1.1 Introduction

The use of ion mobility as an analytical technique to detect and separate biomolecules dates back to the break of the century with the application of the method for proteomics (Valentine et al. 2006; McLean et al. 2005; Gabryelski and Froese 2003), glycomics (Taraszka et al. 2001; Jin et al. 2005; Hoaglund et al. 1997), and metabolomics (Dwivedi et al. 2008). It is a technique that separates gas-phase ions based upon their mobility in a buffer gas. This separation is related to ion size, shape, as well as $m/z$, and charge. The basis for separation by traditional drift tube ion mobility at a low electric limit can be derived from the Mason–Schamp equation:

$$K = \frac{3}{16} \left(\frac{2\pi}{\mu_k T}\right)^{1/2} \frac{ze}{N\Omega}$$

where $K =$ drift velocity, $\nu_d$/electric field strength $E$, $\mu =$ reduced mass of the ion (neutral given by $m_{\text{neutral}}/m_{\text{ion}}$), $\kappa_B =$ Boltzmann constant, $T =$ temperature, $z =$ charge state of the analyte ion, $e =$ charge on an electron, $N =$ number density of the drift gas, and $\Omega =$ average collision cross section. The hyphenation of ion mobility spectrometry (IMS) with MS is often referred to as ion mobility–mass spectrometry (IM–MS). The most common mass analyzer coupled with IMS comprises a time-of-flight (TOF) instrument due to the inherent high sampling rate, although other mass detection systems have been described (Kanu et al. 2008). Four different methods of ion mobility separation are currently used in combination with MS, including drift-time ion mobility spectrometry (DTIMS), aspiration ion mobility spectrometry (AIMS), differential mobility spectrometry (DMS), also called field-asymmetric waveform ion mobility spectrometry (FAIMS), and traveling-wave ion mobility spectrometry (TWIMS). A description of these methods is beyond the scope of this chapter, particularly since they have been reviewed in great detail elsewhere (Kanu et al. 2008; Lanucara et al. 2014).

The innovative demonstration of protein conformer separation by means of IMS by Clemmer et al. 1995 has prompted instrumental IM–MS designs and the broader application of IMS as an analytical tool. The designs by Pringle et al. 2007 and Baker et al. 2007, both orthogonal acceleration time-of-flight (oa-TOF) based IM–MS platform, but utilizing different IMS geometries, have been commercialized and applied for numerous applications and include drug metabolism/metabolites (Dear et al. 2010), lipids (Kliman et al. 2011), trace impurities (Eckers et al. 2007), carbohydrates (Vakhrushev et al. 2008, Schenauer et al. 2009), macromolecular protein species and viruses (Ruotolo et al. 2005, Bereszczak et al. 2014), metal-based anticancer drugs (Williams et al. 2009), and PEGylated conjugates (Bagal et al. 2008). In this chapter, the application of IMS for the identification, quantification, and characterization of proteins is illustrated by application examples that demonstrate the benefits of integrating IMS into the analytical schema in terms of increased resolution and sensitivity, as well as those obtained from collision cross section measurements.

1.2 Traveling-Wave Ion Mobility Mass Spectrometry

The principle of TWIMS is briefly discussed as it forms the basis of subsequent sections. A schematic of the device is shown in Figure 1.1. Details can be found in the papers of Pringle et al. 2007 and Giles et al. 2004. Ions are formed by electrospray ionization (ESI) in the source and subsequently pass through a quadrupole for mass selection before injection into the ion mobility cell. Unlike our other instruments, which use a uniform electric field across the cell for ion mobility experiments, so-called
drift tube IMS, this device uses traveling-wave (T-wave) technology. The T-wave cell consists of a stacked-ring radio frequency (RF) ion guide, which incorporates a repeating sequence of transient voltages applied to the ring electrodes. These voltage pulses result in a traveling electric field that propels ions through the background gas present in the mobility cell. The total time taken for an ion to drift through the cell depends on its mobility, as well as the wave period and height, and the gas pressure. Ions with high mobility are better able to keep up with traveling waves and are pushed more quickly through the cell. Ions with low mobility crest over the waves more often and have to wait for subsequent waves to push them forward, resulting in longer drift times. To measure an arrival time distribution (ATD), ions are gated into the mobility cell using an up-front stacked-ring RF device that traps ions before releasing them into the IMS cell. The oa-TOF pulses in an asynchronous manner, sending ions that have exited the mobility cell into the TOF mass analyzer and finally to the detector. The sum of all detected ions is the ion mobility chromatogram, or mobilogram. Selecting a peak in the ion mobility chromatogram displays the underlying TOF mass spectrum. Resolution enhancements to the device are recently described (Giles et al. 2011).

1.3 IM–MS and LC–IM–MS Analysis of Simple and Complex Mixtures

1.3.1 Cross Section and Structure

By measuring the mobility of an ion, information about the rotationally averaged collision cross section, which is given by shape and size, can be determined. The relationship between the mobility of an ion and its collision cross section has been derived in detail using kinetic theory (Mason and McDaniel 1988). When all experimental IM parameter values are held constant, a dependence of the ion mobility constant results only from the average cross section with $K \sim 1/\Omega$ (Bowers et al.; Henderson et al. 1999; Verbeck et al. 2002), where $K = \text{drift velocity } \nu_d / \text{electric field strength } E$ and $\Omega = \text{average collision cross section}$. A detailed description of kinetic theory is beyond the scope of this discussion. Ruotolo et al. 2005 were among the first describing the use of IM–MS to decipherer protein complex structure. The analysis of the 'Trp RNA-binding attenuation protein (TRAP) provided compelling evidence that many features of protein assemblies, including quaternary structure, can be preserved in the absence of solvent molecules. The researchers made use of TWIMS coupled to a modified TOF mass spectrometer to measure the CCS of four charge states of an 11-mer complex, demonstrating that the lowest charge state exhibited the largest CCS, with a value in close agreement to that estimated for a ring structure determined by X-ray crystallography. To investigate the sensitivity of the various conformers to changes in internal energy, they examined collision cross sections of the apo TRAP complex as a function of activation energy by manipulating their acceleration in the atmospheric pressure interface of the instrument, shown in Figure 1.2. The experiment illustrated that when an internal energy is imparted to 22+ ions, an expansion of the collapsed state occurred, while for 19+ ions they could partially drive the structural transitions observed for the ring structure as a function of protein charge state. IM–MS has proved to be extremely useful for the structural analysis of proteins and protein assemblies as illustrated in a number of recent reviews (Lanucara et al. 2014; Zhong et al. 2012; Uetrecht et al. 2010; Snijder and Heck 2014).

Collision cross section measurements and structure IM–MS experiments are not restricted to the analysis of large molecules but have been applied to other molecule classes and applications as well. For example, Valentine et al. 1999 used IMS to measure collision cross sections for 660 peptide ions generated by tryptic digestion proteins. Measured cross sections were compiled into a database that contains peptide molecular weight and sequence information and can be used to generate average intrinsic contributions to cross section for different amino acid residues. This was achieved by relating unknown contributions of individual residues to the sequences and cross sections of database peptides. Size parameters were combined with information about amino acid composition to calculate cross sections for database peptides. Figures 1.3(a) and (b) summarize the work showing cross sections as a function of molecular weight for the singly and doubly charged database peptides, respectively (Valentine et al. 1999). A strong correlation of increasing cross section with increasing molecular weight was observed, suggesting that (predicted) cross section can be
used as an additional search parameter for peptide identification. A follow-up study proposed that the method that employs intrinsic amino acid size parameters to obtain ion mobility predictions can be used to rank candidate peptide ion assignments. Intrinsic amino acid size parameters were determined for doubly charged peptide ions from the complete annotated yeast proteome. The use of the predictive enhancement as a means

**Figure 1.2** Ion mobility data for selected charge states of apo-TRAP (19+, 21+, and 22+) as a function of activation energy (175, 125, and 50 V) applied in the high-pressure, sampling cone region of the instrument. The light gray and dark gray dashed lines represent the collision cross sections for collapsed and ring structures. (Source: Ruotolo et al. 2005. Reproduced with permission of The American Association for the Advancement of Science.)

**Figure 1.3** (a) Cross sections for 420 [M+H]+ peptides (solid diamonds) as a function of molecular weight. Uncertainties correspond to one standard deviation or to a range. The inset shows variations in cross sections for [M+H]+ peptides over a smaller molecular weight range (defined by the dashed-line box). (b) Cross-sectional measurements for 240 [M+2H]2+ peptides (open diamonds) as a function of molecular weight. (Source: Valentine et al. 1999. Reproduced with permission of Springer.)
to aid peptide ion identification was discussed and a simple peptide ion scoring scheme presented.

1.3.2 Separation

The work of Clemmer and coworkers (Liu et al. 2007; Valentine et al. 2001, 2006) demonstrates the use of IMS for the separation and profiling of plasma proteins. The integration of IMS into an LC–MS schema is described to increase the separation power of a platform. The setup comprised off-line strong cation exchange (SCX) and inline LC–IM–MS separation of trypsin digested plasma proteins. The SCX–LC–IM–MS setup is described in great detail as well as how the additional IMS separation dimension increased the available experimental peak capacity. The experimental two-dimensional LC–IM peak capacity was estimated to be ~6000–9000 obtained from a partial \( t_r(t_d) \) base-peak plot derived from a single LC–IM–MS analysis, which greatly exceeds that of a single LC or IMS experiment. Also discussed is the use and creation of a relational table or database that comprises physico-chemical analyte information such as SCX retention time \( t_{r,SCX} \), reversed-phase (RP) retention time \( t_{r,RP} \), drift time \( t_d \), and \( m/z \). This information can be stored in a multidimensional space as shown in Figure 1.4. Knowledge of the positions of peaks will further corroborate assignments of other data sets. In addition, the accumulation of data provides valuable information for future work that would aim to predict SCX retention times, LC retention times, and mobilities based on sequences and charge states. The contribution of IM for the identification peptides as an additional search and identification parameter has been discussed in detail (Valentine et al. 1999, 2011). These concepts have been applied by Thalassinos et al. 2012 for the identification and quantitation of peptides and proteins across two similar mammalian species and Paglia et al. 2014 for the identification of the key metabolites potentially involved in cancer. The increase in system peak capacity, experimentally derived, for a multidimensional LC–IM–MS system has been described and demonstrated by Rodríguez-Suárez et al. 2013.

Ion mobility-assisted data-independent analysis (DIA) LC–MS (Geromanos et al. 2009; Distler et al. 2014a) can be seen as an extension to the work of Clemmer and coworkers. Here, however, IMS is additionally used to align precursor and product ions to increase the specificity of a DIA workflow using TWIMS. In other words, it not only increases system peak capacity but also enhances the selectivity of DIA. In this experiment, to maximize duty cycle, peptide precursor ions are not isolated by the quadrupole mass analyzer positioned in front of the TWIMS cell. The ions undergo separation first in the mobility section and are either not fragmented or collision induced dissociated (CID) in the transfer region. This process is repeated at a fixed frequency, thereby generating so-called low and elevated energy precursor and product ion spectra, respectively. Thus, precursor and product ions share identical \( t_d \) which can be used to entangle the multiplexed product ion spectra. Briefly, precursor and product ion mass extracted chromatograms are created in the \( t_r \) and \( t_d \) domains. Precursor and product ion that share the same drift and retention time are correlated, which simplifies the multiplexed CID spectra prior to a database search for identification of peptides and proteins. As an example, Yang et al. 2014 applied label-free LC–IM–DIA–MS to demonstrate that RSL3 binds to and inhibits GPX4, which regulates ferroptotic cancer cell death. Figure 1.5 contains a 3D representation of the isotopic clusters of peptide IAFPCNQFGK from GPX4 analyzed by LC–DIA–IM–MS. Detection and

![Figure 1.4 3D dot plot representation of the positions of peaks (in the retention time, drift time, and m/z dimensions) that are obtained from the \( 1 \times 10^5 \) most intense features (light gray) observed during the triplicate LC–IMS–MS analyses of all SCX fractions associated with Sample 1. Superimposed on the plot are the positions for \( >10,000 \) features that have been assigned to peptides (dark gray). The arrows indicate some of the precursor ion positions of peptides identified for the four proteins labeled. This representation is intended to provide the reader with the impression that the possible existence of abundant protein in plasma could be tested at many positions in the map and therefore upon comparison there should be little ambiguity regarding its detection, whereas a low-abundance protein may be represented at only a single position, leading to uncertainty about its detection. (Source: Liu et al. 2007. Reproduced with permission of Springer.)
identification was conducted by dedicated software. The results shown in Figure 1.5 illustrate the presence of GPX4 with RSL3 active probe treatment and its absence when the probe was inactive or a competitor was present. It was derived and concluded that RSL3 to inhibit GPX4, a protein essential for cancer cell viability. Numerous applications describe the use of LC–DIA–IM–MS for the label-free quantification, as described in a recent review describing DIA and its application (Distler et al. 2014b).

### 1.3.3 Sensitivity

A more recent application of IM–MS is described by Helm et al. 2014 who used the technique to increase MS/MS sensitivity in untargeted data-dependent analysis (DDA) and targeted parallel reaction monitoring (PRM) such as proteomic LC–MS experiments on a commercial hybrid quadrupole – ion mobility – time-of-flight mass spectrometer. This technique, as will be demonstrated, enhances the duty cycle of the oa-TOF analyzer and thus sensitivity. Briefly, as shown previously, TWIMS separation is strongly dependent upon ion charge \( z \). Moreover, ions are nested for a given charge state by mass and drift time. This charge state separation and nesting can be used to discriminate against single charge background and to exclusively select multiply charged peptides for tandem MS. Subsequently, precursor ions are sequentially selected by the quadrupole mass analyzer and fragmented by CID in the first stacked-ring ion guide of the triwave device and prior to reaching the ion mobility cell. Product ions are trapped within this first travelling wave region of the triwave device and gated into the high-pressure ion mobility cell where they are separated according to their gas-phase mobility within the cell. As a result, as illustrated in Figure 1.6, fragment ions of the same mobility exit the cell as a series of compact packets. Hence, by synchronizing the pusher pulse that accelerates the fragment ions into the oa-TOF mass analyzer with the arrival of product ions from the TWIMS cell into the pusher region, fragment ions are sequentially injected into the TOF analyzer with greatly enhanced duty cycle (~100%) across the mass scale. This synchronization leads to a concomitant increase in sensitivity, which is reflected by the results shown in Figure 1.7, where the percent identified DDA spectra versus amount protein digest on column is contrasted. On average, a 10-fold increase in peptide MS/MS sensitivity can be observed (Helm et al. 2014). Since the ion mobility time frame is in the order of milliseconds, it nests well between the second time frame of liquid chromatography and that of the oa-TOF mass spectrometer that operates in the microsecond time frame.

An example of an IM-enabled targeted high-resolution multiple reaction monitoring (HR-MRM) experiment is shown in Figure 1.8. In HR-MRM, the last quadrupole of a tandem quadrupole instrument is substituted with a high-resolution mass analyzer to allow parallel detection of all product ions in a single, high-resolution, accurate mass experiment. Here, unlike the previously described experiment, peptide precursor masses, including internal standards, are predefined, along with their retention time and CID collision energy profile. The principle of product ion enrichment to increase duty cycle and MS/MS sensitivity is identical. In this particular example, a number of putative cardiovascular disease plasma proteins were quantified (Domanski et al. 2012). As an example, shown in the top pane of Figure 1.8, are the summed product ion extracted mass chromatograms of ATEHLSTLEK from Apolipoprotein A-1 and its labeled internal standard analog, as well as product ion spectra of both peptides. The calibration curve of heavy labeled ATEHLSTLE[K] is shown in the bottom pane of Figure 1.8 from which an Apolipoprotein A-1 serum concentration of 1.403 mg/mL can be calculated.

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**Figure 1.5** Confirmation of GPX4 binding to an active affinity probe. (a) Cell lysates prepared from cells treated with active probe (A), inactive probe (I), or active probe in the presence of competitor (A + C) that were affinity purified by α-fluorescein antibodies and probed for GPX4 by western blot using GPX4-specific antibody. (b) 3D visualization of isotopic clusters of peptide ILAFPCNQFGK from GPX4 as analyzed by LC–DIA–IM–MS. (Source: Yang et al. 2014. Reproduced with permission of Elsevier.)
Figure 1.6 Principle IM-enabled DDA with asynchronous pusher operation (a) and synchronization of a pusher pulse with product ion drift time (b).

Figure 1.7 Increased MS/MS sensitivity expressed as number of identified peptides for normal DDA (white) and IMS-enabled DDA (black).
This concentration estimation compares well with an average reported literature value of 1.400 mg/mL (Domanski et al. 2012). The linear dynamic range was at least three orders of magnitude; however, the great benefit of high-resolution PRM is its high selectivity in the mass-to-charge domain since high-resolution MS can often separate out interferences from the product ion of interest (Mbasu et al. 2016).

1.4 Outlook

Ion mobility coupled with mass spectrometry has made significant strides since the turn of the twenty-first century. Despite IMS coupled with TOF MS first being described in the 1960s, it is only in the last 20 years that improvements in electronics and the performance of TOF mass analyzers has allowed IMS–MS to become a mainstream platform. As electronic improvements still continue to track Moore’s law (Waldrop 2016), this should allow concomitant improvements in the underlying performance of both IMS and oa-TOF technologies, resulting in higher resolving power, as well as faster sampling rates. Improvements in IMS resolution have already been described and the unique hybrid combination of IMS with TOF should allow the comprehensive profiling of complex heterogeneous samples.

The outlook for the future is promising. The use of rotationally averaged collision cross sections as a means

Figure 1.8 Summed product ion extracted mass chromatograms of “light” labeled ATEHLSTLSEK and “heavy” labeled ATEHLSTLSE[K] (a), corresponding part product ion MS/MS spectra, respectively (b), extracted product ion mass chromatograms “light” ATEHLSTLSEK (c), and calibration curve “heavy” ATEHLSTLSE[K] (d).
for identification and confirmation of compound identity is an intriguing prospect, providing a physicochemical supplement to retention time and tandem MS information. The limiting factor is currently the lack of CCS measurements populated into compound libraries and the lack of computational tools to rapidly generate CCS values from compound structure. Developments in these areas will undoubtedly occur and make the routine use of IMS information for identification purposes a powerful technology. In summary, the next few years should see significant improvements in both the technology and the informatics and workflows to use the information generated for both qualitative and quantitative analyses.

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References


