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Enhancing bottom-up and top-down proteomic measurements with ion mobility separations

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Abstract

Proteomic measurements with greater throughput, sensitivity, and structural information are essential for improving both in-depth characterization of complex mixtures and targeted studies. While LC separation coupled with MS (LC–MS) measurements have provided information on thousands of proteins in different sample types, the introduction of a separation stage that provides further component resolution and rapid structural information has many benefits in proteomic analyses. Technical advances in ion transmission and data acquisition have made ion mobility separations an opportune technology to be easily and effectively incorporated into LC–MS proteomic measurements for enhancing their information content. Herein, we report on applications illustrating increased sensitivity, throughput, and structural information by utilizing IMS–MS and LC–IMS–MS measurements for both bottom-up and top-down proteomics measurements.

Keywords

Ion mobility separations; Ion mobility spectrometry; Mass spectrometry; Proteomics

1 Introduction

Over the last decade, MS-based proteomic analyses have become one of the most informative methods for studying complex mixtures of proteins to enable systems biology approaches. Currently, MS information is being used in a wide range of biological and biomedical applications, including analysis of cellular responses and the development of biomarkers based upon disease-specific posttranslational protein modifications [1,2]. These

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studies are significantly enhancing our understanding of the complex and dynamic nature of the proteome in biology and disease. MS techniques, such as those for targeted analysis, are being successfully applied for biomarker verification [3, 4], whereas others including global quantitative analysis (e.g. for biomarker discovery) are more challenging, generally providing less sensitivity and poorer quantification than targeted analyses, and would benefit from further development of existing capabilities. Thus, increasing the information content, sensitivity, and quantification in both targeted and global analyses would be beneficial for nearly all applications of proteomic measurements.

Recent technological developments in gas-phase ion mobility spectrometry (IMS) separations provide an opportunity to advance MS-based proteomic applications by adding a structure-based separation to the measurements. IMS is appealing because it offers a rapid additional analysis dimension that can be easily coupled with MS after other separations (e.g. LC) for multidimensional measurements [5]. Interfacing IMS with orthogonal TOF mass spectrometers [6, 7] permits simultaneous acquisition of ion mobility structural information and provides high-accuracy mass data with exceptional throughput because IMS typically requires <10–100 ms for the separation, during which a high-resolution TOF mass spectrum can be acquired every ~100 μ s. This high throughput enables IMS–MS platforms to quickly screen conditions while gathering global information about the classes of molecular species present since IMS is able to separate different chemical species such as carbohydrates, peptides, lipids, and “chemical noise” components on the basis of their backbone molecular differences (Fig. 1) [8]. The ability to differentiate molecular isomers with IMS–MS also has important consequences for targeting peptides and proteins and separating out other compounds that may have the same mass but are not a focus in the studies. IMS–MS separations can also be coupled with fast LC separations for addressing highly complex samples [9, 10]. In this case, the additional IMS separation power can allow significant reduction of LC separation times while the depth of coverage characteristic of much longer traditional LC–MS analyses is still maintained [11].

While IMS–MS has been utilized since the 1970s, its practical use was impeded by low sensitivity due to catastrophic losses throughout the ion path from the source to detector, including ~99% at the IMS inlet. Although this problem was solved over a decade ago with the addition of ion funnels both before and after the IMS drift cell [12], IMS also suffered from low duty cycle (~1%) due to the need for short ion pulses to achieve the highest possible IMS resolving power. Other analytical techniques (e.g. MS) solved this problem by shifting to the frequency domain via multiplexing using Fourier or Hadamard transforms where multiple ion packets are dispersed simultaneously and normal time domain spectra are recovered by reverse-transformation of raw data following acquisition [13]. However, multiplexing efforts in IMS failed due to prominent artifact peaks that correspond to slight changes in the IMS packets. This problem was recently overcome by designing a pseudorandom transform [14] using a novel filtering algorithm to separate artifacts from true signal [15]. Utilization of these techniques makes proper, artifact-free separations possible, greatly increasing the IMS duty cycle and thus the sensitivity and dynamic range by approximately tenfold [16]. The success of the enhanced IMS separations has been crucial in enabling high sensitivity and high throughput IMS–MS measurements. In this manuscript,

we highlight both bottom-up and top-down proteomic applications enabled by the recent technological advances in IMS–MS measurements.

2 Materials and methods

2.1 Materials

For the sensitivity and isomer studies, bradykinin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted to 100 pM in 49.75:49.75:0.5 water:methanol:acetic acid. Human plasma (Sigma-Aldrich) was utilized to study the number of features observed by coupling together the IMS and MS measurements. The plasma was depleted of its 14 most abundance proteins and digested with trypsin using protocols defined previously [16]. The final peptide concentrations were normalized to 0.3 µg/µL prior to storage at –80°C and LC–IMS–MS analysis. Two sequence isomers (RRGPFPSPF and GPFRPRFPS) were synthesized from Sigma-Genesys and bradykinin was purchased from Sigma-Aldrich. Each peptide was prepared to a concentration of 100 nM in 49.75:49.75:0.5 water:methanol:acetic acid and each solution was run separately on the IMS–MS instrument. Finally, ten peptides (des-Pro-Ala bradykinin, fibrinopeptide A, Tyr C peptide, human osteocalcin fragment 7–19, syntide 2, diazepam binding inhibitor standard, porcine dynorphin A fragment 1–13, (D-Ala-6) luteinizing hormone releasing hormone, bradykinin fragment 1–7, porcine renin substrate, and bradykinin) were purchased from Sigma-Aldrich. For the dynamic range experiments to understand the linearity of the concentration curves created by IMS-MS, all ten peptides were mixed together and prepared with 49.75:49.75:0.5 water:methanol:acetic acid to create six different solutions with concentrations of 100 pM, 1 nM, 10 nM, 50 nM, 100 nM, and 1 µM for each peptide. Ten replicates of each solution were directly injected into the IMS–MS instrument to determine CV for intensity.

In the aggregation study, heme-containing peptides from horse heart cytochrome c (Sigma-Aldrich) were prepared, purified, and fractionated as detailed in [17]. The detailed preparation for the heme-containing peptides from the *Shewanella oneidensis* protein mixture used in the fragmentation studies is also given in [17]. For the phosphopeptide sample, human plasma was digested with trypsin at room temperature. Tryptic peptides were desalted and methyl-esterified followed by immobilized metal-ion (Fe^{3+}) affinity chromatography to enrich phosphopeptides, as detailed in [18]. After immobilized metal-ion affinity chromatography enrichment, the aliquots were analyzed by LC–IMS–MS.

His-tagged recombinant wild-type transthyretin [19] and Leu55Pro TTR [20] were kindly provided by L. H. Connors and E. S. Klimtchuk in the BUSM Amyloid Center and diflunisal (5-(2,4-difluorophenyl)-2-hydroxybenzoic acid) was obtained from Sigma-Aldrich for the protein ligand studies. The proteins were buffer exchanged into 20 mM ammonium acetate (pH 7.0) using micro Bio-spin six columns (Bio-Rad). For all experiments the concentration of the protein was 6 µM (thus the protein tetramer concentration was 1.5 µM). For the ligand binding study, diflunisal was prepared as a stock solution in DMSO at a concentration of 1.60 mM. It was added to either the wild-type protein or L55P at concentrations of 1.5 or 6 µM to create 1:1 and 1:5 protein tetramer:ligand ratios, respectively, in order to study how the presence of the ligand affects protein assembly.

2.2 Instrumental analysis

Analyses of all samples in this manuscript were performed on an in-house built IMS–MS instrument [21] that couples a 1 m ion mobility separation with an Agilent 6224 TOF MS upgraded to a 1.5-m flight tube (providing MS resolution of ~25 000 [22]). The IMS–MS data were collected from m/z 100–3200 for the peptide studies and m/z 100–10 000 for the transthyretin analyses.

A fully automated in-house built two-column HPLC system equipped with in-house packed capillary columns was used for all LC runs. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile [23]. Both 60-min LC gradients (using 30-cm-long columns with an od of 360 μm , id of 75 μm , and 3- μm C18 packing material) and 100-min LC gradients (using 60-cm-long columns with same dimensions and packing) were performed in this manuscript. Both gradients linearly increased mobile phase B from 0 to 60% until the final 2 min of the run when B was purged at 95%. Five microliters of sample was injected for both analyses and the HPLC was operated under a constant flow rate of 0.4 $\mu\text{L}/\text{min}$ for the 100-min gradient and 1 $\mu\text{L}/\text{min}$ for the 60-min gradient. The analyses of the CHAPs-contaminated samples were performed on both a Thermo Fisher Scientific LTQ Orbitrap Velos MS (Velos) (San Jose, CA, USA) and the IMS–MS platform. The Velos MS data were collected from m/z 400–2000 at a resolution of 60 000 (automatic gain control (AGC) target: 1×10^6).

3 Results and discussion

To investigate the sensitivity increase affiliated with adding the IMS separation (having updated multiplexing sequences) to a TOF mass spectrometer, bradykinin was directly infused into the IMS–TOF MS instrument at a concentration of 100 pM (Fig. 2A). The ion funnel trap was pulsed with a 4-bit multiplexing sequence to release eight packets into the IMS drift cell and the sequence was demultiplexed using the novel filtering approach [15]. A clear bradykinin signal was illustrated with a S/N ratio of 112 for (bradykinin)²⁺ as shown in Fig. 2A. To compare this spectrum with TOF-only mode and remove the IMS separation, the ion funnel trap was operated in a continuous mode where all ions entering the source traveled directly to the detector without being pulsed. In this case, the peak for the 100 pM bradykinin was barely visible in the spectrum and could not be detected above the noise level. By trapping and releasing the bradykinin ions during acquisition of the IMS–MS spectrum, the drift cell was able to separate chemical noise to a different area of the nested IMS spectrum in addition to the improvement achieved by funnel trap's heating and evaporating some of the solvent clusters to reduce chemical noise. The detection limit of bradykinin was also analyzed with the ESI–IMS–TOF MS platform and found to be ~2 attomoles [21] indicating the power of using the additional optimized IMS separation for high sensitivity measurements.

The combination of IMS and MS also substantially increases the amount of information obtained from complex mixtures. Proteomic analyses of blood and other biological fluids have proven to be immensely challenging due to massive sample complexity, since the dynamic range of protein concentrations of potential interest can span >10 orders of magnitude (in blood plasma). Analytes of clinical interest are often present at the low end of

this concentration range making them challenging to detect [24–26]. Recently, it was estimated that although the human genome would predict the generation of slightly fewer than 20 000 proteins [27], the human proteome contains more than 2 million different protein products [28] or “proteoforms,” and at present there are over 12.6 million known proteins representing significant taxonomic diversity spanning prokaryotes, eukaryotes, and viruses [29, 30]. By adding the IMS separation to LC–MS measurements, the multidimensional LC–IMS–MS separations allow peptides to be further separated from other peptides and chemical noise to yield more information and better S/N ratios. As illustrated in Fig. 2B for a 3-s LC snapshot of human plasma, many different peptides are observed in the nested IMS–MS spectrum just in the small mass range of m/z 620–640, but if the MS spectrum is analyzed individually, the low concentration features are lost. However, by plotting both dimensions in the nested IMS–MS spectrum, additional low level features are easily observed.

To further examine how well IMS–MS quantitates the features observed in the nested spectrum, the linear dynamic range was evaluated. In this study, six different concentrations of equimolar mixtures of ten peptides ranging from 100 pM to 1 μ M for each peptide were analyzed on the IMS–MS instrument. Ten technical replicates of each concentration were performed so that CV values for intensity could be calculated. The IMS–MS platform was able to detect all peptides at each concentration (except Tyr C at 100 pM) and a linear dynamic range was observed with all peptides having R^2 values >0.9 (Fig. 2C). CV intensity values from the technical replicates ranged from 15% for the 100 pM peptides to 2% for those at 1 μ M. The low CV values and dynamic range linearity illustrate the potential of IMS–MS measurements for quantitation studies where analytes are present over a large range of concentrations.

The addition of the IMS separation also has important implications in studies focusing on sequence isomers (or molecules present at the same m/z but having a different chemical makeup), aggregation states, and changes in protein structures upon ligand binding. The analysis of isomers has allowed IMS to be used in metabolomic [31,32], lipidomic [33], and proteomic studies [11, 34, 35], with its utility growing every year. When we analyzed three peptide isomers (RRGPRPSPF⁺, RPPGFSPFR⁺, and GPFRPRFPS⁺) with the same nine amino acids but different sequences first individually for confident identification and then as a mixture, IMS was able to separate them from each other (Fig. 2D) as has been observed in other isomeric studies [36–38]. To understand why the peptides separated, NWChem molecular dynamic simulations [39] were performed at 300 K. Representative structures from the lowest energy conformers are shown in Fig. 2D. The RRGPRPSPF⁺ isomer had a more compact “S” form relative to the two larger isomers, with RPPGFSPFR⁺ existing in a more open “S” conformation and GPFRPRFPS⁺ having an alpha helix turn and more extended structure. With IMS–MS these isomers were easily separated, but with MS alone these would have been indistinguishable in the same solution and even using MS/MS, it would have been difficult to characterize the multiple fragmentation patterns being observed.

Quantitating aggregation states is another task that is challenging to accomplish with MS alone. Since monomers, dimers, trimers, etc. all have the same m/z but have different

isotopic spacing, they can be difficult to quantify with MS alone. IMS-MS has been utilized previously to analyze protein aggregation states [40–42] and it has great importance in quantitating the amount of hemepeptide aggregation in a sample containing (CAQCHTVEK-heme)⁺. Using MS, it was clear that there were many different aggregation levels present, but their quantitation was virtually impossible since the amount of tetramer was so low it was indistinguishable from noise in the MS spectrum (Fig. 3). However, in the IMS spectra it was clear that the peptide was predominantly present as a dimer but also existed as a monomer, trimer, and tetramer and each was easily quantitated by calculating the area under the IMS peaks. Obtaining this information would not have been possible without the use of the IMS separation.

IMS-MS is also a powerful tool for probing the stability of native protein structures before and after noncovalent ligand binding to determine the effects of ligand(s) on native protein structure [43, 44]. In many cases, MS alone is not able to detect subtle differences in the structural stability afforded by the binding of ligands to protein assemblies. To study the influence of small molecule noncovalent binding on the His-tag form of the plasma protein transthyretin, both the wild-type (TTR) and Leu55Pro variant (L55P), which is the most amyloidogenic transthyretin variant, were studied with and without the ligand diflunisal. Diflunisal is a drug under evaluation for patient use, where it would be desirable to stabilize the tetrameric form of the protein, thus inhibiting its unfolding that precedes fibril formation [45]. The TTR and L55P proteins were analyzed at a concentration of 6 μ M without diflunisal. At this concentration they existed almost solely as tetramers with only a small amount of octamer observed in the IMS-MS nested spectrum and no monomers, dimers, or trimers. The tetrameric complexes for both TTR and L55P exhibited both a compact and extended conformation, with the extended conformation being more prevalent at higher charge states (Fig. 4). Previous publications have indicated that proteins (and other biological molecules in the gas phase) unfold at higher charge states due to the additional electrostatic repulsion [46, 47], matching these observations. Since the compact form is thought to be more solution-like, TTR appears to be more stable than L55P since a higher proportion of its conformers were compact compared to L55P, which had a higher percentage of extended conformers. This observation confirms that the single nucleotide change (T→C) that produced the Leu55Pro variation caused sufficient structural changes in the expressed protein to effecting the structural stability of the tetramer [43].

Upon addition of 1.5 μ M diflunisal to TTR and L55P (1:1 protein tetramer to ligand concentration ratio), due to the two noncovalent binding pockets of the tetramer [48], the resulting complexes existed in three different binding states: unbound, singly ligated, and doubly ligated. Diflunisal readily bound to both the TTR and L55P tetramers exhibiting a ~30:45:25 ratio of unbound:singly ligated:doubly ligated for both proteins. Increasing the ligand concentration to a 1:5 protein tetramer to ligand concentration resulted in a ~0:10:90 ratio with the protein tetramers having mostly two diflunisal molecules attached, only a small amount of a single diflunisal bound, and essentially no unbound protein remaining. When the ligand-bound spectra (right side of Fig. 4) were compared to those without diflunisal added (left side of Fig. 4), a charge state reduction was also observed for both proteins and the extended conformation vanished, indicating the stabilizing effect of diflunisal on the tetramers. While the charge state shift could have been observed with MS

alone, identifying the increased stability of the ligand-bound structures due to the disappearance of the extended structure in the ligand bound spectra was only possible from the additional IMS dimension.

The previous examples illustrate the benefit of IMS for targeted applications, but it can also be highly advantageous for global studies. LC–IMS–MS measurements for biological samples have shown reduced false discovery identifications due to the additional discrimination afforded by the third dimension [49], while also having higher sensitivity and throughput than LC–MS measurements alone [16, 35]. LC–IMS–MS has also been instrumental in the analysis of complex environmental water, soil, and plant matter samples. In these samples, the three dimensions are particularly useful for separating high concentrations of organics (e.g. humic acid substances in soil), natural contaminants (e.g. abundant salts or polymers), and detergents from peptides in samples where their removal through additional sample processing can result in prohibitive peptide losses [50]. In one case, the tryptic digest of *Arabidopsis* plant samples under different growth conditions had to be analyzed with CHAPS detergent present in the sample because upon its removal hardly any peptides remained. The tryptic plant digests were analyzed with both the Velos Orbitrap MS and the IMS–MS platform to understand the differences observed between the two instrument platforms. The CHAPS contaminants entirely dominated ~20 min of the LC run in the Velos Orbitrap measurements (Fig. 5A) and hindered the detection of almost all peptides eluting during this period. This occurred because the AGC in the linear ion trap limited the number of ions accumulated during that time to reduce space charge effects and achieve high measurement accuracy. However due to the high concentration of CHAPS ions, this limitation did not allow enough time for sufficient plant peptides to accumulate and be observed. While the CHAPS contaminants again dominated this 20-min segment of the LC run for the IMS–MS instrument, plant peptides were still detected since there was no AGC to limit the analysis (Fig. 5B). Furthermore, the IMS separation moved the CHAPS contaminants to a different drift time area than the peptides so both groups could be easily distinguished (Fig. 5B). During the 20-min that the CHAPS contaminants eluted in the LC separation, LC–IMS–MS was able to detect over 400 peptides whereas the Velos only detected ~50. Since these samples were precious and unique, IMS–MS was able to preserve the peptide information, giving important details about the plant chemistry under different conditions while hundreds of the peptides were missed in the conventional analyses.

The ability of the IMS–MS instrument to perform (CID) between the IMS and MS separation stages for data-independent acquisition (DIA) is also an important attribute for global analysis [51–53]. Although there are still some challenges in the analyses of DIA data for complex samples, we utilized a targeted approach to classify modified peptides such as phosphopeptides and hemepeptides. In the targeted analysis for phosphopeptides in a tryptic digest of human serum, parent and fragment spectra were alternately collected during the LC–IMS–MS run. The phosphopeptides in the parent spectrum were assigned by looking for HPO_3 (80 u) or H_3PO_4 (98 u) spacing in the fragment spectra immediately following the parent spectrum (Fig. 6A). Fragmentation patterns that did not have this spacing were considered to be nonphosphopeptides, while those with the correct spacing were assigned as phosphopeptides and their additional fragment peaks were utilized to sequence the peptides.

An example of phosphopeptide identifications from human serum are shown in Fig. 6A, where three out of the possible eleven peptides in the parent spectrum are assigned as phosphopeptides. This targeted strategy was also employed for finding heme-containing peptides in a tryptic digest for *Shewanella oneidensis* MR1, a dissimilatory metal-reducing bacterium that can reduce iron, manganese, and other polyvalent metals and radionuclides, including environmental contaminants such as chromium, uranium, technetium, and plutonium [54,55]. In this sample, parent and fragment spectra were again alternated during the LC–IMS–MS run. Heme⁺ fragments with a small peak at 615.17 Da and larger peak at 616.18 Da [17] due to the presence of multiple Fe isotopes were targeted in the fragment spectra to assign the parent spectra. Figure 6B illustrates two hemepeptides in the parent spectrum with matching heme fragment ions. While analysis of the 4D LC–IMS–(CID)–MS data is difficult due to the many dimensions (LC, IMS, MS, and MS/MS), this targeted strategy worked well for modifications and the future seems promising with Skyline's [56] initially incorporating IMS analysis options in 2014 and further optimizing them in 2015.

4 Concluding remarks

In summary, we analyzed the benefits of adding a rapid IMS separation for various bottom-up and top-down proteomic mass spectrometry measurements in applications ranging from complex biological and environmental samples to targeted peptide and protein studies. The evaluation illustrated:

1. The addition of the IMS separation to MS and LC–MS analyses significantly increased the sensitivity and number of detectable features, and also resulted in improved quantification.
2. IMS–MS was able to distinguish peptide isomers, aggregation states, and protein conformational changes upon ligand binding that were unresolvable by MS alone.
3. IMS–(CID)–MS allowed DIA of all fragment ions and by targeting specific fragments, modified hemepeptides and phosphopeptides were classified.

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Abbreviations

AGC	automatic gain control
DIA	data-independent acquisition
IMS	ion mobility spectrometry

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Statement of Significance

This manuscript details applications utilizing IMS–MS and LC–IMS–MS measurements, which enable improved bottom-up and top-down proteomic results compared to standard LC–MS analyses.

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Figure 1.

Drift time separation for carbohydrates, peptides, and lipids showing carbohydrates traversing the drift cell fastest due to the many ring structures in their backbone and lipids travel slowest due to their rigid linear backbone.

Figure 2.

(A) (Bradykinin) $^{2+}$ directly infused at 100 pM in the IMS-TOF MS instrument with and without pulsing (to simulate no IMS separation). The noise is greatly reduced with pulsing and (bradykinin) $^{2+}$ can be easily distinguished with a S/N of 112 for the IMS-TOF MS spectra (left), however without pulsing the S/N is <3:1 (right). (B) IMS-MS nested spectra illustrate the rich density of peptide features in a 3-s LC window for human plasma. By using MS (left) or IMS (top) alone, the number of resolvable features is much lower than in the IMS-MS nested 2Dspectrum. (C) The intensities for ten peptides plotted at six concentrations from 100 pM to 1 μ M (100 pM, 1 nM, 10 nM, 50 nM, 100 nM, 1 μ M for each peptide) illustrating linear response over concentrations. Tyr C was the only peptide not observed at 100 pM. (D) The drift time spectrum for the three peptide isomers (RRGPRPSPF $^{+}$, RPPGFSPFR $^{+}$, and GPFRPRFPS $^{+}$) illustrating three distinct peaks due to their structural differences.

Figure 3.
The IMS-MS nested spectra for the heme peptide (CAQCHTVEK-heme)+ allowing quantitation of different aggregation levels from monomers (M) to tetramers (4M) as shown by the zoomed in area on the right.

Figure 4.

Wild-type transthyretin (TTR) and Leu55Pro variant (L55P) (left) were combined with 1.5 μ M diflunisal (right). In both cases, one and two diflunisal molecules bound to the tetramer of each, reducing the charge state and stabilizing the compact conformation. The area in the nested spectra corresponding to the extended conformations is shown by the red oval.

Figure 5.

A peptide extract contaminated with CHAPS surfactant analyzed using both the (A) Velos Orbitrap and (B) IMS-MS platform. The CHAPS interferes in both chromatograms (left) but the IMS-MS was able to still detect 49 peptides in the spectra shown (right) while the peptides were not detected in the Velos mass spectrum due to AGC limitations.

Figure 6.

(A) Parent (left) and fragment (right) spectra for a phosphopeptide/peptide mix. Phosphopeptides were determined by the HPO_3 (80 u) or H_3PO_4 (98 u) loss mass differences. All three phosphopeptides shown in this spectrum had H_3PO_4 losses. (B) Parent (left) and fragment (right) spectra for a hemepeptide/peptide mix. Hemepeptides were identified by the presence of the heme fragment.