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Development of a high-throughput IMS-IMS-MS approach for analyzing mixtures of biomolecules

Ruwan T. Kurulugama¹, Stephen J. Valentine², Rena A. Sowell¹, and David E. Clemmer^{1,*}

¹Department of Chemistry, Indiana University, Bloomington, IN 47405

²Predictive Physiology and Medicine, 409 W. Patterson Dr., Bloomington, IN 47403

Abstract

A high-throughput approach for biomolecule analysis is demonstrated for a mixture of peptides from tryptic digest of four proteins as well as a tryptic digests of human plasma. In this method a chip-based electrospray autosampler coupled to a hybrid ion mobility (IMS) mass spectrometer (MS) is utilized to achieve rapid sample analysis. This high-throughput measurement is realized by exploiting the direct infusion capability of the chip based electrospray with its rapid sample manipulating capability as well as a high sensitive IMS-MS with a recently developed IMS-IMS separation technique that can be multiplexed to provide greater throughput. From replicate IMS-MS runs of known mixtures, the average uncertainty of peak intensities is determined to be $\pm 7\%$ (relative standard deviation), and a detection limit in the low attomole range is established. The method is illustrated by analyzing 124 human plasma protein samples in duplicate, a measurement that required 16.5 hours. Current limitations as well as implications of the high-throughput approach for complex biological sample analysis are discussed.

Keywords

Ion-mobility spectrometry (IMS); mass spectrometry (MS); Two-dimensional IMS; High-throughput proteome analysis; chip-based electrospray

Introduction

Rapid sample analysis is central to many areas, including the emerging field of proteomics.^{1–5} This is especially true for molecular profiling experiments where many measurements are required to ascertain normal abundance variabilities within sample cohorts. Mass spectrometry (MS) based platforms have attracted considerable attention because of the intrinsic speed and sensitivity of these methods. The current preferred approach for analyzing complex biological protein samples consists of multiple steps of sample separation methods prior to MS analysis. Although these separations provide detailed coverage for complex samples, they are still relatively time consuming.⁶ For example, in a recent study we have examined 5 human plasma samples in triplicate using two-dimensional (2D) chromatography coupled with ion mobility

*To whom the correspondence should be addressed. E-mail: clemmer@indiana.edu.

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In the interest of full disclosure, one of the authors from Indiana University (DEC) is also a co-founder of Predictive Physiology and Medicine.

spectrometry (IMS) and MS analysis; this experiment required more than 500 hours of instrument analysis time.⁷

An alternative to multidimensional separation methods is to sacrifice protein coverage in order to analyze many samples rapidly. One approach for doing this is to eliminate the time-intensive chromatography steps and analyze the sample by direct infusion into the mass spectrometer.⁸ The characterization of complex biological samples by direct infusion electrospray ionization (ESI)-MS is limited due to considerable chemical noise as well as the phenomenon of ionization suppression.^{9–12} Although mass spectrometry is a highly sensitive approach, an inherent limitation is the relatively small dynamic range that can be achieved with current technology which is often much smaller than the analyte concentration range in proteomics studies.¹³ The problems of chemical noise and ion suppression which are associated with the simultaneous analysis of all mixture components exacerbate this technological weakness.

Besides the use of time-consuming separation steps, several MS instrumentation techniques have been developed to mitigate the effects of chemical noise. Such methods include modes of operation in which high-abundance ions are discriminated against based on mass-to-charge (m/z) selection. Methods include the dynamic range enhancement applied to mass spectrometry (DREAMS) approach which is used with Fourier Transform MS and multiple reaction monitoring (MRM) used extensively in tandem MS instrumentation.^{14–19} We have demonstrated a different approach that combines a gas-phase separation, ion mobility spectrometry (IMS),^{20–25} with time-of-flight (TOF) MS.^{26, 27} Here, the initial separation of ions according to their mobilities reduces spectral congestion associated with chemical noise.²⁸ Thus incorporation of an IMS separation step prior to MS analysis provides a means of increasing coverage and sensitivity while maintaining high analysis speeds.^{29–31} This is a distinguishing feature of the IMS-MS approach; the former methods are limited because they involve an ion selection process.

Recently IMS-MS techniques have been extended to two IMS dimensions that take advantage of the ability to alter the structures (and thus the mobilities) of ions inside sequential drift tubes.^{32–35} The 2D approach has exhibited relatively large peak capacities and has recently been demonstrated in a multiplexing mode (described below) improving the overall experimental duty cycle and diminishing the effects of mobility selection of ions. Here we describe the coupling of an ESI chip-based autosampler device with the IMS-IMS-MS instrumentation. Figures of merit for the analysis of tryptic digest mixtures are reported in terms of sensitivity, detection limits, reproducibility, and protein/peptide coverage. Additionally, an example application of high-throughput molecular profiling is presented. Finally, the limitations of the technique are discussed and future directions for development and application are proposed.³⁶

Experimental

General

The instrumentation used in the current studies involves the coupling of a NanoMate (Advion Biosystems, Ithaca, NY) autosampler with an IMSMS instrument as shown in Figure 1. The details of the IMS-MS instrumentation have been published elsewhere^{9, 28, 37–42} as well as the descriptions of the NanoMate device and its capabilities.^{43–48} Only a brief introduction to the current instrumentation and method is presented here. Briefly, tryptic digests of protein samples were loaded onto the 96-well tray of the autosampler. The NanoMate was positioned at the front of the IMS-MS instrument such that the electrospray nozzle was aligned with the ESI source orifice of the instrument. Sample was drawn from a single well and electrosprayed using the 400 nozzle nanospray chip. Electrosprayed ions were accumulated at the back of the ion funnel in the source chamber and were subsequently pulsed into the drift tube for mobility

separation. The ions inside the drift tube separate according to their mobilities through an inert buffer gas under the influence of a weak electric field.^{49, 50} The mobility-separated ions were then detected using an orthogonally pulsed reflectron geometry TOF mass analyzer. The instrument was primarily operated in two modes; two-dimensional IMS-MS mode and multiplexed three-dimensional IMS-IMS-MS mode. These operational modes will be discussed in detail below.

Preparation of standard four protein digest samples. Four yeast protein samples (alcohol dehydrogenase, enolase, glutathione reductase and hexokinase) were purchased from a commercial vendor (Michrom Bioresources, Auburn, CA) as dried peptides in amounts of 500 pmoles of total protein sample. These protein samples were digested to completion resulting in 500 pmoles of each peptide. The dried samples were dissolved in 200 μL of HPLC grade (EMD Chemicals, Gibbstown, NJ) distilled water to obtain 5 $\text{pmol}\cdot\mu\text{L}^{-1}$ stock solution. Using stock solutions, a mixture of four proteins was prepared in H_2O /methanol/acetic acid (49:49:2%). The concentrations used for each protein in the solution were as follows: alcohol dehydrogenase (5 $\text{pmol}\cdot\mu\text{L}^{-1}$), enolase (0.5 $\text{pmol}\cdot\mu\text{L}^{-1}$), glutathione reductase (0.05 $\text{pmol}\cdot\mu\text{L}^{-1}$), and hexokinase (0.005 $\text{pmol}\cdot\mu\text{L}^{-1}$). The solution described above was used to obtain sequence coverage for each protein by infusing 3.75 μL of total sample volume. The detection limit study was carried out by using a sample that is 10^3 times more dilute than the four protein sample mentioned above.

Sample preparation for reproducibility study. For the reproducibility experiment, a tryptic digest of phosphoglucose isomerase (Michrom Bioresources, Auburn, CA) protein was used. The sample was purchased as 500 pmole of dried tryptic peptides. A stock solution with a concentration of 5 $\text{pmol}\cdot\mu\text{L}^{-1}$ was prepared in HPLC grade distilled water. The final sample solution was prepared in H_2O /methanol/acetic acid (49:49:2%) with a peptide concentration of 0.5 $\text{pmol}\cdot\mu\text{L}^{-1}$.

Acquisition of plasma samples

Whole blood (~4 mL) was drawn by trained health care professionals using standard venipuncture techniques as proscribed by a protocol approved by an institutional review board. Blood was collected in a tube containing the anticoagulant lithium heparin (BD vacutainer). To obtain platelet poor plasma, the blood sample was centrifuged at 4000 rpm. Subsequently, 30 μL of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added to each sample. Next the samples were centrifuged at 12000 g to obtain platelet free plasma. Then sample aliquots (1 mL) were stored in a -86°C freezer until sample workup is performed.

Depletion of abundant proteins

The Agilent High Capacity Multiple Affinity Removal System (MARS, Agilent Technologies, Inc., Palo Alto, CA) was used to remove the top six proteins (albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin). A 90 μL aliquot from each sample was diluted with 270 μL of binding buffer (A). The mixture was then filtered (0.22 μm spin filter, Agilent) to remove particulates. The entire filtrate was injected onto the MARS column. Abundant protein removal was carried out using a HPLC system (600 series pump and 2487 dual λ detector, Waters) as follows: 1) 100% buffer A (0.5 $\text{mL}\cdot\text{min}^{-1}$ for 10 min) and, 2) 100% B (1.0 $\text{mL}\cdot\text{min}^{-1}$ for 7 min). Buffers A and B are proprietary buffers (composition unknown) provided by Agilent for MARS. The second step served to elute the bound fraction containing abundant proteins. Typically, the flow-through fraction (lower abundance proteins) elutes at 1.5 to 7 min. Between runs, the column was equilibrated with buffer A (1.0 $\text{mL}\cdot\text{min}^{-1}$ for 11 min). The elution of the bound and unbound protein fractions was monitored at $\lambda=280\text{ nm}$.

Enzymatic digestion of the plasma proteins to create mixtures of peptides. Plasma proteins were digested with trypsin according to the procedure outlined below. Briefly, the *bottom-up* approach was used to allow for the generation of ions that will produce useful precursor-ion and fragment-ion datasets necessary for peptide/protein identification. The flow-through fraction (~5 mL) from the MARS column was concentrated (~200 to 300 μ L) using a 5K Da MWCO membrane concentrator (4.0 mL Vivaspın, Agilent). From previous studies that utilized a subsequent Bradford assay, it was estimated that each sample contains ~1 mg of total protein. The sample was then diluted (~2 mL) with ammonium bicarbonate buffer (200 mM, pH = 8.0) containing 6M urea. Dithiothreitol (DTT) was added using a 40:1 molar ratio (DTT:protein) and the sample was incubated at 37 °C (2 hours) to reduce disulfide bonds. The sample was then cooled to 0 °C. Next iodoacetamide (IAM) was added to the sample at a 80:1 molar ratio (IAM:protein). The reaction occurred for 2 hours in darkness. Subsequently, cysteine (40:1 molar ratio) was added to react with residual DTT and IAM. This step was carried out for 30 min at room temperature. Then ~4 mL of ammonium bicarbonate buffer was added to drop the urea concentration to ~2 M. Finally, TPCK-treated trypsin (Sigma-Aldrich) was added to the sample (1:50 wt/wt ratio) and digestion proceeded at 37 °C for 24 hours. Tryptic peptides were desalted (Oasis HLB cartridge, Waters) and subsequently dried on a centrifugal concentrator (Labconco).

IMS-MS instrumentation. The IMS-MS instrument used for these experiments was similar to an instrument described previously.^{29, 51} A brief introduction describing some modifications to the original instrument is provided here. The ESI source ion funnel employed in this instrument was modified to improve the trapping efficiency to enhance the overall sensitivity of the measurement. The funnel consisted of 100 stacked brass electrostatic lenses. Each lens was ~0.50 mm thick and the spacing between adjacent lenses was ~0.75 mm. Funnel Lenses were held together with two zero insertion force (ZIF) sockets (3M, Austin, TX) in a stacked arrangement. The inner diameter (ID) of the first funnel lens was 2.54 cm. The following lenses had progressively smaller inner diameters until reaching 0.254 cm around $\frac{3}{4}$ of the length of the funnel. The back section of the funnel gradually opened up to 1.68 cm ID. The first ninety eight lenses were connected to each other using 5 M Ω resistors (1% tolerance). The last two lenses were not connected to the resistor chain allowing the potential of these two lenses to be controlled independently; they were used as the trap and the extraction lens regions, respectively. Also, these last two lenses were not connected to the capacitor series, thereby keeping them free of rf potential. The trap lens contains a 90% transmission Ni mesh grid (Buckbee-Mears, St. Paul, MN) to facilitate ion trapping. The dc potential for this funnel was applied as two separate sections which can be adjusted independently. The front section consisted of the first 88 electrostatic lenses while the back section consisted of the last 10 lenses. The first region was typically operated at 12 V $\cdot$ cm⁻¹ dc field, whereas the back region was operated at 0.05–0.1 V $\cdot$ cm⁻¹ dc field. This very low dc field at the back of the funnel helped to reduce the velocity of the ions and thereby improved the trapping efficiency. When the ion funnel was in trapping mode, the voltage at the gate was only 15 V above the previous ring electrode (funnel last lens). This created a trapping potential where the ions were trapped closer to the center of the last funnel lens. Due to the positive voltage gradient throughout the funnel (even though small at the back of the funnel) the ions were believed to be accumulated closer to the last funnel lens. When the funnel was in the ion extraction mode, the field between the gate lens and the funnel last lens was maintained at 228 V $\cdot$ cm⁻¹. The distance between the gate lens and the funnel last lens was 0.131 cm. The region right after the gate lens (0.211 cm) called the extraction region was kept at 47 V $\cdot$ cm⁻¹ field during the ion extraction period. The higher electric fields used during extraction seem to help extract the ions during the short ion ejection time.

Also, a jet disrupter was placed 1.5 cm from the front lens of the funnel.^{52, 53} The jet disrupter reduced the air flow into the funnel and improved the flow dynamics in the funnel. This also

reduced the pumping load and helped to keep the drift tube pressure low for higher ion transmission through the drift tube via shorter transmission times (less loss to diffusion). The rf field throughout the funnel was maintained at an amplitude of $\sim 160 V_{p-p}$ using a frequency between 390–420 KHz.

IMS-MS and multiplexed IMS-IMS-MS experiments

The current generation of IMS instrumentation can be operated in a variety of modes.³¹ Briefly, it can be operated as an IMS-MS instrument where the electrosprayed ions are separated in the drift tube and detected at the back of the instrument with a mass analyzer. In this method, the 2 drift tube sections are used as a combined single drift tube. Second, this instrument can be used to mobility select certain ions at the back of the first drift tube and allow that ion packet to separate in the second drift tube. Third, the mobility selected ions can be activated by applying a relatively high field before they are introduced into the second drift tube. Under favorable conditions, the applied high field can cause structural changes to the selected ions thus separating ions whose structures are similar prior to the mobility selection and activation. This IMS-IMS technique increased the peak capacity of the drift dimension compared to the IMS-MS mode of operation. Finally, the mobility separated ions can be subjected to collision induced dissociation (CID) at the back of the drift tube to perform IMS-CID-MS experiments. This parallel dissociation can be employed with any of the first three operational methods described above.

The instrument used for this work consisted of two drift tube sections which allowed performing two-dimensional IMS (IMS-IMS) experiments. The first drift tube section (D1) is 79.7 cm long and was connected through a regular ion funnel (F2, 12.7 cm long) to the second drift tube section (D2) which was 76.2 cm long. The D1 and D2 regions were operated at $9 V \cdot cm^{-1}$ drift field while the funnels were operated at $10 V \cdot cm^{-1}$ dc field. The drift tube and the funnels were filled with He buffer gas at a pressure of 2.59 Torr. The operating temperature for the drift tube was 298 K. An ion gate was employed at the end of the first drift tube which was used to select mobility separated ions. The ion gate consisted of two adjacent lenses both containing 90% transmission Ni mesh grids. The distance between the two lenses was 0.32 cm. Drift tube funnels were constructed with 0.23 cm thick stainless steel ring electrodes and 0.32 cm thick Teflon spacers. When the instrument was operated in IMS-IMS mode, a negative step wave ($\sim 3.5 V_{p-p}$) was applied to the gate lens (second lens with the mesh grid) to select ions with a particular drift time range. The potential of the gate lens was maintained at a high value ($\sim 1.5 V$ above preceding lens) to block those ions of unwanted mobilities. Periodically it was lowered to let the selected ions pass to the next drift tube section. The gated ions enter the F2 funnel and can be activated ($\sim 200 V \cdot cm^{-1}$) or fragmented ($> 300 V \cdot cm^{-1}$) at the back of the funnel. Lower activation voltages can be used to transmit precursor ions without activation. At the back of D2, another funnel (F3) was employed to focus the radially diffused ion cloud. At the end of this third funnel, a split-field drift tube design was employed to facilitate efficient extraction of ions as well as parallel fragmentation of precursor ions.⁴¹ Ions exiting the drift tube traverse a differentially pumped region and were focused into the source of the mass analyzer using an einzel/dc quadrupole lens assembly. The mass analyzer was placed orthogonally to the ion beam axis and consisted of a two stage reflectron. A bipolar TOF detector (Burle Electro-Optics, Sturbridge, MA) with a 25 mm microchannel plate was employed as the ion detector.

Previous IMS-IMS experiments were relatively inefficient due to the mobility selection and activation process. For example, the instrument used for this experiment produced an effective drift range of ~ 10 ms for the D1 region. If the selection pulse width was 150 μs , approximately 67 experiments were needed to be carried out to cover the entire drift range. Although this method produced more information, it took 67 times longer to perform the experiment. A

recently developed multiplexed scanning method significantly improved the efficiency of this method.³² The multiplexed scanning method coined as combing-IMS utilized a series of pulses to select multiple ion packets in a given experiment. The series of pulses used for one scan was called a comb, and each pulse was called a tooth. For successive scans, the starting time of the initial pulse was incrementally varied such that eventually the mobility selection covered the entire drift range of the experiment. Furthermore, these multiplexed IMS-IMS experiments can be carried out in a single run, thereby increasing the throughput of the technique. The combing-IMS method utilized in this study employed a ~150–250 μ s wide selection pulse and the pulse period is varied for different experiments (~900–1000 μ s).

Coupling of chip based electrospray autosampler to the IMS-MS instrument. The autosampler was positioned directly in front of the ESI source by mounting it on a translation stage which can be moved in all three dimensions. The maximum ESI voltage setting for the electrospray was 2000 V, and for the IMS-MS experiments the source chamber voltage can be as high as ~2600 V. Under these conditions, electrosprayed positive ions must travel against the electric field created by the potential difference between the chip based electrospray and the ESI source chamber to enter the mass spectrometer. This problem was solved by biasing the autosampler with the source chamber voltage of the IMS-MS instrument. The autosampler was controlled by a dedicated computer system which was connected through a controller unit. Since the computer system cannot be biased with the same high voltage, it was electrically isolated using a model 9PFLST fiber optics modem (B & B Electronics, Ottawa, IL) from other components. Additionally, the communication between the controller unit and the data acquisition system was achieved through a fiber optics system constructed in house. The pneumatic pressure required by the instrument to manipulate the solvents and samples was obtained through a regular compressed air outlet operating at maximum pressure of 80 psi. A moisture trap was employed between the autosampler and the air outlet to prevent any electrical leakage into the plumbing system through the gas connections.

The schematic timing diagram for the operation of the autosampler device is shown in Figure 1. The chip based electrospray generated a contact closure signal after it started electrospraying the sample. A short delay (typically 5–15 s) was employed between the start of the electrospray and the generation of the contact closure signal. This contact closure signal triggered the start of the acquisition system. Two algorithms (in house written) controlled the automated data acquisition and file storage. A pulse timing program controlled the duration, frequency, level, and timing of each pulse utilized in the experiment. Pulse diagrams for typical IMS experiments have been published elsewhere.³¹ After each run the program waited for the next contact closure signal from the autosampler to initiate the next run. The spray time for the chip base electrospray was calculated such that there was a short time gap between the end of the data acquisition and the actual end of the sample spray, typically 5–15 s. Additionally, the autosampler required about 30 s to switch between samples. Therefore, the total time for each run consisted of data acquisition time, delay times employed at the beginning and the end of each run and the sample switching time.

Instrumental conditions for reproducibility and detection limit studies. Tryptic digest of phosphoglucose isomerase protein was directly infused into the IMS-MS instrument using chip based electrospray. The electrosprayed ions were subjected to IMS separation and analyzed by MS. For this experiment, 5 μ L of peptide sample was loaded into each of the 10 wells of the 96 well plate. The electrospray was operated at 1500 V ESI potential and 0.3 torr gas pressure. At these pressure settings and the sample solution conditions, the calculated flow rate was approximately 200 nL·min⁻¹. The data acquisition time for each run was 2 minutes. For this experiment each drift scan was 32 ms long. Therefore, during a 2 minute run a total of 3750 scans were accumulated. For this experiment the autosampler was programmed to pick 3 μ L from the sample well and discard the excess sample after each run.

For the detection limit study, the four protein tryptic digest mixture was directly infused into the ESI source of the IMS-IMS MS instrument using the autosampler running at the same conditions as above. The data was acquired in multiplexed IMS-IMS mode. For this experiment five combs were used with a 900 μ s tooth spacing between two pulses. Each tooth was 180 μ s wide, which gated the ions into the next drift tube. For each successive scan, starting position of the first tooth was shifted by 180 μ s until the entire drift space was scanned.

IMS-IMS-MS analysis of plasma samples

Dried samples were resuspended with 200 μ L of water. The final working samples were prepared by 1:1 dilution of the above stock solution with methanol (with 4% acetic acid) to obtain H₂O/methanol/acetic acid solution (49:49:2%). Also to each sample, two internal standards (bradykinin and angiotensin 1 each 5×10^{-3} mg·mL⁻¹) were added. Samples were worked up in batches of 24 (12 separate samples run in duplicate). The volume of each sample loaded on to the autosampler well plate was 40 μ L. The adhesive foil covering was used to minimize solvent evaporation during data acquisition. Samples were electrosprayed directly into the IMS instrument as described previously. The IMS-IMS-MS instrument was operated such that the selection pulses were 250 μ s wide with a periodicity of 1 ms. Thus there were a total of 4 comb pulse settings. We have recorded each comb pulse setting for 45 seconds which led to the overall experimental time of 3 minutes.

Few representative plasma samples were analyzed using reversed phase (RP) liquid chromatography and tandem mass spectrometry to obtain protein identifications for these samples. The peak assignments were made based on a data base obtained by running the same samples using LC-Tandem mass spectrometry methods. The gradient elution for the chromatographic separation was carried out using the Ultimate 3000 LC pump with an autosampler (Dionex, Bannockburn, IL). The tandem MS analysis was performed using a LTQ FT hybrid linear ion trap fourier transform ion cyclotron resonance mass spectrometer (Thermo Scientific, Waltham, MA). The peak assignments were also verified by cross referencing the data base obtained from LC-IMS-CID-MS experiments for 5 human plasma samples.

Results and discussion

Reproducibility of IMS-MS Measurements

The ability to reliably reproduce peak intensities for a given sample is of utmost importance in quantitative proteomics. Generally, mass spectrometry data are reasonably reproducible (CVs of 5 to 10%) if the electrospray ionization remains stable. For IMS-MS, the temperature and pressure fluctuation inside the drift tube or mass spectrometer does not usually change the signal intensity considerably. For comparative proteomics studies that utilize an IMS-MS instrument, one needs to determine the reproducibility in both the peak intensity as well as the position of the peak (e.g., drift time and m/z value). Ideally, peak intensity variation should be minimal to obtain a reasonable quantitative analysis. Peak position variation should be at a minimum to be able to compare peaks between samples. Here, we have studied both the peak intensity and the peak position for multiple ions across ten individual runs.

Figure 2 shows a nested drift(flight) time distribution for peptides arising from a tryptic digest of phosphoglucose isomerase. The acquisition time for this run is 2 minutes and the total sample used for each run is 0.2 pmoles. This experiment is carried out using IMS-MS method. Here ions are allowed to separate using the whole drift tube followed by mass analysis. This figure also shows the integrated signal intensity for the drift dimension across the entire mass range. The mass spectrum shown is obtained by integrating the signal intensity across the whole drift range. This figure clearly depicts the considerable separation achieved in the drift dimension. The peaks are identified using the peptide mass finger print technique. An *in silico* digestion

of the phosphoglucose isomerase is used to obtain the peptide database. This database consisted of $[M+H]^+$, $[M+2H]^{2+}$, and $[M+3H]^{3+}$ peptide ions as well as up to two missed cleavages. Monoisotopic masses of peptides are used, and post translational modifications are not considered when preparing this database. The mass tolerance used for the peak picking is ± 0.5 Da. A wide mass tolerance is used for this experiment due to the fact that this is a single protein digest with a relatively low sample complexity. However, the average mass tolerance for picked peaks is ± 0.17 Da. A program developed in house is used to pick peaks from the dataset and record the m/z , drift and intensity (peak height) values for each peak. Once the peak list is obtained, another program aligns the database peaks which contained the peptide identity, charge state and m/z information with the picked peak information. After obtaining the peak assignments, each peak is manually checked to verify the assigned charge state to eliminate false assignments.

It is important to note that for these studies, each protein is individually electrosprayed into the instrument and the identified peaks (peptide mass fingerprint) are included in a database. These peptides are identified based on the m/z value and charge state. Additionally, identified peptides are associated with a drift time. These database entries for individual proteins are used to identify the peptides from the protein mixture. Here we note that with the addition of drift time alignment, the identities of the peptides are of higher confidence than those that would be obtained with a peptide mass fingerprint alone. As mentioned earlier, if two or more peptides have the same m/z value, charge state, as well as drift time, they are not included in the final peptide list to avoid ambiguous assignments.

As described previously separation of charge state families according to mobilities is observed (Figure 2). Singly-charged peptides have relatively low mobility compared to the doubly and triply charged ions with the same m/z value (e.g. $[TSLVK+H]^+$ vs $[LATELPAWSK+2H]^{2+}$). Among the peaks that have been identified across all ten runs, the average intensities vary from 75 (for $[IYESQGK+H]^+$) to 2021 (for $[AVYHVALR+2H]^{2+}$). For these 2-minute runs a total of 33 peaks are identified corresponding to 25 unique peptides. The sequence coverage obtained for this protein is 48.6% using $0.5 \text{ pmol} \cdot \mu\text{L}^{-1}$ sample. The flow rate for direct infusion is $0.2 \mu\text{L} \cdot \text{min}^{-1}$. Thus total sample used for each run is 0.2 pmoles. The nested drift(flight) time distribution shows many more peaks than we were able to assign. In part this may be explained as features from peptides containing post-translational modifications or those arising from non-specific enzymatic cleavage.

Typically the peak position in the drift dimension is very reproducible and the variation from run to run is $<1.0\%$ RSD. For this experiment the average variation in drift time across all peaks is 0.034 ms which corresponds to a RSD of 0.15%. The pressure is not normalized when calculating the RSD of drift times. However, the pressure fluctuations during these experiments are typically less than 1% over the course of multiple experimental runs. The mass resolving power of this instrument is $\sim 6000\text{--}8000$ under optimum conditions, but a resolving power of $\sim 3500\text{--}5000$ can be achieved on a regular basis. The native time resolution for the TOF analyzer is 0.1 ns and the total flight time is 60 μs . The raw data from this instrument is binned to 4 ns time resolution for data processing. This instrument produces highly reproducible peak positions for both the drift and mass dimensions allowing one to use this information reliably for high-throughput qualitative analysis. This is especially advantageous when performing high-throughput comparative studies where peak intensities from multiple samples are evaluated.

Table 1 shows the reproducibility of peak intensities for peptides arising from a tryptic digestion of phosphoglucose isomerase. The experiment is repeated 10 times and the peak heights for 28 different ions are reported here. The intensities (ion counts per 2 minute run) for those 28 peaks range from 75 ± 6 to 2021 ± 83 . A peak picking threshold of 25 counts is used for

this analysis. Since this experiment is performed using the IMS-MS method, low intensity peaks (<25 counts) are often surrounded by signal noise making it difficult to unambiguously assign the charge state. The peak intensities reported here are normalized to the maximum sum of all peaks from all ten runs. The peak heights range from 2% to 13% RSD, while the average is 7%. The standard deviation is highest when the signal intensity is low. Therefore, this fluctuation in peak intensities between runs can be attributed to the sampling variations arising from low intensity signals.

Multiplexed IMS-IMS-MS technique for analyzing complex peptide mixtures. Figure 3 shows a typical drift(flight) time distribution for a multiplexed IMS-IMS separation. For this experiment, the total drift time for D1 is ~12.7 ms and five combs are used with 900 μ s tooth spacing. The gating pulse width is 180 μ s. The sample used here is a four protein tryptic digest with peptides having a concentration range of three orders of magnitude. The concentrations of aldehyde dehydrogenase, enolase, glutathione reductase and hexokinase peptides are 0.5 pmol· μ L⁻¹, 0.05 pmol· μ L⁻¹, 0.005 pmol· μ L⁻¹ and 0.5 fmol· μ L⁻¹ respectively. For this experiment, data is collected for a total of 15 min. where the duration of each comb is 3 minutes. The total sample volume used for a single run is 3 μ L. Therefore, the total amount of sample used for aldehyde dehydrogenase, enolase, glutathione reductase and hexokinase are 1.5 pmoles, 0.15 pmoles, 0.015 pmoles and 1.5 fmoles respectively. For this instrument each drift section gives a drift resolving power of ~40–60. If the two drift tube sections are used as a single drift tube, the overall drift resolving power is ~70–90.²⁹ The two dimensional peak capacity for this instrument is estimated to be ~400–600 [40–60 $\times$ 10 (average estimated shift in drift for a drift tube typical of this resolving power and the type of sample used)²⁹]. The dotted line on Figure 3 corresponds to the drift time where mobility selections are performed. The drift spectrum above the plot shows sharp peaks where the selections are made. Also peaks can be seen in between selection times, which are due to activated and drift shifted ions. When the activation voltage (~65 V) is not applied during the experiment the space between dotted lines shows significantly fewer peaks. The peaks labeled as [VVGLSTLPEIYEK+2H]²⁺, [EALDFFAR+2H]²⁺ and [EELFR+2H]²⁺ correspond to peptides from the protein alcohol dehydrogenase.

Table 2 lists the peptides identified from each protein and their $t_D(m/z)$ values. The drift times reported here are for the non-activated species and the m/z values are the theoretical monoisotopic values. All peptide masses are matched to the theoretical masses with a tolerance better than ± 0.5 Da. For the results presented in Table 2, the following mass accuracies are obtained; alcohol dehydrogenase ± 0.22 Da, enolase ± 0.19 Da, glutathione reductase ± 0.18 and hexokinase ± 0.23 Da which correspond to 220, 190, 180 and 230 ppm mass errors respectively at m/z 500 ($z = 2$). The sequence coverage obtained for aldehyde dehydrogenase, enolase, glutathione reductase and hexokinase are 62.9%, 50.6%, 19.0% and 16.5% respectively.

Sensitivity of the IMS-IMS-MS Instrument

One of the major considerations in a high throughput mass spectrometry analysis is the sensitivity of the instrument used. We have studied the sensitivity of this instrument using a four protein tryptic digest. The concentration range used is 0.5 fmol· μ L – 0.5 amol· μ L. This sample is similar to the four protein digest used in the previous experiment, but the concentration is lowered by three orders of magnitude. This peptide mixture is run in multiplexed IMS-IMS mode to assess the detection limits. For this experiment data is collected for total of 15 min. where the duration of each comb is 3 min. Each drift scan is 32 ms and therefore 5624 total scans are accumulated during this 3 min. acquisition time for each run. The total sample volume used for a single run is 3 μ L. This experiment is repeated ten times and the average signal to noise ratio (S/N) is calculated. Figure 4 shows the nested drift(flight) time 2D plot for the third comb from the second run. The mass spectra labeled A–F show the

isotopic distribution and the signal intensity for the [ATDGGAHGVINVSVEAAIEASTR+3H]³⁺, [SIGGEVFIDFTK+2H]²⁺, [VNQIGTLSESIK+2]²⁺, [ANELLINVK+2H]²⁺, [IGDYAGIK+2H]²⁺ and [TFAEALR+2H]²⁺ peptide ions respectively. Peptides A, B, D and E are from alcohol dehydrogenase and peptides C and F are from enoase. To determine the noise associated with each peak, a solvent samples is electrosprayed without the analyte. To obtain the integrated intensity for a given peak the ion counts for an integration box size of 20% full width at base in the drift dimension and 20% full width at base for the monoisotopic peak in the mass dimension are used. For each peak identified in the sample, an area with the same dimensions and the same position is integrated in the blank sample to obtain the noise for that peak. Three blank runs are performed and the average noise is obtained for each peak. The detection limits are calculated by extrapolating the S/N for a given peptide to the S/N = 3 threshold. Table 3 lists the peaks identified from alcohol dehydrogenase and enolase, and the detection limits for those peptides. Peptides from the other two proteins, glutathione reductase (5.0 amol·μL⁻¹) and hexokinase (0.5 amol·μL⁻¹) are not identified. Although the detection limits listed in Table 3 appear to be relatively low, it is important to discuss them in the context of the sample. That is, detection limits are impacted by sample matrix effects. Two examples of such effects include ionization efficiency (see below) as well as chemical noise. Thus a true measure of detection limits is sample and analyte dependent and the best method for determining such values is to spike in serial dilutions of analytes of interest. Limits of detection are then determined from concentration levels corresponding to dataset features observed with an S/N value of 3. Currently we are evaluating the detection limits for peptides spiked into human plasma digests as part of a comparative proteomics study (see below).

For the current study we have chosen to use the extrapolation method. And, although serial dilutions of analytes have not been spiked into the sample, an estimate of the detection limits can be obtained from the existing data. Overall, the best detection limit obtained using extrapolation is determined to be 0.8 attomoles for the peptide ion [SIGGEVFIDFTK+2H]²⁺ from the protein alcohol dehydrogenase (Figure 4D). Another dataset feature providing the lowest (6±1) S/N (i.e., the value closest to S/N = 3) is observed. The feature is obtained from a peptide with an upper limit concentration of 30 attomoles (an extrapolated detection limit of 11.3 – Table 3). Therefore, having analyzed a relatively simple sample, we estimate the detection limit for this instrumentation to be in the low attomole region. As mentioned before, detection limits for peptides from a three-dimensional experiment such as described here highly depend on the noise around each peak. For example, the average integrated signal intensity for peak E in Figure 4 is 1386 and average noise is 3.5 counts. The calculated detection limit for this peptide is 1.9 attomoles. But for peak B which gave a detection limit of 0.8 attomoles, the average integrated intensity is 810 counts and the noise averaged 1 count.

Human plasma sample analysis

To illustrate one application of this method, human plasma samples from 124 different individuals have been analyzed in duplicate runs constituting an experiment that required a total of 16.5 hours (8 minutes per sample). Figure 5 shows nested drift(flight) time distributions for two plasma samples (top) and the variation of intensities among individual samples (bottom) for the peptide ion [ATEHLSTLSEK+H]²⁺ from the protein apolipoprotein A1. The experiment has been performed on two different days and the data corresponding to those two days are labeled as experiment 1 and 2. Here we note that the plots in Figure 5 represent raw data; that is, peak normalization to account for variation in instrument performance has not been performed. The data processing steps of dataset normalization and alignment have been performed for a number (72 –limited by current algorithms) of the 124 samples as part of a separate comparative proteomics study (Culbertson AW, Williams WB, Kurulugama RT, Clemmer DE, March K, Naylor S, Valentine SJ, unpublished data). It is noteworthy that the

maximum variation in normalized peak intensities for the peptide mentioned above is at least 4 fold.

Assignments for features observed in the IMS combing experiments are obtained from comparisons with data from LC-MS/MS analyses of the same samples. Here assignments are made based on matches to m/z values. A threshold of 0.07 Daltons has been used for the comparison; therefore, m/z values from the 2 experiments must be within 0.07 Daltons to be considered a match. Matches are confirmed based on charge state determinations from a mass spectrum. Typically, for each 4-minute sample collection, ~100 to 250 features are assigned in this fashion.

As noted above, a concern is the problem of ion suppression as it relates to experimental sensitivity and overall molecular coverage. This is especially problematic for comparative plasma proteomics where the samples contain a very large number of interfering species. To better understand the role of ion suppression in these IMS combing experiments, analyses of serial dilutions of two known peptides doped into the plasma digest matrix have been performed. Examination of ion signals for the triply-charged angiotensin 1 and singly-charged leucine enkephalin peptides has revealed a dynamic range approaching 3 decades with limits of detection reaching 200 attomoles (Culbertson AW, Williams WB, Zhang X, Kurulugama RT, Clemmer DE, Naylor S, Valentine SJ, unpublished data). One explanation for these remarkable figures of merit is that the relatively high resolving power of the IMS-IMS-MS approach helps to mitigate the ion suppression effects of coexisting species. That is, the 2 dimensions of mobility dispersion help to remove interfering species allowing the detection of many different peptide ions.

To evaluate the utility of the IMS combing technique for comparative proteomics, the throughput and coverage obtained with conventional technology should be considered. A current study indicates a relatively high degree of overlap between identified species obtained from LC-MS/MS analyses and observed peptide ions in the IMS combing studies. Here identical plasma digest samples have been subjected to each analysis. On average, 524 ± 29 peptide ions corresponding to 83 ± 6 proteins are identified from LC-MS/MS analyses of the multiple samples. The estimated false positive rate obtained from numerous reverse protein database searches is 1 to 2% (Culbertson AW, Williams WB, Kurulugama RT, Clemmer DE, March K, Naylor S, Valentine SJ, unpublished data). On average, a significant percentage of the peptide ion (~50%) and protein (~75%) assignments are matched to those in the IMS combing datasets (m/z and z match). At this time it is difficult to estimate the number of false matches. It is worth noting that the identified features from the LC-MS/MS are from higher signal species because of the process used in data dependent experimental techniques. Therefore, it is likely that higher-signal species from the LC runs correlate to higher-signal (or observable) species for the IMS combing experiments as well. Additionally, to reduce the number of false matches, a tighter m/z match tolerance has been used (0.07 Da).

The limitations of such a comparison should be noted. First, the comparison is between all peaks observed from the IMS combing experiments and the identified features from the LC-MS/MS analysis. Clearly many dataset features from the latter technique are not compared with peaks observed in the former. Additionally, the fact that only half of the identified peptides are matched to dataset features in the IMS combing experiments may indicate the effects of ion suppression; that is, there is a clear benefit to sequentially introducing different species to the ionization source. Having said that, the fact that so many matches to identified species can be obtained on such a short experimental timescale (30 to 40 times faster than the LCMS/MS experiment) raises the question of whether or not the resolution of the combing technique is sufficient to distinguish sample populations in comparative proteomics studies. Preliminary comparisons of the data reported here (depicted in Figure 5) with that for samples from

cardiovascular compromised patients has revealed the ability to distinguish the two sample cohorts. (Culbertson AW, Williams WB, Kurulugama RT, Clemmer DE, March K, Naylor S, Valentine SJ, unpublished data) Here we note that the true utility of IMS combing for comparative proteomics remains to be determined; however, these preliminary studies suggest that further investigation is warranted.

Conclusion

This work shows the use of chip based electrospray coupled to a high sensitive IMS-IMS-MS instrument to achieve high-throughput analysis of complex protein samples. The key components of this method are the use of an autosampler with its capability of direct infusion and high-throughput sample handling and the high sensitive mass spectrometer with the capability to perform IMS-IMS experiments. The average reproducibility of peak intensities is $\pm 7\%$ RSD, thus qualitative experiments can be carried out for comparative proteomics. The sensitivity of the instrument is in the low attomole range making it possible to study dilute samples, or use very small amounts of samples. Assignments of peaks are currently carried out by manually scrutinizing the isotopic distribution of the assigned peaks and also comparing peak positions with other data that utilized more rigorous assignments. This is tedious and time consuming. However, algorithms for automation are under development.

As mentioned above one disadvantage of this method is the difficulty to perform CID-MS to identify peptides. Although peaks are separated in drift time, for complex samples often multiple peaks are observed at similar drift times even when a two-dimensional IMS separation is employed. In such cases the use of parallel fragmentation is complicated because fragment ions may arise from multiple parent ions. Due to the inability to use CID information in direct infusion experiments, we have employed separate experiments to confirm the identity of individual components in the sample. But once the constituents of the samples have been identified, high-throughput experiments can be carried out for a large population of samples.

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Figure 1. Schematic diagram showing the coupling of the NanoMate autosampler to the IMS-TOFMS instrument. This instrument can be operated in several modes such as IMS-MS, IMS-IMS-MS, and Multiplexed IMS-IMS-MS. Also, parallel CID can be performed at the back of the drift tube for MS/MS analysis. Shown below is the timing diagram for the operation of the instrument. See text for detail.

Figure 2.

Nested drift(flight) time $[t_D(t_F)]$ 2D plot obtained for Yeast phosphoglucose isomerase protein digest. The 2D plot is shown on a false color map where red color represents the most intense peaks and dark blue color represent the least intense peaks. The drift distribution is shown above the 2D plot. Drift distribution is obtained by integrating the intensity across all m/z values. The mass spectrum shown here is obtained by integrating the intensity across all drift times. The instrument is operated in direct infusion IMS-MS mode. A representation of $[M+H]^+$, $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions identified in this 2 min. data acquisition are labeled on the graph. Charge state lines have been labeled as dotted lines. The total amount of sample used for this experiment is 0.2 pmoles. A total of 33 peptides are identified which correspond to 25 unique peptides. The sequence coverage obtained for this experiment is 46.8%.

Figure 3.

Nested drift(flight) time $[t_D(t_F)]$ 2D plot obtained for a tryptic digestion of four proteins from Yeast. This experiment is performed in direct infusion multiplexed IMS-IMS-MS method. This 2D plot is shown on a false color map where red color represents the most intense peaks and dark blue color represents the least intense peaks. The four proteins used are alcohol dehydrogenase, enolase, glutathione reductase and hexokinase with $0.5 \text{ pmol} \cdot \mu\text{L}^{-1}$, $0.05 \text{ pmol} \cdot \mu\text{L}^{-1}$, $0.005 \text{ pmol} \cdot \mu\text{L}^{-1}$ and $0.5 \text{ fmol} \cdot \mu\text{L}^{-1}$ respectively. The dotted lines correspond to the drift times where the selections are made (tooth positions). The 2D plot shown here is the third comb out of five total combs. The drift distribution is shown above the 2D plot and it is obtained by integrating the intensity across all m/z values. The mass spectrum shown here is

obtained by integrating the intensity across all drift times. Peak positions for three representative peptides from alcohol dehydrogenase have been labeled on the 2D plot.

Figure 4.

Nested drift(flight) time [$t_D(t_F)$] 2D plot obtained for a tryptic digestion of four proteins from Yeast. This experiment is performed in direct infusion multiplexed IMS-IMS-MS method. This 2D plot is shown on a false color map where red color represents the most intense peaks and dark blue color represents the least intense peaks. The four proteins used are alcohol dehydrogenase, enolase, glutathione reductase and hexokinase with $0.5 \text{ fmol} \cdot \mu\text{L}^{-1}$, $0.05 \text{ fmol} \cdot \mu\text{L}^{-1}$, $0.005 \text{ fmol} \cdot \mu\text{L}^{-1}$ and $0.5 \text{ amol} \cdot \mu\text{L}^{-1}$ respectively. Peak labels A, B, C, D, E and F correspond to $[\text{ATDGGAHGVINVSVEAAIEASTR}+3\text{H}]^{3+}$, $[\text{SIGGEVFIDFTK}+2\text{H}]^{2+}$, $[\text{VNQIGTLESISK}+2]^{2+}$, $[\text{ANELLINVK}+2\text{H}]^{2+}$, $[\text{IGDYAGIK}+2\text{H}]^{2+}$ and $[\text{TFAEALR}+2\text{H}]^{2+}$ peptide ions respectively. Peptides A, B, D and E are from alcohol dehydrogenase while peptides C and F are from enolase. The mass spectra for each peptide show the isotopic peak distribution which enabled the confirmation of the charge state.

Figure 5.

Nested drift(flight) time 2D plots for tryptic digests of plasma samples from two individuals and the peak intensity vs. sample number for $[\text{ATEHLSTLSEK}+\text{H}]^{2+}$ peptide from apolipoprotein A1. A small section from the 2D plot is shown here ($m/z = \sim 585\text{--}633$ and $t_D = 13.5\text{--}18.3$ ms) which includes the $[\text{ATEHLSTLSEK}+\text{H}]^{2+}$ peptide peak. Sample numbers given here are arbitrary numbers corresponding to the sequence they are analyzed. LC-tandem mass spectrometry experiments have been used to confirm the identity of the peptide. For this experiment 124 Human plasma samples are used and each sample has been run in duplicate. The total time for the experiment is 16.5 hours.