-ESI-MS/MS benefits more from the APD algorithm than LC-ESI-MS/MS presumably due to co-migration of peptides with similar m/z in CZE experiments.

We also investigated the effect of the isolation window on the number of IDs. We observed a ~15% increase in the number of peptide IDs for a 0.4 *m/z* isolation window compared with a 0.7 *m/z* isolation window. Decreasing the isolation window reduces co-fragmentation of the precursors and improves the quality of the MS/MS spectra, which leads to an increase in the numbers of IDs. Therefore, a 0.4 *m/z* isolation window was used in subsequent studies.

The mass spectrometer’s injection time had a limited effect on the number of IDs. Use of an 11 ms injection time generated 6% more peptide IDs compared to a 60 ms injection time. While there was not a significant difference in the number of identified peptides, there was a large decrease in the MS/MS scan rate when the injection time was increased from 11 ms to 60 ms. 323,303 tandem mass spectra were collected for the 11 ms injection time, but number of tandem mass spectra decreased to 131,524 when the injection time was increased to 60 ms. The injection time was kept at 11 ms in subsequent studies.

Figure 1 (top) presents a representative base-peak electropherogram of the CZE-ESI-MS/MS analysis of a K562 cell proteome digest. The separation window is approximately 100 min wide, which is two times wider than a previous report using a linear polyacrylamide coated capillary prepared by free radical polymerization.[18] Figure 1 (bottom) presents the number of peptide IDs per second. The system generated a peak of 20 peptide IDs/sec, stayed above 10 IDs/sec for much of the time between 25 and 70 minutes, and dropped below 5 peptide IDs/sec at 85 min.

The effect of the sample loading amount on the number of IDs is listed in Table 1. The numbers of protein groups and peptides IDs increased when the sample loading amount

increased from 48 ng to 220 ng, and then plateaued for higher loading amounts. We determined the peak widths for different sample loading amounts (S-Figure 1). It seems that the peak broadening for larger injections is not a serious problem when the sample loading amount increased from 220 ng to 772 ng and when employing the pH junction for injection.[20–24] The plateau in IDs for loading amounts greater then 220 ng may be because sample overloading leads to co-migration of the peptides and compromises the quality of the MS/MS spectra used for identification. Nevertheless, these results suggest that the CZE is particularly useful for comprehensive proteomics analysis using small amounts of sample.[25,26]

The previous state-of-the-art for single-shot CZE-ESI-MS/MS was 10,274 peptide IDs, which was generated from 440 ng of a HeLa digest using a commercial linear polyacrylamide-coated capillary and an Orbitrap Fusion mass spectrometer operating with the SPD algorithm.[10] The number of identifications presented in this paper is 2.7 times larger than the previous record. This improvement results from the wider separation window produced by the improved capillary coating, the faster MS/MS spectra collection rate of the Orbitrap Fusion Lumos Tribrid platform, and the use of the APD algorithm. We summarize recent CZE-MS/MS results for analysis of complex proteome digests in S-Table 1 in the supporting information.

There are a few limitations in the CZE experiment. We noticed that the slowly migrating components undergo significant longitudinal diffusion, leading to the repeated acquisition of data from the same precursor and waste of a significant amount of mass spectrometer time. Optimization of the CZE conditions and MS parameters will be required to improve the identification results.[7] In addition, the first 20 minutes of the CZE separation generated very few IDs, and the number of IDs drops significantly after 85 minutes. The identification rate will be improved by using a multi-segment sequential injection method that has been recently reported.[27,28]

We note that state-of-art RPLC-MS/MS analysis has generated approximately 53,000 peptide IDs in a 90 min gradient from 1 μg of HeLa sample with the APD algorithm using the same mass spectrometer that was used in this paper.[14] Additional optimization will be required for CZE to match the performance of RPLC for bottom-up proteomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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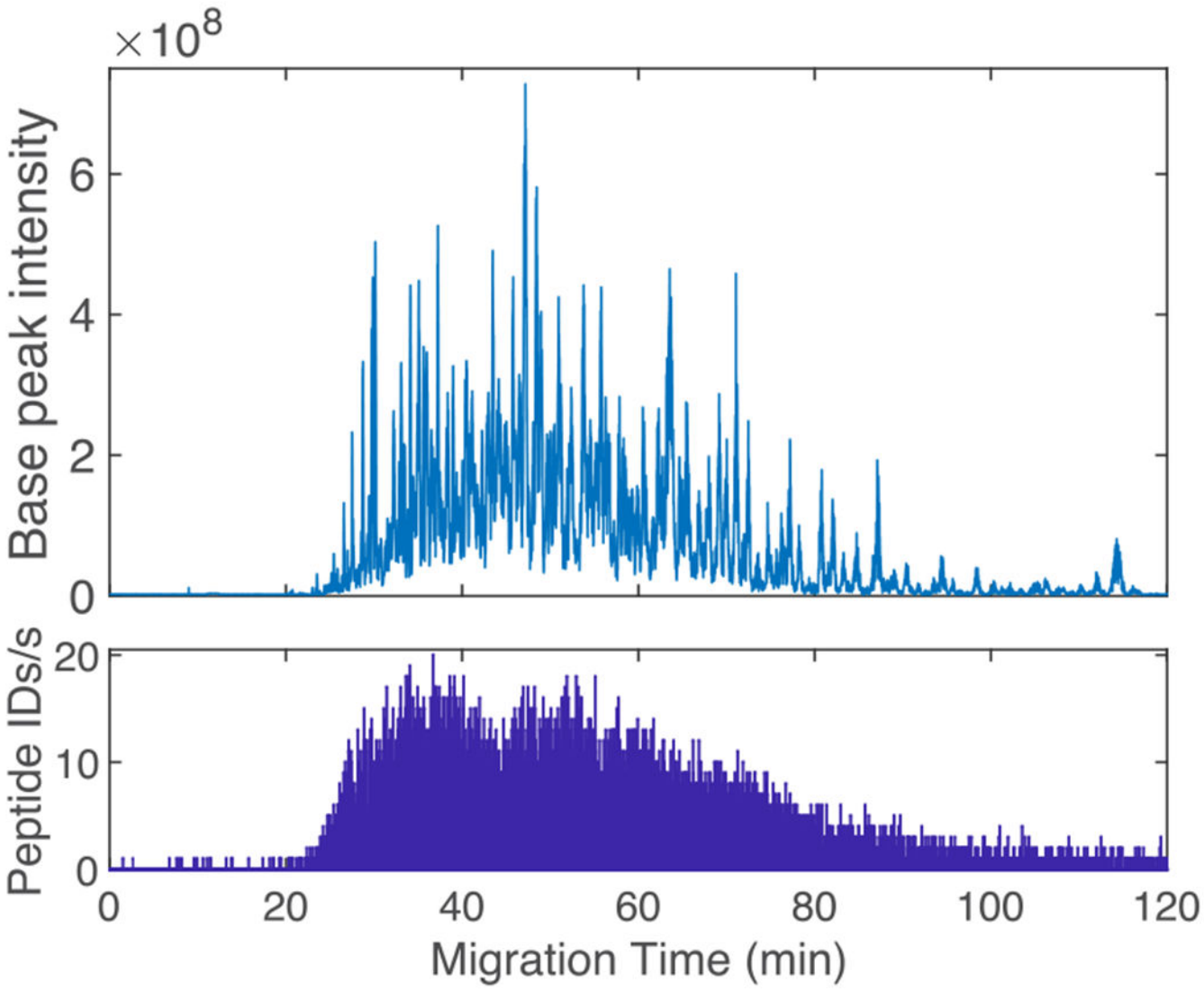

Figure 1.

Single-shot CZE-ESI-MS/MS analysis of K562 cell lysate digest. Approximately 220 ng of the digest was loaded for CZE analysis. Top-base peak electropherogram. Bottom-unique peptide IDs per second.

Table 1–

Numbers of peptide and protein IDs for CZE-ESI-MS/MS

Algorithm	Isolation window	Injection time	Loading amount ng	Peptide IDs	Protein IDs
Effect of algorithm					
SPD	0.4 m/z	11 ms	770 ng	14,144	2,963
APD	0.4 m/z	11 ms	770 ng	27,678	4,387
Effect of isolation window width					
APD	0.7 m/z	18 ms	386 ng	20,355	3,454
APD	0.4 m/z	18 ms	386 ng	22,433	3,720
Effect of injection time					
APD	0.4 m/z	60 ms	220 ng	25,553	3,828
APD	0.4 m/z	11 ms	220 ng	27,113	4,397
Effect of loading amount					
APD	0.4 m/z	11 ms	48 ng	19,874	3,566
APD	0.4 m/z	11 ms	95 ng	20,707	3,618
APD	0.4 m/z	11 ms	220 ng	27,113	4,397
APD	0.4 m/z	11 ms	770 ng	27,678	4,387