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Large-scale collision cross-section profiling on a travelling wave ion mobility mass spectrometer

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Abstract

Ion mobility (**IM**) is a gas-phase electrophoretic method that separates ions according to charge and ion-neutral collision cross-section (**CCS**). Herein, we attempt to apply a travelling wave (**TW**) **IM** polyaniline calibration method to shotgun proteomics and create a large peptide **CCS** database. Mass spectrometry methods that utilize **IM**, such as **HDMS^E**, often use high transmission voltages for sensitive analysis. However, polyaniline calibration has only been demonstrated with low voltage transmission used to prevent gas-phase activation. If polyaniline ions change conformation under higher transmission voltages used for **HDMS^E**, the calibration may no longer be valid. Thus, we aimed to characterize the accuracy of calibration and **CCS** measurement under high transmission voltages on a **TW IM** instrument using the polyaniline calibration method and found that the additional error was not significant. We also evaluated the potential error introduced by liquid chromatography (**LC**)-**HDMS^E** analysis, and found it to be insignificant as well, validating the calibration method. Finally, we demonstrated the utility of building a large-population peptide **CCS** database by investigating the effects of terminal lysine position, via **LysC** or **LysN** digestion, on the formation of two structural sub-families formed by triply charged ions.

Introduction

Ion mobility (**IM**) is a gas-phase electrophoretic separation technique wherein a weak electric field pulls analyte ions through a drift region filled with inert buffer gas. As ions traverse this region, they undergo collisions with neutral gas molecules. Ion drift velocity is dependent on charge state and number of collisions with gas molecules [1]. Therefore, the time that an ion arrives at the end of the drift region can be largely determined by ion charge (**z**) and collision cross-section (**CCS**).

Although **IM** was first combined with mass spectrometry (**MS**) in the 1960s [2, 3], the additions of soft ionization and nested time-of-flight (**TOF**) analysis transformed **IM-MS** into a powerful probe for biomolecules nearly three decades later [4–6]. Despite lower resolution than methods such as high-performance liquid chromatography (**HPLC**), **IM-MS** has found increasing analytical utility and applications as evidenced by extensive literature

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[7–9]. Electrodynamic ion funnels provided a sizeable enhancement to sensitivity [10], and the cyclic drift tubes [11] and field-asymmetric ion mobility spectrometry [12] provided IM resolutions from 500 to 1000. In some IM-MS methods, ion CCS values can be measured and compared with candidate structures obtained through molecular dynamics (MD) simulations, allowing the investigation of three-dimensional structures [13–16].

The development of commercial instrumentation has greatly expanded the reach of IM-MS, particularly in the area of intact protein-protein and protein-ligand complexes [17–20]. The majority of structural investigations on commercial instruments have utilized some form of the first [21] or second [22, 23] generation SYNAPT HDMS (Waters, Milford, MA). Unmodified SYNAPT instruments incorporate a travelling wave (TW) ion guide in the mobility cell [24], creating a non-uniform electric field during separation [25]. Consequently, a TW IM drift time (t_D) is not linearly proportional to CCS, and the instrument must be calibrated with ions of known CCS from drift tube analysis [17, 26, 27].

Bush et al. have recently characterized a TW IM calibration strategy focused on peptides [28]. Singly, doubly, and triply protonated polyalanine CCS were directly measured on a modified drift tube SYNAPT G1, and then used as calibrants to measure the CCS of tryptic peptides on a standard TW IM SYNAPT G2. The tryptic peptides were also directly measured on the G1 to evaluate TW IM CCS accuracy. Since previous strategies were primarily aimed at proteins and protein complexes, the polyalanine method was a significant step forward for TW IM peptide analysis. In the current investigation, we extend the previous study by evaluating the potential utility of the polyalanine calibration strategy for measuring CCS from large-scale proteomic datasets.

Peptide CCS databases have been previously used to calculate the size contributions and volumes of specific amino acids [29–32], conformations of peptide-metal complexes [33, 34], and intrinsic structural preferences of peptides in the gas-phase [35–37]. Typically, biologically relevant structures are thought of as the native-like solution structures, but the gas-phase offers a glimpse at the innate, solvent-independent intramolecular interactions that are potentially present in anhydrous environments, such as membranes [38]. Peptide CCS databases have commonly come from the proteolytic digestion of protein mixtures, and to the best of our knowledge, all but one peptide CCS database have come from static-field home-built IM instruments. The 2011 data by Valentine et al. was acquired on a SYNAPT G2, though reduced mobility was recorded instead of CCS [32]. Here, we evaluate the TW IM polyalanine calibration method applied to peptides identified by LC-HDMS^E on a SYNAPT G2 mass spectrometer [39–41]. HDMS^E has the ability to identify thousands of peptides in a single experiment. In order to increase the throughput and speed of large-scale CCS peptide database construction, we created custom software called *pepCCS* to measure CCS values from proteomic database search output.

In an optimal high-throughput method, *pepCCS* would be the only additional step outside of the HDMS^E workflow. Polyalanine could simultaneously be used for lock spray correction and concurrent CCS calibration. However, instrument parameters and transmission voltages used for sensitive HDMS^E analysis can be significantly different from what is traditionally employed for CCS measurement, and calibration accuracy has never

been validated under such settings. Therefore, the validity, accuracy, and reproducibility of polyalanine calibrants for LC-HDMS^E were evaluated. Additionally, the possible complications of using on-line LC when measuring CCS are assessed, given the very strong evidence that ions can retain some structural elements from their electrospray solutions [42]. Finally, the utility of large-scale HDMS^E CCS profiling is demonstrated by comparing the CCS of C-terminal lysine and N-terminal lysine peptides derived from enzymatic digestion of yeast and human cell lysates.

Experimental

Chemicals and biological samples

Trypsin, endoprotease Lysine-C (**LysC**), whole cell yeast protein extract from *Saccharomyces cerevisiae*, whole cell protein extract from human K562 cells, and DL-dithiothreitol (**DTT**) were obtained from Promega in Madison, WI. MS-grade Pierce LysN protease (**LysN**) was obtained from Thermo Fisher in Rockford, IL. Bovine serum albumin (**BSA**), bradykinin, poly-DL-alanine, iodoacetamide (**IAA**), and trifluoroacetic acid (**TFA**) were purchased from Sigma Aldrich in St. Louis, MO. Optima grade water, acetonitrile (**ACN**), formic acid (**FA**), and acetic acid (**HAc**) were purchased from Fisher Scientific in Pittsburgh, PA.

Digestion of proteins and cellular extracts

Approximately 500 µg of protein was used for each digestion. The samples were denatured in 8M urea and 50 mM tris•HCl (pH ~8), then subsequently reduced and alkylated. DTT was added to 5 mM and incubated at room temperature for 60 minutes, followed by IAA addition to 15 mM and room temperature incubation for 30 minutes. Alkylation was quenched by a second aliquot of DTT and the samples were diluted with 50 mM tris•HCl. For trypsin and LysN digestions, urea was diluted to less than 1 M. For LysC digestions, urea was diluted to 4 M. Enzyme was added to create specific enzyme:protein ratio (w/w) for trypsin (1:50), LysC (1:50), and LysN (1:75). Trypsin and LysC digests were incubated at room temperature for 17 hours. LysN digests were incubated at 37 °C for 4 hours. All digests were quenched by adding TFA to lower the sample pH to 1 and then stored at -80 °C. Reversed-phase (**RP**) solid-phase extraction (**SPE**) was performed by Sep-Pak cartridges (Waters, Milford, MA) according to the manufacturer's protocol.

Off-line strong cation exchange fractionation

Yeast proteins digested by LysC and LysN were fractionated by strong cation exchange (**SCX**) on an Alliance HPLC (e2695 separation module, 2489 UV/visible detector, Waters, Milford, MA). Samples were reconstituted in Solvent A (20% ACN, 10 mM KH₂PO₃, pH 2.6) and loaded onto a polysulfoethyl A column (200 × 2.1 mm, 5 µm, 300 Å, PolyLC Inc., Columbia, MD) by 100% Solvent A for 3 minutes. Separation occurred by ramping Solvent B (20% ACN, 10 mM KH₂PO₃, 500 mM KCl, pH 2.6) at 0.2 mL min⁻¹ to 48% over 36 minutes, then to 100% B over the next 12 minutes. Fractions were collected every three minutes and then combined to make a total of 8 fractions per digest. SCX salts were removed by Sep-Pak SPE cartridges.

CCS calibration and measurement

The CCS calibration strategy is similar to that described by Bush et al. [28], but we used a t_D /CCS power-relationship instead of a quadratic relationship [17]. A detailed explanation of our calculations and our *pepCCS* software can be found in the next section and in Supplemental 1.

Polyalanine was suspended in 50% ACN, 1% HAc at 0.1 mg mL⁻¹ and acquired under five different TW IM wave velocity (**WV**)/wave height (**WH**) ratios (m s⁻¹ V⁻¹): 500/30, 500/35, 600/35, 700/35, and 800/40. WH refers to the magnitude of the sequential voltage pulses that make up the travelling wave, and WV refers to the propagation speed of those pulses. Instrument parameters used for soft transmission, hereafter referred to as “low voltage”, had the spray voltage (nano ESI capillary voltage), cone voltage, extraction cone voltage (orthogonal skimmer cone), trap bias (energy for injection into helium cell), and helium cell DC (energy for injection into IM cell) set to 1.2 – 2.0 kV, 20 V, 4 V, 35 V, and 15 V, respectively. Instrument parameters used for HDMS^E analysis, hereafter referred to as “high voltage”, had complementary settings of 3.0 kV, 30 V, 2 V, 48 V, and 35 V, respectively. Direct infusion bradykinin and BSA CCS measurements were acquired with a method and tune page identical to the calibration. The listed $\pm$ variations represent a single standard deviation of the measurement. Absolute errors must include the average calibration error (approx. 0.5%) and errors associated with the calibrants themselves (approx. 3%) [28].

Direct infusion MS

MS was performed with the Nanolockspray source of the SYNAPT G2 HDMS. The average pressures (mbar) of the instrument backing, source, trap, helium cell, and IMS were 2.73, 1.22×10^{-3} , 2.66×10^{-2} , 1.46×10^3 , and 3.67, respectively. For the bradykinin acquisitions, analyte was dissolved in 100% water at concentrations of 0.9 μ M to 9 μ M. The ion of interest was isolated by the quadrupole and the maximum m/z in the mass range was decreased to minimize the number of milliseconds per IM bin. The CCS distributions were converted from extracted t_D distributions with a window of 0.001 Th. For BSA experiments, the digest was concentrated to 1 μ M in 50% ACN, 1% HAc. All peptides were analyzed simultaneously with an m/z range held at 100 to 1800. The CCS distributions were converted from extracted t_D distributions with a window of 0.001 Th.

CCS calculation by *pepCCS*

The *pepCCS* software was created to calculate peptide CCS values from SYNAPT G2 measurements. As of publication, it is not compatible with enhanced duty cycle (**EDC**) measurements. The process begins with a comma separated values (**csv**) list of user-defined CCS calibrants (polyalanine). Once the calibrant IM-MS spectra are acquired, the file was opened in DriftScope for peak detection. A lockspray-corrected, non-chromatographic peak list csv was then exported. The *pepCCS* software then parsed the peak list for the m/z of the calibrants and extracted their corrected and centroided t_D . After creating a best-fit linear regression for CCS versus reduced t_D (for details on calculation, see Supplemental 1), the equation is used to recalculate the calibrants' CCS from t_D , and the calculated CCS is compared with the known CCS to determine the mean calibration error (%) and RMSE ($\text{\AA}^2$).

After calibration, measuring unknown CCS can be done in two ways. In direct infusion mode, the user creates a list of peptide ion sequences and charge states for which to calculate CCS. DriftScope peak lists are exported and searched by *pepCCS* in an analogous way to the calibration peak list. In HDMS^E mode, *pepCCS* utilized the Final Peptide csv created by PLGS. Our software extracts mass, charge, and t_D to calculate the CCS of every identified peptide.

LC-HDMS^E acquisition

Online RP-LC was performed on a nanoAcquity UPLC (Waters). Columns with integrated emitters were pulled from fused silica tubing (360 μm O.D., 75 μm I.D.) with a laser puller (P-2000, Sutter Instrument Company, Novato, CA). Emitter tips were etched with hydrofluoric acid for 2.25 minutes, cut to 12 cm, and then packed with 1.7 μm , 150 Å, BEH C18 material obtained from a Waters UPLC column (part no. 186004661). Approximately 1.5 μg of peptides dissolved in Solvent A (water, 0.1% FA) was loaded on-column without trapping. Solvent B (ACN, 0.1% FA) was ramped from 0% to 7% over 2 minutes, and then to 30% during the following 118 minutes. The flow rate was set to 0.4 $\mu\text{L min}^{-1}$. WV was set to 800 m s^{-1} and WH was set to 40 V for all HDMS^E acquisitions. Collision energy in the transfer was ramped from 28 V to 48 V in the high energy scan. Tryptic BSA peptides were acquired in triplicate. Tryptic HK digests, fractions of yeast LysC digests, and fractions of yeast LysN digests were acquired in duplicate. A CCS calibration was acquired before each set of analyte acquisitions.

PLGS search parameters and CCS list curation

HDMS^E raw files were processed and searched on PLGS version 2.5.1. Low energy, elevated energy, and intensity thresholds were set to 100, 20, and 1000 counts, respectively. SwissProt reference protein databases for *Saccharomyces cerevisiae*, *Bos taurus*, and *Homo sapiens* were downloaded from UniProt (www.uniprot.org) and used for searching. Precursor and fragment mass tolerance was set to automatic to obtain a protein false discovery rate (FDR) of 1%. Minimum number of ion fragments per peptide was set to 3, minimum number of ion fragments per protein was set to 7, and minimum number of peptides per protein was set to 1. Carbamidomethyl cysteine and oxidized methionine were selected as fixed and variable modifications, respectively. The maximum number of miscleavages was set to 2. The mean apparent peptide FDR was $(0.6 \pm 0.1)\%$.

To ensure we only report highly confident IDs and CCS values from K562 and yeast samples, peptides had to meet specific curation criteria. Only first-pass and miscleavage identifications found in both duplicates were used for CCS analysis. The list was then purged of all identifications with less than 95% confidence (“yellow” and “red” peptides). Finally, any peptide whose CCS varied 1% or more between analyses was also removed.

Results and Discussion

Accuracy of CCS calibration under high transmission voltages: bradykinin

Our low voltage parameters result in significantly less HDMS^E identifications relative to high voltage settings. In Figure S1, two chromatograms are shown for the LC-HDMS^E

analysis of tryptic yeast protein extract. The data in Figure S1A was acquired under low voltage settings and resulted in only 1578 total peptide IDs, whereas data from Figure S1B was acquired with high voltage settings and resulted in 6061 total peptide IDs. We hypothesize that this is primarily due to inefficient transport in the ion optics when voltages are lower.

Inefficient transfer seems to affect ions across the entire m/z range, but it may be more significant for larger ions of lower charge. In Figure 1A, direct infusion with low transmission voltages produces polyalanine spectra that drop off near m/z 900. In contrast, high transmission voltages show ions above m/z 1200 and notably more singly and doubly charged ions. Although the base-peak intensities are similar for each spectrum, the soft transmission acquisition required nearly twice as much time. This suggests that greater loss could occur as ions increase in mass and decrease in charge. In Figure 1B, the intensities of doubly charged A11 – A24 calibrants are shown, each normalized to the most intense doubly charged calibrant in their respective high or low voltage spectrum. Although the high voltage settings yield overall intensities much higher than low voltage, the high voltage settings also have a slight bias against ions of low m/z .

On the SYNAPT G2, transmission voltages and gas flow rates can cause the collisional activation and unfolding of proteins prior to IM analysis [23, 43]. LC-HDMS^E CCS profiling only seeks information on gas-phase structural preferences, and therefore it is more important to have sensitive transmission over soft transmission to retain structural elements from solution. However, if high transmission voltages are sufficient to change the conformation of the polyalanine ions, then the drift tube calibration values may not be valid and could introduce large errors to future measurements. The measurement of peptide CCS must not cause a significant drop in accuracy under our high voltage settings.

Validation of polyalanine calibration under high transmission voltages was first tested by measuring the CCS of bradykinin (**BK**), a neuropeptide that has been well studied by drift tube IM-MS. Pierson et al. have characterized two peaks for the gas-phase distribution of BK^{2+} , with the primary peak having a CCS of $246 \text{ \AA}^2$ [44]. A CCS for the secondary peak was not given, but it appears to be similar to the BK^{2+} CCS published by Bush et al., $237 \text{ \AA}^2$ [18]. For BK^{3+} , Pierson et al. have established three gas-phase quasi-equilibrium (**QE**) structures at $269 \text{ \AA}^2$, $285 \text{ \AA}^2$, and $305 \text{ \AA}^2$ [44], and three similar structures were also observed by Kemper et al. [45]. These values will serve as a reference to our calibrated TW IM CCS values. The acceptable error will be 1.8%, the average discrepancy between TW IM values and drift tube values obtained by Bush et al. for tryptic peptides [28].

CCS distributions for BK^{2+} and BK^{3+} acquired with a 500 m s^{-1} wave velocity and 30 V wave height are shown in Figure 2, and Table S1 lists the measured CCS at all WV and WV used. Overall, the profiles from low and high voltages are very similar. Slight differences in the secondary peaks may indicate some activation from higher voltages, though nothing much more significant than what was already present under low voltages. However, the mean calibration error under high voltage was $(0.44 \pm 0.03) \%$ while the error for low voltage settings was $(0.34 \pm 0.03) \%$. This implies that larger errors should be expected with high transmission voltages.

The mean CCS of the primary and secondary BK^{2+} peaks were $(247.4 \pm 0.8) \text{ \AA}^2$ and $(238.5 \pm 0.6) \text{ \AA}^2$, respectively, under low voltage settings. This corresponds to an error of 0.7% for the primary peak and 0.6% for the secondary peak. The high voltage settings yielded a primary peak at $(249.8 \pm 0.3) \text{ \AA}^2$ and a secondary peak at $(240.6 \pm 0.6) \text{ \AA}^2$, placing the errors of the mean at 1.6% and 1.5%. This is a noticeable increase from low voltage settings, but still below 1.8%.

Proteomic settings also produce higher errors for BK^{3+} , but to a lesser magnitude. The distributions in Figure 2 represent the best resolution we achieved of the multiple peaks present. The mean CCS of the primary peak measured at low voltage was a near perfect match to Pierson et al.'s structure C at $(305 \pm 1) \text{ \AA}^2$. The primary peak from the high voltage settings was $(307 \pm 1) \text{ \AA}^2$, an error of only 0.7%. The respective mean CCS measured under high and low voltage settings for a second peak was $(290 \pm 2) \text{ \AA}^2$ and $(293 \pm 2) \text{ \AA}^2$. We hypothesize that there are several features with similar CCS in the second peak, and that the overlap leads to poor resolution. In Figure 2, the partially resolved peak in this region was measured at $287 \text{ \AA}^2$, very similar to Pierson et al.'s structure B [44]. QE structure A was not observed at appreciable signal-to-noise ratio in any of our acquisitions. It should be noted that Pierson et al. only observed this structure at approximately 2.3% relative abundance [44].

The BK experiments suggest that high transmission voltages will introduce larger error relative to low voltages, but that the difference is not significant. The only errors larger than 1.8% belonged to features that could not be reproducibly resolved. Perhaps the most interesting aspect of this data is the fact that the BK^{3+} distributions resemble the QE structures even though they were electrosprayed from water. In another study by Pierson et al., structures A, B, and C initially had approximate relative abundances of 39%, 70%, and 100%, respectively, when electrosprayed from 100% water [46]. They had to be deliberately activated to reach their QE abundances. Therefore, Figure 2 suggests that BK^{3+} may undergo substantial ion heating even at low voltage settings and lose evidence of its solution preferences. Using EDC, we were able to acquire a BK^{3+} t_D distribution at even lower transmission voltages (Figure S2). The abundance of structure B seems slightly increased relative to structure C, though structure A is still not observed.

The SYNAPT G2's low pressures prior to IM separation have been shown to lead to higher E/N values than typically seen with static-field drift tubes [25, 43]. In this investigation, SYNAPT G2 pressures dropped as low as 0.77 mTorr in the source region. In contrast, pressures on home-built instruments are commonly maintained near 3 Torr [44] and as high as 10 – 12 Torr [45].

Accuracy of CCS calibration under high transmission voltages: tryptic peptides

The BK data represents the behavior of only one peptide. To further validate the accuracy of high voltage polyaniline calibration, we performed a tryptic digest on bovine serum albumin (BSA) with carbamidomethylated cysteines. The same peptide ions used by Bush et al. in their evaluation of polyaniline calibrants were used here [28]. It will be helpful to compare our high voltage TW IM CCS values with their published TW IM values, as it is most

important that high transmission voltages not introduce significant error beyond what is already introduced by TW IM.

Table 1 lists all the BSA peptides we were able to identify during direct infusion via accurate mass matching. Many of the CCS pairs are indistinguishable within a single standard deviation. Two peptides, GACLLPK²⁺ and KVPQVSTPTLVEVSR³⁺, displayed anomalously high errors and standard deviations. From their CCS distributions in Figure 3, it is clear that they favor multiple conformations in the gas-phase, possibly due to the presence of cis/trans proline isomerization [47]. Bush et al. mention that they also observed several BSA peptides with multiple IM features [28]. The average CCS of GACLLPK's two peaks is approximately 207 Å², which is the published TW IM value [28]. As for KVPQVSTPTLVEVSR, we hypothesize that the poor resolution of the two more compact peaks leads to a greater variation in the centroid arrival time picked by DriftScope, producing the observed standard deviation.

When GACLLPK²⁺ and KVPQVSTPTLVEVSR³⁺ are omitted, the average difference between published TW IM values and our TW IM values are approximately 0.4% under both low and high voltage settings. When compared to CCS obtained on the modified drift tube SYNAPT G1, the average errors are approximately 1.4% and 1.7% for proteomic voltages and soft voltages, respectively.

The combined results of the BK and BSA data lead to the confident assertion that peptide CCS values can be calibrated and measured with higher transmission voltages. The proteomic settings will greatly increase the sensitivity of analysis without introducing significant error, despite evidence of minor calibrant and analyte activation under high voltages. We cannot completely discount the possibility that activation is greater than we suspect, but error in calculated CCS is reduced due to analyte activation to a similar extent. This would be a less desirable situation, as the CCS values used for calibration would no longer be accurate. Ions that are not similarly activated would contain large errors in their calculated CCS values, whereas the similarly activated ions would contain undetectable inaccuracies.

Potential complications of using on-line LC during CCS measurement

Structural elements from solution have previously been observed to persist in the IM distributions of small proteins and peptides [46, 48]. Our method relies on LC-HDMS^E to aid in measuring CCS values of peptides in the gas-phase from complex samples, and it is likely that structures from LC gradient solutions and non-native structures mediated by the column's stationary phase would be of little interest, if not an outright interference. Their presence could affect the run-to-run or gradient-to-gradient reproducibility of CCS measurement. However, we have evidence that the high voltage settings may lead to peptides losing their "native" structures from solution and adopting gas-phase preferences, even without deliberate collisional activation.

In Figure 4, the mass spectra of BK³⁺ and BK²⁺ after quadrupole isolation with an approximate *m/z* window of 3 Th is shown. The trap collision energy was set to the minimum value required for transmission. Activation is immediately apparent for the BK³⁺

acquired under voltage settings by the highly abundant sequence-specific fragments. The BK^{2+} spectrum contains a few very low abundance fragments. Lowering transmission settings to soft voltages did not completely stop BK^{3+} fragmentation, but did greatly decrease it. BK^{2+} spectra did not contain any detectable fragments. With this amount of ion activation, it is not surprising that the BK CCS distributions from Figure 2 resembled the QE gas-phase distribution instead of the distribution Pierson et al. observed when electrosprayed from 100% water [46].

We hypothesized that most peptide ions will behave similarly to BK in this respect. It is difficult to directly test this without multiple mobility cells, so we instead opted to measure the CCS of BSA peptides identified by LC-HDMS^E and compare them to the direct infusion results. All BSA direct infusion CCS values were electrosprayed in 50% ACN, 1% HAc, while peptides from LC-HDMS^E are sprayed in solutions ranging from 5% to 30% ACN, 0.1% FA.

The results are summarized in Table S2. Of the 11 direct infusion peptides also identified by HDMS^E, the average deviation from the mean direct infusion CCS value was 0.9%, with no deviation being larger than 1.4%. HDMS^E acquisitions were only performed at 800 m s⁻¹ WV and 40 V WH, values that were optimized for number of identifications. Using a single wave velocity/height ratio may be responsible for observed error with such a small standard deviation. However, an average discrepancy of 0.9% is of low significance. We cannot discount the possibility of solution structure elements appearing in HDMS^E CCS of other peptides, but they will likely not be a common occurrence under high transmission voltages.

Large-scale CCS profiling

Following the validation of accurate CCS measurement with high transmission voltages, we aimed to demonstrate the utility of bulk structural comparisons in a large-scale LC-HDMS^E CCS profiling experiment. Proteolytic digests of total protein from cell lysates can produce tens of thousands of peptides with a common N- or C-terminal residue. Digest peptides from protein mixtures have been used in many previous CCS databases, but to our best knowledge the mixture has never been as large or as complex as a total protein extract.

In the initial experiment, extract from human K562 cells was digested by trypsin, an enzyme that cleaves proteins into peptides with a C-terminal lysine or arginine residue. After duplicate LC-HDMS^E acquisitions, each dataset was searched on PLGS and the Final Peptide csv was submitted to pepCCSca to measure the CCS of identified sequences. To our knowledge, there is no other software that can couple CCS measurement to protein/peptide database search output. Figure 5 shows a plot of CCS versus m/z for 2814 doubly protonated peptides and 1172 triply protonated peptides, all of which passed the strict curation criteria listed in the **Experimental** section. Initially, 5511 2+ and 2954 3+ peptides were identified. The majority of deletions from curation was from our insistence only using first-pass and miscleavage identifications, and necessitating they be identified in both runs. The 2+ family exhibits a single, linear, relatively narrow distribution, which is in strong agreement with several previous studies of doubly charged tryptic peptides [29, 30, 39, 49]. The 3+ family has been less characterized in literature, but is known to possess a wider range of CCS values than the 2+ family [49]. Our data is in agreement, but we note an

interesting phenomenon that occurs as the 3+ family m/z increases. At roughly m/z 650, the 3+ family begins to cluster into two distinct sub-families, one compact and one highly extended. In contrast, the 2+ family appears to display a narrow band in its CCS distribution at the same m/z range. Counterman and Clemmer have previously observed two sub-families of triply protonated polyanines, the compact sub-family attributed to hinged helix coils (HHC) and the other sub-family attributed to extended helices (EC) [50, 51].

For the next experiment, we aimed to determine if the N- and C-terminal amino acids could affect the clustering of ions into a particular sub-family. Total protein extracts from yeast (*Saccharomyces cerevisiae*) were subjected to Lys-N or Lys-C digestion, which created peptides with N-terminal or C-terminal lysines, respectively. Since tryptic digests often produce the most peptide and protein IDs in shotgun proteomics [52], we performed off-line SCX-HPLC to separate peptides into eight fractions and performed two HDMS^E analyses on each. Although this method greatly increased our analysis time, it also increased our chances of obtaining a number of CCS measurements comparable to the trypsin experiments.

The CCS versus m/z plot in Figure 6A contains 1463 doubly charged and 1007 triply charged LysN peptides. Figure 6B contains 1295 doubly charged and 1072 triply charged LysC peptides. Interestingly, both sets resulted in the same two sub-families for triply charged ions, but the LysN peptides displayed a stronger preference for the compact sub-family. As expected, the LysC sub-family split was very similar to that seen from tryptic peptides. We would like to note that miscleavages are included in these plots to achieve a higher sample population. We initially thought internal lysines may be a significant factor in sub-family clustering, but the miscleavages seemed to make no difference to the overall distribution. Complementary plots for Figure 5, Figure 6A, and Figure 6B that omit miscleavages can be found in Figure S3.

The visual differences between Figure 6A and 6B are obvious, but deeper search of the data provided quantitative evidence of the compact preferences for peptides with an N-terminal lysine. Fourteen internal peptide sequences are listed in Figure 6C, each belonging to the same protein and found in both LysN and LysC curated peptide lists. In the protein, these sequences contain a lysine at each end, leading to peptides that only differ by terminal lysine position when cleaved. In all but two pairs, the LysN partner had a significantly smaller CCS. Finally, Figure 6D shows that triply charged LysC peptides are consistently larger than LysN at all m/z .

Lacking MD simulations to properly interpret the CCS data, we can only make conjectures as to why LysN peptides tend to populate the compact sub-family. The result would appear to fall in line with earlier IM-MS studies by Hudgins et al. on the gas-phase helix preferences of protonated polypeptides [53, 54]. They showed that a positive charge located at the C-terminus will interact favorably with a helix macrodipole and stabilize extended helical ion structures. Conversely, a positive charge at the N-terminus will destabilize a helix and could shift preference to a globular structure with intramolecular solvation of the charge. Tao et al. showed that a helical region of singly charged tryptic peptide would adopt a turn motif when HK was added to the beginning of the sequence [35]. It is possible that the charge-macro-dipole interaction is also significant for triply charged ions. N-terminal lysines

would certainly increase the likelihood of a charge being located at the N-terminus. However, the widely varied internal sequences from our proteomic samples introduce a much more complicated landscape than what was previously characterized by Hudgins et al.

The triply protonated polyaniline studies by Counterman and Clemmer show that transitions between HHC and EC structures are temperature-dependent, with EC being favored as temperature increases [51]. Higher temperatures may facilitate proton transfers to shift the net charge from N-terminus to C-terminus, resulting in EC formation. All of our peptide ions were formed at the same source temperature of 70 C, and so the presence of compact and extended structures may point to the role of proton sequestration. For example, Figure 6C's RAILERNAAYQ internal sequence may strongly sequester the charge at the N-terminus when an N-terminal lysine is followed by arginine, even at higher temperatures. When the lysine is at the C-terminus, the third proton may prefer that position over an N-terminal amine to reduce coulombic repulsion, leading to the large difference between the LysN and LysC CCS. In contrast, the internal sequence LSGVTLSSELLR might strongly sequester a proton with its arginine and keep a positive charge at the C-terminus regardless of lysine position.

Conclusion

This investigation has built upon the polyaniline calibration method detailed by Bush et al. and applied it to peptide CCS database creation from large, complex populations. After several levels of validation, we have found that high transmission voltages and electrospray from LC gradients do not introduce significant error to polyaniline calibration and CCS measurement. We also showed the utility of a large-scale CCS profiling study, the results of which are currently under investigation with MD simulations.

As in any method, there are drawbacks to its use. Some are unavoidable and some could potentially be mitigated. First, TW IM calibration unquestionably introduces error to CCS measurements, even under the very best calibration schemes. We showed that high transmission voltages cause insignificant error for the peptides in this study, but we cannot assert this will be true for all peptides. Some peptides may have distinct theoretical conformations whose difference in CCS is only slightly larger than the absolute error under low voltage settings. The usually insignificant additional error from high voltage settings could then render the two theoretical CCS experimentally indistinguishable. Second, all peptide CCS lists have an associated identification FDR from database searching, meaning there are necessarily incorrect entries in the database. CCS calculation may have its own FDR that is independent of the PLGS identification FDR, though we assume this would be low given our curation criteria and the very small run-to-run CCS deviations. The creation and validation of a CCS FDR may be very helpful for future large-scale studies. Finally, HDMS^E only outputs a single drift time for identified ions, making it impossible to identify multiple IM structures. An alternative approach that extracts the complete IM drift profile at the apex LC retention times of identified peptides could mitigate this issue.

As we stated previously, our original motivation for calibration under high transmission voltages was to perform high-throughput concurrent HDMS^E analysis and CCS calibration

with the lockspray. In the current protocol, calibration at various WV/WH ratios must be performed separately. If polyalanine was instead used as a lockspray, CCS calibration could be performed simultaneously with LC-HDMS^E. The SYNAPT G2 does not record mobility data for the lockspray ions, and so we are currently working on acquisition modifications to facilitate this. Future work will also include building large-scale CCS databases for endogenous signaling peptides and searching for bulk structural patterns intrinsic to peptide families, followed by correlating the structural information to their binding behavior and physiological function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Polyalanine CCS Standards

Figure 1.

(A) Mass spectra of DL-polyalanine were directly infused under high and low transmission voltages. (B) This bar graph displays the relative intensities of doubly charged A₁₁ – A₂₄ CCS calibrants. The intensities are normalized to the most abundant doubly charged calibrant in the high and low voltage mass spectrum, respectively.

Figure 2.

The CCS distributions of doubly and triply protonated bradykinin were measured under high and low transmission voltages. The wave velocity and wave height used for these particular acquisitions was 500 m s^{-1} and 30 V, respectively.

Figure 3. The CCS distributions under high and low transmission voltages for (A) GACLLPK²⁺ and (B) KVPQVSTPTLVEVSR³⁺ reproducibly show multiple features.

Figure 4.

The quadrupole-isolated mass spectra under high transmission voltages for (A) BK^{3+} and (B) BK^{2+} show various levels of fragmentation. The low transmission voltage spectra for (C) BK^{3+} and (D) BK^{2+} show less fragmentation.

Figure 5.
The doubly and triply charged tryptic peptides from LC-HDMS^E analysis of K562 total protein extract are plotted CCS versus m/z .

Figure 6.

The CCS of (A) LysN and (B) LysC yeast protein digests are plotted against m/z . (C) Fourteen pairs of peptides with identical internal sequences were reproducibly identified in both LysN and LysC analyses, and their CCS values were compared. (D) Identified peptides were grouped into 100 Th bins and the mean CCS of each bin from LysC peptide was compared to the mean CCS from each bin of LysN peptides.

Table 1

CCS of tryptic BSA peptides under high and low transmission voltages

Sequence	z	Lit. TW CCS (Å ²)	High Voltage CCS (Å ²)	Error (%)	Low Voltage CCS (Å ²)	Error (%)
GACLLPK*	2	207	203.1 ± 0.8	1.88	203.0 ± 0.6	1.93
GACLLPK*	2	207	212.3 ± 0.7	2.56	211.8 ± 0.9	2.32
LVTDLTK	2	205	206.9 ± 0.5	0.93	206.5 ± 0.7	0.73
ATEEQLK	2	209	209.9 ± 0.5	0.43	209.6 ± 0.6	0.29
LCVLHEK	2	228	227 ± 1	0.44	227.6 ± 0.6	0.18
AEFVEVTK	2	219	220.2 ± 0.4	0.55	219.8 ± 0.8	0.37
YLYEJAR	2	237	238.3 ± 0.5	0.55	237.8 ± 0.7	0.34
LVVSTQTALA	2	234	235.8 ± 0.7	0.77	235.6 ± 0.3	0.68
QTALVELLK	2	245	245.3 ± 0.6	0.12	245.0 ± 0.7	0.00
LVNELTEFAK	2	262	262.2 ± 0.6	0.08	261.8 ± 0.2	0.08
HLVDEPQNLIK	2	288	286.7 ± 0.6	0.45	286.8 ± 0.3	0.42
TVMENFVAFVDK	2	297	295 ± 1	0.67	295.3 ± 0.5	0.57
SLHTLFGDELCK	2	297	296.0 ± 0.7	0.34	296.0 ± 0.2	0.34
YICDNQDTISSK	2	298	295.8 ± 0.9	0.74	296.2 ± 0.4	0.60
LGEYGFQNALIVR	2	307	306.1 ± 0.6	0.29	305.8 ± 0.4	0.39
DDPHACYSTVFDDK	2	313	312.2 ± 0.5	0.26	311.7 ± 0.6	0.42
MCPTEDYLSILNR	2	338	337.8 ± 0.6	0.06	336.9 ± 0.7	0.34
YNGVFQECQAEDK	2	325	324.0 ± 0.4	0.31	323.5 ± 0.6	0.46
HLVDEPQNLIK	3	319	321.2 ± 0.5	0.69	321 ± 1	0.50
SLHTLFGDELCK	3	334	334.3 ± 0.6	0.09	333.7 ± 0.8	0.09
TCVADESHAGCEK	3	340	342 ± 1	0.59	341 ± 1	0.29
DDPHACYSTVFDDK	3	323	324.8 ± 0.6	0.56	324 ± 1	0.31
KVPQVSTPTLVEVSR*	3	338	341 ± 3	0.86	339 ± 4	0.30
DAIPENLPPLTADFAEDKDVCK	3	422	422 ± 2	0.00	421 ± 1	0.24
Mean[‡]				0.42		0.36

[‡]Peptides marked with asterisk were omitted from calculation.