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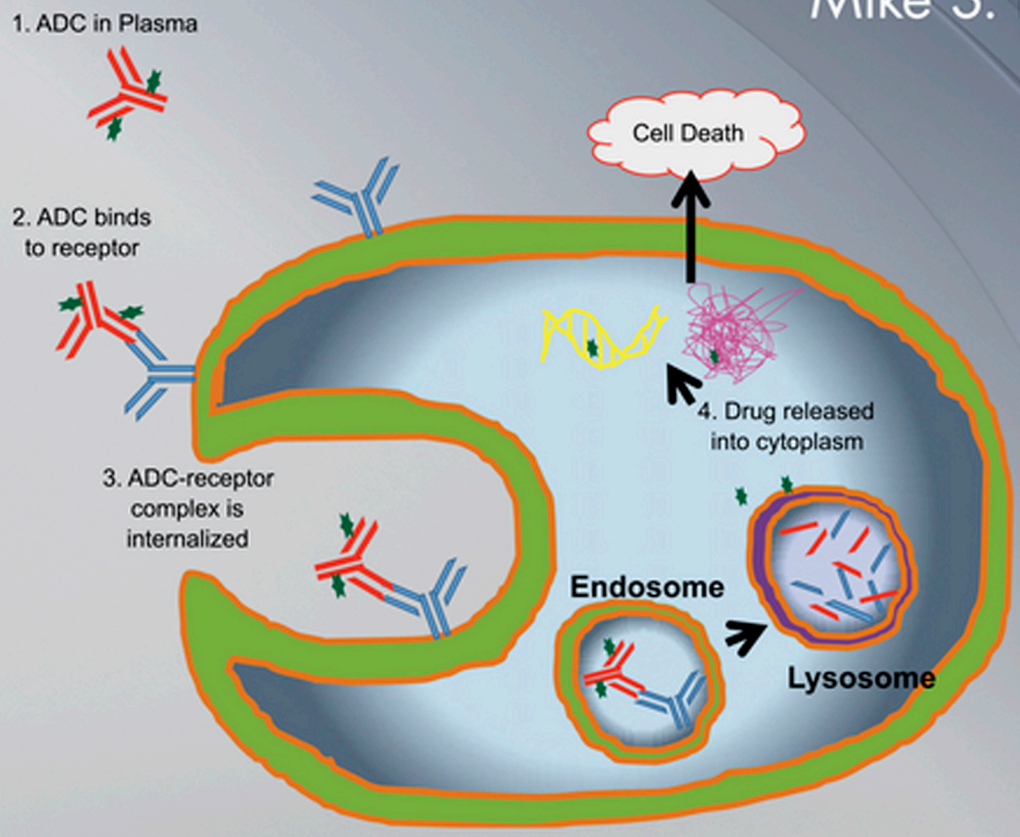
PROTEIN ANALYSIS USING MASS SPECTROMETRY

Accelerating Protein Biotherapeutics from Lab to Patient

Mike S. Lee and Qin C. Ji



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Protein Analysis using Mass Spectrometry

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Foreword

This book explores recent advances in mass spectrometry and related technology, and the innovative approaches used in measuring and characterizing peptides and proteins as part of bringing new medicines to patients in need. Qin and Mike have brought together a wide range of leading scientists to provide a clear picture of the variety and depth of technology and techniques.

As you will see in each chapter, fundamental LC–MS knowledge has been used in each innovative advance. Sample preparation techniques for peptides and proteins rely on the core of historic approaches used for small molecule drug analyses but have been expanded to address a host of requirements related to protein structure, including reduction and alkylations, acid dissociation, protein digestion, and the specificity possible with immunocapture. Liquid chromatography techniques from regular to ultrahigh-performance approaches and downward to micro- and nanoflow are covered, as well as utilization of 2-D chromatography. Triple quadrupole and high-resolution mass spectrometers, with their recent advances in sensitivity and selectivity, are prominent in the discussions as their advances are central to making possible many advances in peptide and protein analyses.

I hope that the readers find this book to be an engaging learning experience; one that provides insights and causes a cascade to the discovery of further advances in peptide and protein analysis by liquid chromatography mass spectrometry.

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Preface

We had a discussion on LCMS analysis of proteins for drug development dating back to the early 2000s. At that time, Qin's group at Abbott Laboratories had just published a manuscript in analytical chemistry for an LCMS bioanalytical method for a small protein (MW > 10 kDa). Through the years, multiple discussions on the topic continued at various conferences, including conversations held at several Annual Land O'Lakes Bioanalytical Conferences where Mike was invited to give lectures. Although mass spectrometry protein analysis has been a popular topic in proteomic research for several decades, it was only in the late 2000s it started to receive increasing attention of scientists in drug development. In this book, we present 16 chapters from industry leaders who have first-hand experience in developing new mass spectrometry technologies, knowing the issues and needs of the analysis in drug discovery and development, forming assay strategies, and interpreting assay results with their respective project teams.

The authors of Chapters 1–4 have experience and expertise with mass spectrometry instrumentation as well as with analytical research and development. Johannes and James from Waters discussed extensively the history and theory of ion mobility mass spectrometry and its application in protein analysis. As they pointed out, "The next few years should see significant improvements in both the technology, and the informatics and workflows to use the information generated from ion mobility mass spectrometry for both qualitative and quantitative analyses." In Chapters 2 and 3, Jessica, Zhiqi, and their colleagues discuss the characteristics and capabilities of high-resolution mass spectrometry, especially, the Thermo Orbitrap mass spectrometry and its application in protein therapeutics bioanalysis and the characterization of posttranslational modifications in therapeutic proteins. In Chapter 4, Suma and her colleagues from SCIEX discuss the workflow of quantitative analysis of proteins using mass spectrometry, especially the triple quadrupole time-of-flight mass spectrometry system. Although the benefit of using low flow liquid chromatography mass spectrometry has been well understood theoretically and widely used in the proteomic research area, the application of this technology in quantitative analysis of proteins in biological matrix is still not widely accepted. In Chapter 5, Shane and Gary describe the success and routine usage of New Objective's integrated nanoflow LC column and nanoelectrospray emitter system for the bioanalysis of proteins in biological matrices with excellent assay ruggedness and high assay throughputs. Jiang at Shire is one of the industry leaders in drug discovery mass spectrometry. Jiang comments that understanding relative expression and structure–function relationship of the splice isoforms are essential for the discovery and development of more specific therapeutics and biomarkers. In Chapter 6, Jiang describes the advanced mass spectrometry characterization of gene splice variants in conjunction with high-throughput transcriptomics as an example of protein mass spectrometry analysis in proteomic research for supporting drug discovery. Bradley and Michael from Lilly are among the pioneers in mass spectrometry biomarker analysis. In Chapter 7, they provide a comprehensive review of the immunoaffinity mass spectrometry technology and its application in protein biomarkers and biotherapeutics characterization. Immunogenicity refers to immune responses of humans or animals to antigens, such as biotherapeutics. The technologies, methodology, and regulatory requirements for the immunogenicity test evolved rapidly in recent years. In Chapter 8, Jianing and her coworkers at BMS describe recent advances in using immunocapture LCMS for immunogenicity assessment from "semiquantitative analysis of antidrug antibody" to "assisting the method development of cell-based neutralizing antibody assays." Keqi is well known in the mass spectrometry field for his design of mass spectrometry ionization sources and ion optics for high ion transfer efficiency. In Chapter 9, Xuejiang Guo and Keqi from PNNL discuss recent advances in methodology and mass spectrometry instrumentation for the sensitive and high-throughput mass spectrometry biomarker analysis. In Chapter 10, Tong-Yuan and his coworkers at JNJ describe the mass spectrometry ligand binding assay reagent

characterization, which is one of the fast growing areas in the bioanalytical scientific field and has shown significant impacts on improving ligand binding bioanalytical assays. In Chapter 11, Stanley and his coworkers at JNJ describe the recent advances in using high-resolution mass spectrometry in improving selectivity for the mass spectrometry bioanalysis of proteins in biological matrices. In Chapter 12, Hongyan and his coworkers at Amgen discuss the advantages and their assay development strategy of LCMS quantitative analysis of therapeutic monoclonal antibodies (mAbs) in biological matrices in supporting preclinical studies. In Chapter 13, Michael at PPD discusses generic peptide strategies (he is one of the pioneers who developed this approach) for LC–MS bioanalysis of human monoclonal antibody drugs and drug candidates. The advantages of this strategy include significant cost saving and accelerated progress for drug discovery and early drug development. In Chapter 14, Y-J and his coworkers at Celgene describe comprehensively the strategy and methodology of mass spectrometry support of antidrug conjugate (ADC) drug development, one of the most active areas recently in drug development. In Chapter 15, Long and Qin at BMS provide a survey of the sample preparation strategies for LCMS protein bioanalysis, which range from traditional organic solvent protein precipitation, solid-phase extraction to more advanced chemical derivatization, and immunocapture sample preparation. In Chapter 16, Wei and his coworkers at BMS describe the mass spectrometry characterizations of protein therapeutics in drug manufacturing process to ensure the quality and integrity of dug product ingredients.

We would like to take this opportunity to thank all the authors for their diligent work in describing the advances in the protein mass spectrometry analysis in supporting from early-stage basic researches to delivering the safe, efficacious drug to patient bedside. We also would like to thank Wiley for the opportunity to bring this book to our readers, which will further stimulate the advances of mass spectrometry technology and methodology to benefit patients' lives.

Mike S. Lee and Qin C. Ji
December 2016
Princeton, NJ

1

Contemporary Protein Analysis by Ion Mobility Mass Spectrometry

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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:

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

where K = drift velocity v_d/electric field strength E, μ = reduced mass of the ion (neutral given by $(m_{neutral}m_{ion})/(m_{neutral}+m_{ion})$, k_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 Ω = 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

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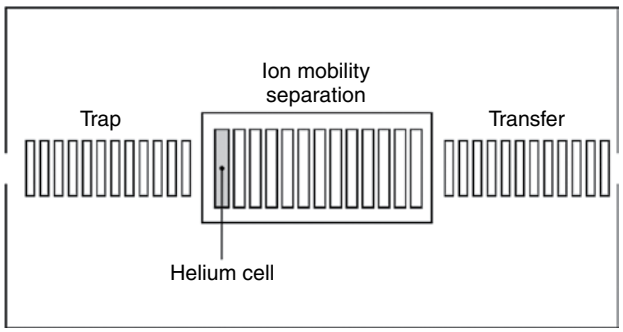

Figure 1.1 Triwave ion mobility optics detail comprising a trap, helium cell, ion mobility separator and transfer. (*Source*: Williams et al. 2012. Reproduced with permission of GIT.)

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 = drift velocity v_d/electric field strength E and Ω = 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

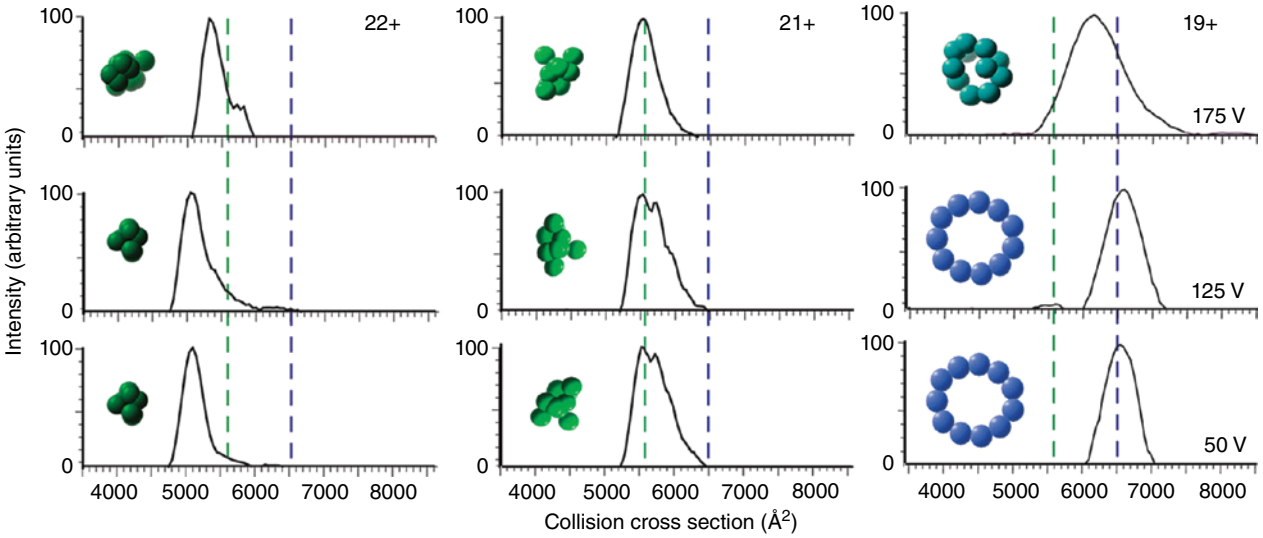

Figure 1.2 Ion mobility data for selected charge states of apo-TRAP (19+, 21+, and 22+) as a function of activation energy (175, 125, and 50 V) applied in the high-pressure, sampling cone region of the instrument. The light gray and dark gray dashed lines represent the collision cross sections for collapsed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

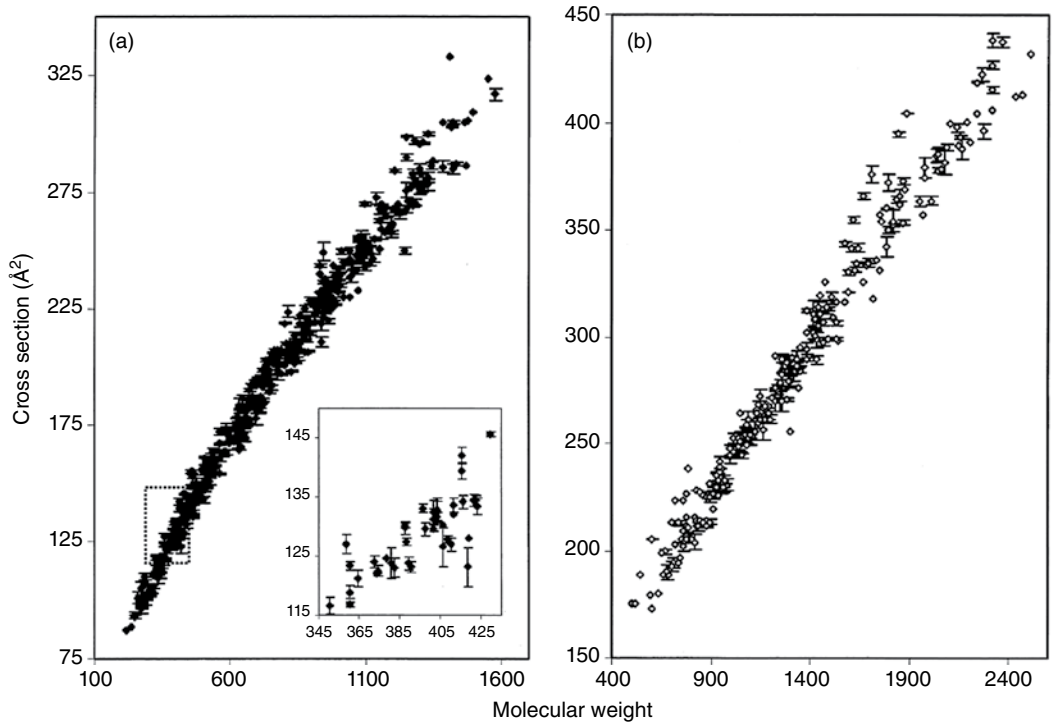

Figure 1.3 (a) Cross sections for 420 $[M+H]^+$ peptides (solid diamonds) as a function of molecular weight. Uncertainties correspond to one standard deviation or to a range. The inset shows variations in cross sections for $[M+H]^+$ peptides over a smaller molecular weight range (defined by the dashed-line 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.).

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

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 in-line 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 physicochemical 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 ILAFPCNQFGK from GPX4 analyzed by LC–DIA–IM–MS. Detection and

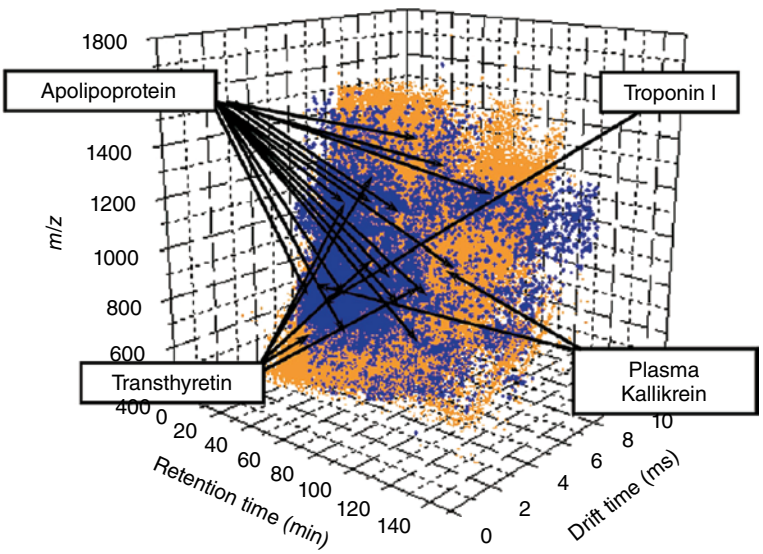

Figure 1.4 3D dot plot representation of the positions of peaks (in the retention time, drift time, and *m/z* dimensions) that are obtained from the 1×10^5 most intense features (light gray) observed during the triplicate LC–IMS–MS analyses of all SCX fractions associated with Sample 1. Superimposed 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.).

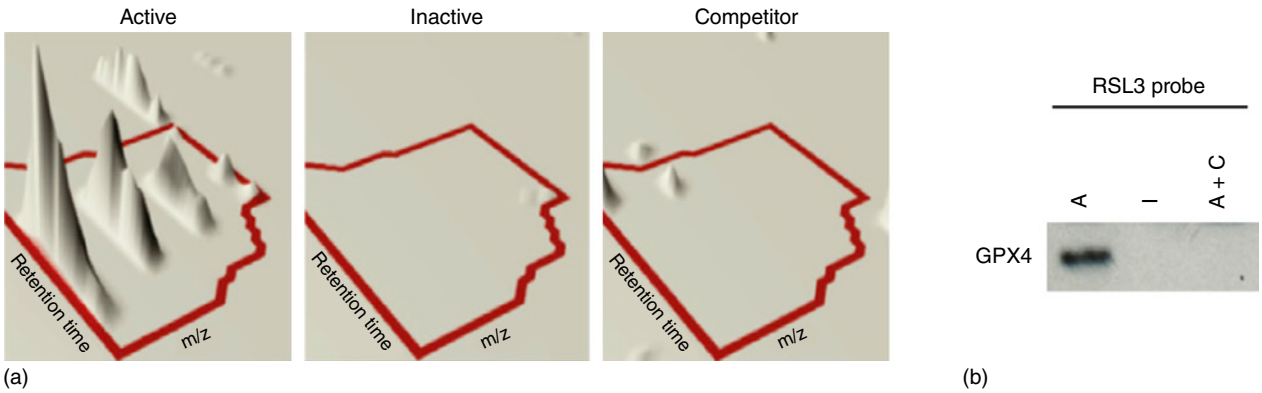

Figure 1.5 Confirmation of GPX4 binding to an active affinity probe. (a) Cell lysates prepared from cells treated with active probe (A), inactive probe (I), or active probe in the presence of competitor (A + C) that were affinity purified by α-fluorescein antibodies and probed for GPX4 by western blot 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.)

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 ATEHLSTLSEK 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 ATEHLSTLSE[K] is shown in the bottom pane of Figure 1.8 from which an Apolipoprotein A-I serum concentration of 1.403 mg/mL can be calculated.

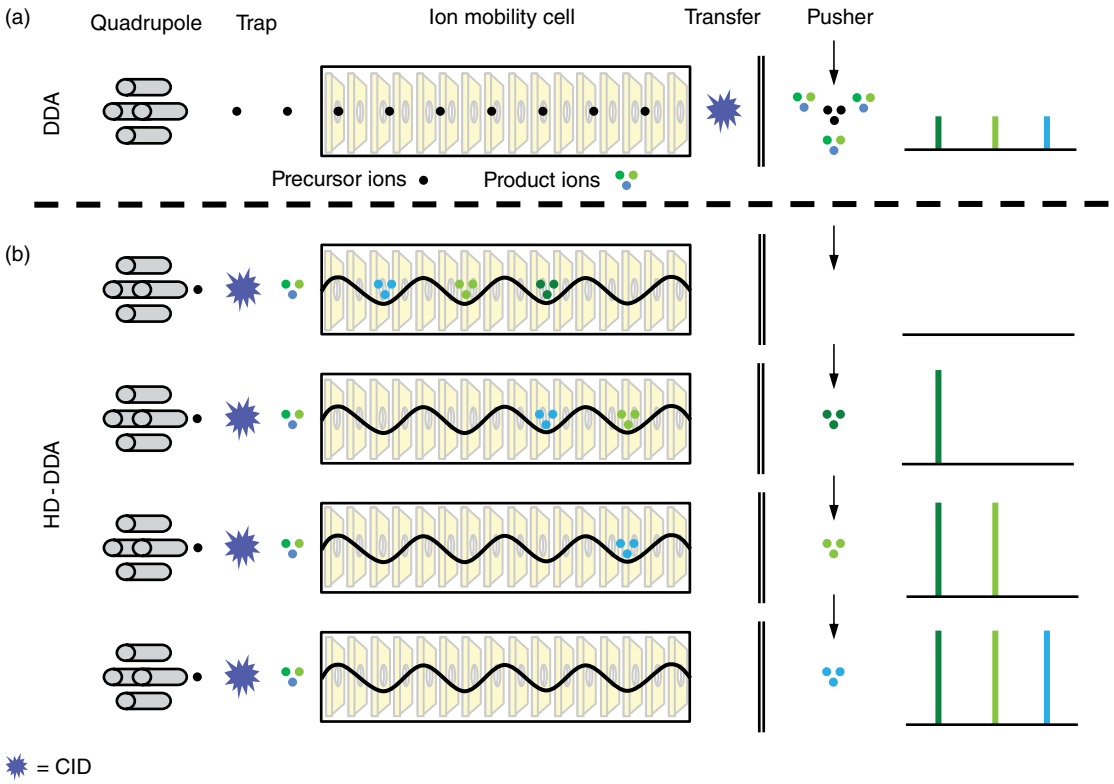

Figure 1.6 Principle IM-enabled DDA with asynchronous pusher operation (a) and synchronization of a pusher pulse with product ion drift time (b).

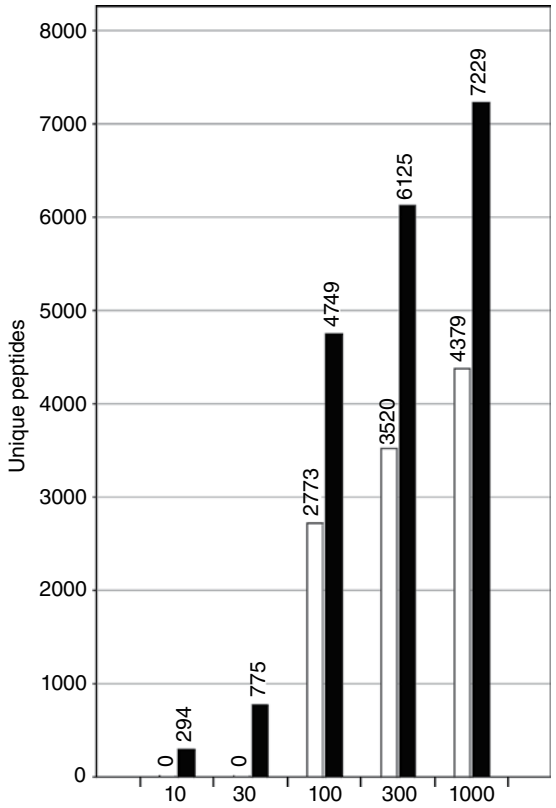

Figure 1.7 Increased MS/MS sensitivity expressed as number of identified peptides for normal DDA (white) and IMS-enabled DDA (black).