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Adding a new separation dimension to MS and LC-MS: what is the utility of ion mobility spectrometry?

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RUNNING TITLE: IM-MS and its applications

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28 **LIST OF ABBREVIATIONS**

- 29 2D-LC: two-dimensional liquid chromatography
- 30 AD: alzheimer disease
- 31 ADC: antibody drug conjugate
- 32 AmtB: ammonia channel
- 33 APCI: atmospheric pressure chemical ionization
- 34 APols: amphipatic polymers
- 35 AqpZ: aquaporin Z
- 36 A·T: adenine thymine
- 37 ATD: arrival time distribution
- 38 bsAb: bispecific antibodies
- 39 BSM: bovine sumaxillary mucin
- 40 CCS: collisional cross section
- 41 CDL: cardiolipin
- 42 C·G: cytosine-guanine
- 43 CID: collision induced dissociation
- 44 CIU: collision induced unfolding
- 45 CRISP : clustered regularly interspaced short palindromic
- 46 CRISPR : clustered regularly interspaced short palindromic repeats
- 47 CZE: capillary zone electrophoresis
- 48 DAR: drug-to-antibody-ratio
- 49 DBD: DNA-binding domain
- 50 DC: differential current
- 51 DDA: data-dependent acquisition
- 52 DESI: desorption electrospray ionisation
- 53 DFT: density functional theory
- 54 DHSS: diffuse hard spheres scattering
- 55 DIA: data-independent acquisition

56 DIMS: differential ion mobility spectrometry

57 DMA: differential mobility analyser

58 DMS: differential mobility spectrometry

59 DNA: deoxyribonucleic acid

60 $^{DT}CCS_{He}$: collisional cross section calculated with drift tube ion mobility spectrometry and
61 helium as buffer gas

62 $^{DT}CCS_{N_2}$: collisional cross section calculated with drift tube ion mobility spectrometry and
63 nitrogen as buffer gas

64 DTIMS: drift tube ion mobility spectrometry

65 DTM: diffuse trajectory method

66 EED: electron excitation dissociation

67 EHSS: exact hard sphere scattering

68 ESI: electrospray ionization

69 ETD: electron-transfer dissociation

70 FAIMS: field asymmetric waveform ion mobility spectrometry

71 FDA: food and drug administration

72 FID: flame ionization detector

73 FRET: fluorescence resonance energy transfer

74 GC: gas chromatography

75 HCP: host cell proteins

76 HD-DDA: high definition data direct analysis

77 HDX-MS: hydrogen/deuterium exchange mass spectrometry

78 HIC: hydrophobic interaction chromatography

79 HMGA: high-mobility group A

80 HRMS: high resolution mass spectrometry

81 IAPP: islet amyloid polypeptide

82 IDP: intrinsically disordered proteins

83 IgG: immunoglobulin G

84	IM: ion mobility
85	IM-MS: ion mobility - mass spectrometry
86	IMS: ion mobility spectrometry
87	IMS-QTOF: ion mobility spectrometry quadrupole time-of-flight
88	IR: infrared radiation
89	IRMPD: infrared multiphoton dissociation
90	LC: liquid chromatography
91	LC-IMS-MS: liquid chromatography - ion mobility - mass spectrometry
92	LC-MS: liquid chromatography-mass spectrometry
93	LCPA: local collision probability approximation
94	LOD: limit of detection
95	mAb: monoclonal antibody
96	MALDI: matrix-assisted laser desorption ionisation
97	MC: Monte Carlo
98	MD: molecular dynamics
99	MS: mass spectrometry
100	MS/MS: tandem mass spectrometry
101	MscL: mechanosensitive channel of large conductance
102	NMR: nuclear magnetic resonance
103	PA: projection approximation
104	PASEF: parallel accumulation–serial fragmentation
105	PDB: protein data bank
106	PG: phosphatidylglycerol
107	PSA: projection superimposition approximation
108	PTM: posttranslational modifications
109	QM: quantum mechanical
110	QqTOF: quadrupole-time-of-flight
111	QTRAP: triple-quadrupole linear ion trap mass spectrometer

112	RF: radio frequency
113	rhGH: recombinant human growth hormone
114	RNA: ribonucleic acid
115	SASA: solvent-accessible surface area
116	SEC: size exclusion chromatography
117	SFC: supercritical fluid chromatography
118	SID: surface induced dissociation
119	SLIM: structures for lossless ion manipulations
120	sno: small nucleolar
121	SRIG: stacked-ring ion guide
122	sRNPs: small nucleolar ribonucleoprotein particles
123	T ₁₀ : thymine 10
124	T2DM: type 2 diabetes mellitus
125	Td: Townsends unit
126	TIMS: trapped ion mobility spectrometry
127	TIMS-MS: trapped ion mobility spectrometry - mass spectrometry
128	^{TIM} CCS _{N₂} : collisional cross section calculated with trapped ion mobility spectrometry and
129	nitrogen as buffer gas
130	TM: trajectory method
131	TOF: time-of-flight
132	TRAP: trp RNA binding attenuation protein
133	TTR: tetrameric transthyretin
134	^{TW} CCS _{N₂} : collisional cross section calculated with travelling wave ion mobility spectrometry
135	and nitrogen as buffer gas
136	TWIMS: travelling wave ion mobility spectrometry
137	VUVPD: vacuum ultraviolet photo dissociation
138	VT-IM-MS: variable temperature ion mobility-mass spectrometry
139	wt: wild type

140 XUV: extreme ultraviolet light

141 **Abstract**

142 Ion mobility spectrometry is an analytical technique known for more than 100 years, which
143 entails separating ions in the gas phase based on their size, shape and charge. While IMS
144 alone can be useful for some applications (mostly security analysis for detecting certain
145 classes of narcotics and explosives), it becomes even more powerful in combination with MS
146 and HPLC. Indeed, the limited resolving power of ion mobility spectrometry alone can be
147 tackled when combining this analytical strategy with MS or LC-MS.

148 Over the last few years, the hyphenation of ion mobility spectrometry to MS or LC-MS attracted
149 more and more interest, with significant progresses in both technical advances and pioneering
150 applications. This review describes the theoretical background, available technologies and
151 future capabilities of these techniques. It also highlights a wide range of applications, from
152 small molecules (natural products, metabolites, glycans, lipids) to large biomolecules (proteins,
153 protein complexes, biopharmaceuticals, oligonucleotides).

154

1. Introduction

During the last decade, ion mobility spectrometry (IMS) has been applied in several fields, starting from the analysis of chemical warfare agents, explosives and illicit drugs to the analysis of biological samples [1–4]. Nowadays, the increased complexity of the samples (e.g. natural products analysis, biopharmaceutical characterization, -omic samples such as proteins mixture in proteomics or biofluids in metabolomics or lipidomics, etc) demands for analytical methods able to offer very high resolving power to achieve analytical goals and acquire sufficient information from the sample.

The addition of IMS dimension to the existing analytical setup can represent an attractive strategy to achieve this goal. [4] Indeed, IMS is a gas-phase separation technique that allows the separation of ions generated by a source (frequently by electrospray ionization, ESI) driven through an ion mobility cell filled with an inert gas (generally nitrogen or helium) under the influence of an electric field. Once in the IM drift cell, ions are separated based on their mobility that accounts for their size, shape and charge. Then, based on the type of IMS separator, the mobility can also allow the determination of the collisional cross section (CCS), a physical property related to the shape of the ion for a given compound in a specific gas environment. The CCS is an important property that could provide an improved characterization of different compounds, such as small molecules, proteins, lipids or glycans, as well as a better understanding of the intramolecular forces [5,6]. A detailed discussion about CCS determination will be addressed in section 2.

As illustrated in Figure 1, IMS combined with mass spectrometry (MS), which is also known as IMSxMS or IM-MS, has seen a spectacular growth in the last 20 years. Indeed, the information obtained from IMS and MS can be considered as complementary and IMS can potentially allow the separation of isobaric ions (unresolved by MS) based on their shape differences [7]. Besides IMS and MS, a third separation dimension, such as liquid chromatography (LC) or another front-end separation technique such as supercritical fluid chromatography (SFC), gas chromatography (GC), or capillary zone electrophoresis (CZE) [8] can also be included in the analytical setup. As shown in Figure 1, this three-dimensional separation (LC-IM-MS) is still

less widespread than IMS alone or IM-MS, but is beginning to attract increasing interest for the analyses of complex samples. As highlighted in Figure 2, LC-IM-MS allows the separation of the different constituents of a mixture based on their lipophilicity (LC), shape (IMS), mass (MS) and charge (IMS and MS). It also offers the possibility to visualize the analytical results in several dimensions, using a plot of MS spectra information vs. IM drift times, for each chromatographic peak.

Another important aspect is the time scale of IMS (in the milliseconds range), which fits very well between the chromatographic step (in the minutes range) and MS experiment (in the microseconds time scale). Because of the fast duty cycle of time-of-flight based mass spectrometer (TOF and QqTOF), these instruments appear to be the most suitable analysers for IM-MS [6].

This review paper describes the theoretical background of IM-MS technology, available instrumentation and future capabilities of IM-MS. It also highlights a wide range of recent applications of IM-MS and LC-IM-MS, ranging from small molecules to large biomolecules.

2. Description of the available ion mobility technologies

Several ion mobility technologies have been developed in the last decades, each having its own principle of operation and design. According to May and McLean [9], there are three main separation concepts employed in IMS, namely i) time-dispersive methods, which generate an arrival time spectrum, with all ions drifting along the same pathway (i.e. drift tube ion mobility spectrometry (DTIMS), travelling wave ion mobility spectrometry (TWIMS)), ii) space-dispersive methods, which separate ions along different drift paths, based on their mobility differences (i.e. field asymmetric waveform ion mobility spectrometry (FAIMS), differential mobility analyser (DMA)) and iii) ion trapping followed by selective release, which traps ions within a pressurized region and selectively ejects the ions based on their mobility differences (i.e. trapped ion mobility spectrometry (TIMS)). All the IMS techniques described in this section can be coupled with MS [7], but performance and possibilities may vary, particularly in terms of achievable limits of detection (LOD), resolving power (peak capacity) and possibility to

accurately calculate CCS values. The main advantages, limitations and a list of available commercial instruments for the different IMS technologies are described in this section and summarized in Table 1. Last but not least, it is important to keep in mind that DTIMS and TWIMS are the main choice for proteins, but have also been successfully used for small molecules. On the other hand, FAIMS, DMA are only dedicated to the analysis of small molecules. TIMS is a relatively new strategy, which also has the potential to be used for both proteins and small molecules. However, it has only been scarcely employed for proteins and more investigations are needed to prove its applicability for large biomolecules.

2.1. Drift tube ion mobility spectrometry

Drift tube ion mobility (DTIMS) is the historical and simplest form of IMS that was assembled for the first time in the 1970s, originally in combination with a single quadrupole mass analyser [10]. The schematic design of DTIMS is shown in Figure 3a. This technology is composed of a tube (the drift cell) filled with an inert gas, namely the buffer gas. [2] Nitrogen or helium are the most common buffer gas in DTIMS [11]. The DTIMS cell is composed of a series of piled electrodes, and each electrode generates a weak uniform electric field (5-100 V) [2] able to induce the movement of the ions through the tube. For example, on the Agilent DTIMS-MS instrument, 1000-1400 V is typically used in a 78 cm drift cell, corresponding to a field strength of around 13-18 V/cm. The mobility of ions along the cell is determined by the number of collisions with the buffer gas, which slows down the ions: For a given charge, compact ions drift faster than elongated ones, because they experience fewer collisions with the buffer gas. [5] The time required to cross the cell is defined as the drift time and the separation of the ions occurs as a function of their shape, which is physically related to the mobility (K) or rotationally averaged CCS value. However, the electric field has to be maintained sufficiently low to observe a good correlation between mobility and shape/size.[7] As ions exit the tube, they are analysed and detected as a function of time, yielding an arrival time distribution (ATD). The first home-made DTIMS generally suffered from an inherent lack of sensitivity, mainly due to diffusion issues and loss in duty cycle that was related to the pulsed analysis. However, in

2014 a commercial DTIMS, using nitrogen drift gas and offering a good compromise between sensitivity, length of drift tube, and optics, was developed by Agilent Technologies. In particular, the instrument incorporates a tandem ion funnel interface before the drift tube that acts as a focusing and accumulation region, and a second ion funnel after the drift tube that refocuses the ions through an hexapole ion guide prior to reaching the QqTOFMS. The Agilent DTIMS-MS instrument includes a QqTOF mass analyser, offers a high precision of CCS values (below 0.5% [12]), a high resolving power (thanks to the length of the tube extended to 78 cm combined with the precision electronics) and sensitivity compatible for protein/peptide analyses [11,13]. Another DTIMS instrument commercially available is manufactured by TOFWerk (Switzerland). This instrument contains a 20 cm drift cell operated at high pressure (i.e. 1-1.4 atm) and elevated temperature (e.g. 60°C), which provides greater resolution, while maintaining low-field conditions, but is limited to low molecular weight molecules.

2.2. Travelling wave ion mobility spectrometry

Traveling wave ion mobility (TWIMS) is a more recent IMS technology that was first described in 2004 [14] and made commercially available in 2006 (Waters Synapt HDMS systems) [15]. This high performance instrument has significantly advanced the field of IM-MS thanks to its versatility and widespread availability [16]. Similarly to DTIMS, TWIMS is a time-dispersive method, even though it uses a dynamic application of the electric field to separate the ions in gaseous phase (often nitrogen and occasionally helium). It consists of a stacked-ring ion guide (SRIG) where positive and negative radio frequency (RF) voltages are periodically applied to adjacent ring electrodes to provide radial ion confinement while a pulsed differential current (DC) voltage is applied to axially propel the ions [2]. Therefore, rather than applying a linear electric field through the full length of the mobility cell, like in DTIMS, the electric field in TWIMS forms a wave whose magnitude and speed determine the ions separation, as illustrated in Figure 3b. By surfing on the waves, larger ions experience larger friction with the buffer gas and slip more often behind the waves, thus taking more time to exit the mobility cell as compared to smaller ions. In TWIMS, CCS can also be determined from the drift time, but only

after calibration by using a set of reference compounds of known CCS values. In addition, the TWIMS technology takes less space than DTIMS (DTIMS needs around 1 meter to be installed in front of the mass spectrometer) and is more versatile, since it allows to perform CID before and after Ion mobility (CID could only be performed after ion mobility with DTIMS) on the commercial instruments (this is a choice of the constructors rather than a theoretical limitation). As described in [9], the original Waters TWIMS technology underwent a major design modification in 2009 (Synapt G2), allowing 4-fold improvement of the accessible resolution, thanks to the use of higher electric fields and higher gas pressure [17]. In 2011 and 2013, two new instruments were released by Waters, namely Synapt G2-S and G2-Si, allowing an improvement of the ion transmission through the IM-MS and a wide range of experimental options [9]. Finally, Waters has released a benchtop instrument in 2016 (Vion IMS QToF), which includes in part the technology of the Synapt, but with a different design (the mobility cell is located before the first quadrupole of the QqTOF/MS). Indeed, this instrument is dedicated to non-experts who want to add an IMS dimension (determination of CCS values, removal of spectral interferences) to routine analyses often performed in MS or LC-MS.

2.3. Field asymmetric waveform ion mobility spectrometry

Field asymmetric waveform ion mobility spectrometry (FAIMS) is also known as differential ion mobility spectrometry (DMS or DIMS). The principle of operation of FAIMS is illustrated in Figure 3c [18]. FAIMS utilizes two electrodes between which a high asymmetric electric field (up to 10000 V/cm, also called dispersion field) is applied at reduced pressure (but can also operate at atmospheric pressure) [19,20] and room temperature. The carrier gas (often nitrogen, but there is a lot of flexibility to use some alternative gas or even dopants) is injected with the ions in the same direction. A compensation voltage is then superimposed to the dispersion field and it can be tuned for ions with a particular mobility [21]. By applying a suitable compensation voltage, the trajectory of the desired ions can be corrected along the axis of the cell to permit these ions to leave the cell. All other ions will migrate away from the straight line

due to their different mobility. A full spectrum can finally be collected by scanning the compensation voltage.

Unlike DTIMS and TWIMS, FAIMS does not separate ions based directly on their mobility, but rather according to the ratio of mobilities in a low-field and high-field. Therefore, the FAIMS separation is much more orthogonal to m/z than other IMS modes (see section 8.2 for more explanations). However, FAIMS is actually considered to be a filtering technique (or selective scanning technique) rather than structure characterization tool, since calculation of CCS is not possible [2]. Very high resolutions of up to several hundreds were reported in FAIMS, but this was only possible when a mixture of gases was employed (He/N₂) [22]. In addition, FAIMS resolution cannot be directly compared to that of any other IMS technique described in this review, since the separation phenomenon is different as is the scaling of the axes [16].

Several companies have commercialized FAIMS devices, including Thermo Fisher Scientific, AB SCIEX and Owlstone. However, the most active provider is certainly SCIEX which utilises FAIMS technology (SelexION) in several MS platforms, including QTRAP (Sciex 5500, 6500 and 6500+ series) and QqTOFs (Sciex TripleTOF 5600+ and 6600) analysers. On Thermo Fisher Scientific instruments, the FAIMS interface can be added to existing triple quadrupole, ion trap MS analysers and high-resolution orbital ion trap mass analysers [23].

2.4. Differential mobility analyser

Differential mobility analyser (DMA) is another form of IMS where the ions with different mobilities are separated in space (rather than in time) at atmospheric pressure and relatively low electric field. Two cylindrical and concentric metal electrodes constitute the usual DMA design. A planar scheme of the DMA design is shown in the Figure 3d [24]. As illustrated in this figure, the ions are injected along an electrical field (directed from top to bottom of the scheme) between the two electrodes. An orthogonal flow of sheath gas from the left to the right of the scheme pushes ions down from an inlet slit to the exit slit. Then, the ions whose mobility matches the length from inlet slit to exit slit, at the used buffer gas flow and the electric field, will reach the exit slit and will be directed towards the MS analyser [25].

The other ions, which do not match this mobility, will be eliminated on the lens that acts as a wall. Only the ions with the targeted mobility will leave the DMA and the variation of the electric field permits to select ions with different mobilities. Similarly to FAIMS, DMA is also considered as a selective filter, but operates in the low-field regime. An important limitation of DMA is that it does not allow the transmission of ions having diverse mobilities. The commercial availability of DMA technology is relatively limited, and only a company called SEADM sells DMA systems that can be directly coupled to MS.

2.5. Trapped ion mobility spectrometry

Trapped ion mobility spectrometry (TIMS) is a technology that appeared very recently on the market, which is based on the use of a non-uniform electric field [16]. In contrast to DTIMS or TWIMS, TIMS uses a high gas flow (exclusively nitrogen) to carry the ions of interest into the drift cell. A low electric field is applied in the meantime in the opposite direction with the aim of trapping and separating the ions as a function of their size-to-charge ratio. Then the electric field is gradually decreased allowing the release of the ions from high (large CCS) to small (small CCS) size-to-charge ratios [5]. A schematic of the separation process is shown in Figure 3e.

The TIMS-MS approach is still in early development and commercial devices have only been released recently by Bruker (timsTOF) [26].

The advantages and limitations of these different IM-MS techniques above described are summarized in Table 1.

3. The importance of collision cross section

3.1. Basic collision cross section equation

Ion mobility is a separation technique that allows analytes to be identified according to their mass, charge and shape. The principle of IMS is often compared to that of electrophoresis, even though the separation is conducted in the gas phase within a millisecond timescale.

Considering a pool of gas-phase ions moving in a chamber filled with a buffer gas and under the influence of an electric field (E), the mobility (K) is defined as the proportionality coefficient between the strength of the electric field E and the velocity v_d attained by the ions:

$$v_d = K \cdot E \quad (1)$$

K is usually reported as the reduced mobility (K_0), upon normalization to standard pressure and temperature ($p_0 = 760$ Torr and $T_0 = 273.15$ K):

$$K_0 = K \cdot \frac{p}{p_0} \cdot \frac{T_0}{T} \quad (2)$$

In addition, K_0 can be related to the collision system parameters, accounting for the analyte characteristics under the experimental conditions, by the Mason-Schamp modification of the fundamental zero-field equation [27]:

$$K_0 = \frac{3q}{16N_0} \cdot \left(\frac{2\pi}{k_B T} \right)^{\frac{1}{2}} \cdot \left(\frac{1}{m} + \frac{1}{M} \right)^{\frac{1}{2}} \cdot \frac{1}{\Omega} \quad (3)$$

where $q = ze$ is the ionic charge (z is the nominal ion charge and e is the elementary charge), N_0 is the gas density number at p_0 and T_0 , k_B is the Boltzmann's constant, T is the gas temperature, m is the mass of the buffer gas, while M is the one of the ion, and Ω is the collision cross section (CCS).

While q and M take into account the charge and the mass of the ion, respectively, CCS (Ω) is the physical quantity taking into account its "size and shape". CCS represents indeed the effective area of the ion colliding with the molecules of the buffer gas. The higher the CCS, the higher will be the number of collisions and thus more pronounced the effects on the ions that will be slowed down by friction upon collisions. The CCS is thus a distinctive characteristic of each ion (this is true for small molecules) and it is considered as an important structural

descriptor obtainable by IMS. However, it is important to keep in mind that the case of proteins is more complex, since the activation conditions can influence the CCS and different CCS of the same ion can co-exist. CCS can also be compared with other structural data obtained with different analytical techniques, such as nuclear magnetic resonance (NMR), small-angle X-ray scattering (SAXS), electron microscopy and X-ray crystallography. This point will be further discussed in section 3.3. It is worth noticing that, according to Equations 1 – 3, the mobility (K) depends on the density number of the drift gas (N), and the proportional linearity between v_d and E is only valid in the low electric field limit (E/N ratio ≤ 2 Townsends (Td)). As the electric field is increasing, K is no longer constant and becomes field-dependent. Thus, the simple relationship between K_0 and $1/\Omega$ breaks down. This point is particularly important to understand the limits of the experimental CCS determination and how to properly deduce it from measured drift times on different types of IMS.

3.2. Experimental collision cross section determination

As described in section 1, several types of ion mobility separators can be coupled to MS. All of these configurations allow the separation of the analytes based on their conformations, but only a few of them can be used to determine CCS values. In this section, our goal was to critically consider whether the IMS hardware allows the determination of experimental CCS. In general, if the analyser operates in the low field limit (E/N ratio < 2 Td) at all times and if the field applied is constant, CCS might be directly determined from measured drift times (like in DTIMS). If the IMS analyser operates in the low field limit, but with non-constant field due to the application of additional radio frequencies (RF) or potentials, then a calibration with ions of known CCS is required (like in TWIMS, DMA and TIMS). Finally, CCS cannot be derived at all from the mobility data if the analyser operates at high field (like in FAIMS). The first two scenarios allowing experimental CCS determination are discussed in further detail.

(i) *Low field limit at all times and application of constant field.* The best example of this IMS configuration is the DTIMS. Knowing the drift time (t_d , time spent by the ions in the drift tube) and that $v_d = L/t_d$ (where L is the drift tube length), Equation 2 can be expressed as follow:

$$K_0 = \frac{L}{t_d E} \cdot \frac{p}{p_0} \cdot \frac{T_0}{T} \quad (4)$$

with consequent CCS determination obtainable by K_0 following Equation 3. Theoretically, DTIMS analysers offer the most accurate CCS measurement using a stepped-field approach without the need for calibration, assuming that all the parameters involved are accurately known and carefully controlled. If not, a calibration with ions of known CCS could be necessary also in this case. For online analysis (e.g. LC-IM-MS), a single-field calibration with ions of known CCS is used to provide a regression model for converting drift time and mass into CCS. In addition, the drift tube can operate with a wide range of neutral gases (e.g. nitrogen, helium, carbon dioxide), thus CCS values are a conditional property. In this regard, users are beginning to ensure appropriate reporting of CCS values with specific reference to the gas and type of IM used (e.g. $^{DT}CCS_{He}$ or $^{DT}CCS_{N_2}$).

An important inter-laboratory study has been recently published [12] to highlight the trueness and reproducibility of CCS measurement with DTIMS. In this work, 120 unique ion species (i.e. metabolites, peptides, lipids and proteins, representing a wide range of masses, chemical compositions and structures) were monitored in both positive and negative ionization modes, and the reproducibility of $^{DT}CCS_{N_2}$ values were evaluated across three laboratories equipped with commercial DTIMS-MS instruments. It appears that the average RSD on CCS values was equal to only 0.29% for all ion species across the three laboratories equipped with the same type of instrument. The trueness of the values, obtained with the commercial DTIMS-MS instruments, was comprised between 0.34% and 0.54%, depending whether a calibration was applied or not. These results clearly illustrate the potential of DTIMS-MS for providing a

molecular identifier for a broad range of applications, including untargeted screening workflows.

(ii) *Low field limit with non-constant field.* The best examples for this IMS configuration are TWIMS, DMA and TIMS. Due to the non-linearity of the electric field that complicates the equations describing the ion trajectories in TWIMS, CCS cannot be directly measured from the drift times, but they can be evaluated after calibration with ions of known CCS and under defined conditions of the drift cell (gas pressure and temperature, drift cell length, wave height and speed). Ruotolo *et al.* reported a detailed TWIMS calibration protocol to which readers may refer to [28]. Generally, proper calibrants should cover the IM drift time range of the ions of interest, but also being physically and chemically related to the analytes and best bracket the m/z of the compound to be analysed, especially when small differences in CCSs are expected [29]. The most reliable calibration is obtained by using reference CCS values obtained by DTIMS operating with nitrogen as buffer gas ($^{DT}CCS_{N_2}$), since TWIMS cannot operate with helium and is more often commercialised with nitrogen, thus giving access to $^{TW}CCS_{N_2}$ measures. The accuracy of CCS values is an important topic now that these values are becoming an additional reference parameter in automated peak assignment of database for -omics sciences.

Similarly, a calibration is needed for obtaining CCS values in DMA. As described in [30], a single calibrant ion (such as tetraheptylammonium⁺) could be used to link the applied voltage to the mobility of the ions transmitted, assuming that pressure and temperature inside the DMA classification zone are known and controlled.

TIMS is another example of ion mobility analyser operating in the low field limit, but with non-constant field. In TIMS, an axial electric field is applied to accumulate, trap and elute the ions according to their Ω/z ratio (see section 1.5 for additional hardware details). Unlike the configurations introduced previously, larger ions (high CCS) migrate first with respect to the smaller ions (low CCS). Moreover, similar to TWIMS, CCS determination can be obtained only

upon proper calibration with ions of known CCS as TIMS also operates with nitrogen as buffer gas. Since the commercialisation of this analyser is still relatively recent, there are still very few sources reporting $^{TIM}CCS_{N_2}$ values [5].

3.3. Prediction of collision cross section values

Even though the experimental CCS values represent an interesting structural parameter obtainable by IMS, it is not sufficient to allow any structural interpretation on its own. To disclose the conformation and shape adopted by the ions in the gas-phase, the experimentally derived CCS needs to be compared with computationally derived CCS values obtained from atomistic models. Nowadays, this approach is the gold-standard if using IM-MS as analytical technique for “conformation analysis” [2,4,5,31–41].

The first challenging task is the achievement of reliable atomistic models. A plethora of computational approaches might be applied for this purpose, including quantum mechanical (QM) methods such as (but not limited to) density functional theory (DFT), *ab-initio* or *semi-empirical* methods and classical methods such as Monte Carlo (MC) and molecular dynamics (MD). The method of choice is at the discretion of the user, but it is mainly driven by the size of the system under investigation. Generally, QM methods offer the highest level of accuracy, but they are extremely time-consuming and usually viable only for small systems, whereas classical methods are instead more suitable for large systems such as biomolecules [42]. A detailed evaluation of the computational approaches is beyond the scope of this review, but it is worth noting that extensive proficiency in the field of computational chemistry is required to obtain reliable atomistic models, thus several research groups (using IMS as structural tool) have already taken into account the possibility to have a modeller in their group or to work in close collaboration with a modelling team.

The second challenging task is the reliable determination of computationally derived CCS. Indeed, once a pool of atomistic models, presumably representing the experimental gas-phase conformations, have been selected through a proper computational method, a CCS calculation algorithm needs to be applied to finally obtain theoretical CCS values (CCS_{THE} or CCS_{CAL})

which can be compared to the experimentally derived CCS (CCS_{EXP} or CCS_{IMS}). Several CCS calculation models might be applied for this purpose. The “historical” models (still widely known and used) are the trajectory method (TM) [43,44], the exact hard sphere scattering (EHSS) [44], and the projection approximation (PA) [43,45,46], all initially parametrised for helium as buffer gas and available in the MOBCAL suite of programs [47]. In general, the three algorithms allow simulation of the collisions between the buffer gas and the randomly oriented structure of the ion under investigation. However, during the collisions, multiple scattering events and long-range interactions also occur, and the three models differ in the assumptions and the grade of approximations of all these events, as depicted in Figure 4. TM [43,44] is an explicit model which can be considered as the most rigorous and physically appealing method, since it accounts for both long-range interactions and multiple scattering events. It is the method of choice for small molecules calculations, but it becomes prohibitively slow if applied to large molecules (> 1000 atoms), except if parallelization of computation is available. EHSS [48] is a hard sphere collisions based method, meaning that both buffer gas and ion under investigation are modelled as hard spheres. It computes the multiple scattering events, but it does not take into account the long-range interactions, offering a reasonable balance between CCS accuracy and time requested for the calculation, also for large molecules (even if an over-estimation of CCS could arise in this latter case). Finally, PA [43,45,46] is a non-explicit method that does not consider the multiple scattering events, or long-range interactions, but works by projecting the “shadow” of the ion onto a plane to find the average area of all the possible orientations of the molecule under investigation. It is the fastest method, but can lead to a systematic underestimation of the CCS, especially for molecules presenting cavities. PA is generally recommended for CCS determination of small molecules (10-200 atoms) with convex surfaces, even though a good correlation has been reported between TM and PA when dealing with CCS calculation of macromolecules [49].

During the last few years, optimizations of these three algorithms and also their parametrizations have been reported, especially to answer to the need of including nitrogen as collision gas [50–54]. In the meantime, new algorithms have also been proposed, such as

the projection superimposition approximation (PSA) [55–57], and the local collision probability approximation (LCPA) [58], both non-explicit methods; the diffuse hard spheres scattering (DHSS) [59] that is a hard sphere collisions based method; and the diffuse trajectory method (DTM) [59] that is a physically explicit method. Furthermore, new CCS calculators are still being developed with the goal to provide the most reliable CCS calculation possible, especially for large biomolecules and with nitrogen as collision gas. This is the case of the IMPACT software [49] that has been specifically developed for the CCS calculation of large biomolecules. Indeed, IMPACT calculates CCS using an octree-augmented projection approximation with an exceptional boost to speed for large targets. “Collidoscope” [60] is an alternative TM based CCS calculator using parallel processing and optimized trajectory sampling, implementing both helium and nitrogen as collision gas option.

It is hard to compare the performance of all these approaches, but accuracy and computing time are clearly strongly dependent on the molecular size. What is clear is that this *gold rush* indicates the increasing and intriguing involvement of IM-MS in the field of structural characterization.

4. Resolution and separation performance of ion mobility spectrometry

4.1. Basic resolution equations in ion mobility

The resolving power of IM is defined in the same fundamental way as for mass spectrometry, but using mobility related parameters (e.g. $t_d/\Delta t_d$ instead of $m/\Delta m$). IM resolution has also been historically described using a variety of approaches beginning with chromatographically-equivalent terms including plate height theory [61,62]. However, due to the fundamental differences in ion mobility analyser principles, different models are necessary to describe the achievable resolving power. As a number of recent reviews have comprehensively described fundamentals behind IMS separation and resolution capabilities [8,9,16], only key theoretical equations for describing IMS resolution relevant to commercially available IM-MS instrumentation and their underpinning studies are highlighted in this section.

The nature of low-field drift tube ion mobility lends itself particularly well to understanding IM resolution as the diffusion-based band broadening can be described by similar equations to that used for isocratic linear chromatographic separations. Following historical development of DTIMS instrumentation and seminal theory [27,63], the conditional resolution model [64] provides a fundamentally-sound basis for describing the evolution of zone width in an ideal case and the maximum obtainable sample peak capacity can thus be readily calculated according to critical experimental parameters (e.g. drift gas, tube length, temperature, pressure, sample type). However, limitations for this model are well-known, including the influence of ion trapping and release processes, as well as additional broadening of the ion packages between the IM and mass analysers. A semi-empirical model designed to account for these effects was developed by the group of Hill [65] and offers a means to better understand instrumental contributions to non-ideal performance, as shown in the recent study of May *et al.* [66]. Resolution on current commercially available DTIMS instrumentation is in the order of 40-60 for low pressure cells for singly charged compounds (Agilent) and up to 250 for high pressure cells (TOFWerk). In Table 2 [21,27,64–71], various aspects of these experimental differences can be rationalised according to the relevant parameters influencing DTIMS resolution. Nevertheless, attempts to improve resolution should also be considered alongside other practical considerations. For example, use of above ambient pressure results in improved resolution, but will also result in substantial losses in transmission [72].

In the case of TWIMS, the fundamentals and achievable resolution are described in detail in the publication of Shvartsburg and Smith [67]. Unlike in DTIMS, the measured drift velocity is proportional to the square of the ion mobility. This yields a resolution term that is dependent upon the ion mobility (scaling as $K^{1/2}$) and allows the IM resolution to be practically optimized according to the spread of mobilities to be measured. Further technical changes in the design of commercially available TWIMS devices has allowed improved resolution (40-60) to be achieved with consideration to CCS measurement stability and the optimization of the ratio of wave height and wave velocity given thorough investigation [17,68]. Furthermore, exciting developments in building serpentine paths of several meters in length seem to be the way

forward to achieve resolution of several 100s for next generation TWIMS devices as effective IM length can be increased enormously without increasing the instrument footprint [73].

A resolution equation for TIMS was derived by Hernandez *et al.*, whereby the resolution for a given ion is proportional to the gas velocity (v_g) and to the fourth root of the length of the electric field gradient [69]. As ions of different mobilities will be trapped using different gas velocities, the achievable resolution will differ accordingly. Finally, the achievable velocity is primarily controlled by v_g , the electric field ramp speed, and the RF confinement. Experimental studies have demonstrated good agreement with this model and resolution of up to 300 under optimized conditions [74]. However, it must be noted that this very high resolution is only achievable for a narrow m/z (respectively mobility) range. When used for generic, full mass scan IM-MS experiments then the resolution will be in the range of 50-70.

Finally, as described in the review of May and McLean, IMS resolution has limitations as a standalone metric for comparing different types of IMS analysers [9]. In particular, the achievable resolution for DMA and FAIMS are more difficult to compare. An excellent overview of DMA resolution theory is found in the aerosol science literature [75], while some recent examples have demonstrated resolving powers in the order of 65 using DMA-MS with optimized plate designs [76]. The fundamentals of compensation field-based resolving power for FAIMS were outlined in a computational study from Shvartsburg and Smith [77] with some recent contributions also highlighting some of the limitations of using classical resolving power to describe the performance of FAIMS devices [78]. Although not implemented in routine FAIMS analysis, one recent study demonstrated an improvement in the resolving power of FAIMS from 20 up to 400-500 by the use of optimized gas mixtures and extended separation times [22], while another recent work utilized ion focusing strategies to yield up to 37% improvement in resolution [79].

A summary of the most relevant IMS resolution equations is provided in Table 2.

4.2. Separation performance in ion mobility

Based on established differences in CCS values of molecules and IM resolution models, the theoretical limitations of various ion mobility analysers can be considered according to the instrument design parameters. Many early studies have assessed peak capacity in some form for IM-MS analysis, with some of them focusing specifically on LC-IM-MS [80–82]. Akin to the chromatographic definition [83], peak capacity within IM is simply a theoretical estimate of the maximum number of peaks from the sample class of interest that can be fully resolved side-by-side within a single scan. In combination with resolution and/or peak capacity models for different types of ion mobility devices, improvements in separation potential can be modelled and practically verified. An excellent example of the improvements from increasing path length can be seen in the development of serpentine paths for TWIMS separation of ions [73]. With effective lengths of several meters, quite elevated resolution separations have been realised, while still maintaining sub-second IMS separation times.

One of the great promises of combining IM with MS is the possibility to resolve and individually interrogate isobaric species present in a single sample. For such considerations, the IM resolving power and measurement precision are the most critical factors. The majority of publications on IM-MS continue to use experimentally determined IM resolving power as a means to broadly describe instrument performance, but it is critical to point out that the nature of the ion (charge state), ion gating/trapping, and the IM principle used can all influence the measured resolving power for a given measurement. In many cases, more relevant information for users can be gained by focusing on critical pair (peak-to-peak) resolution within the molecular class of interest, as originally postulated by Karasek and Kim [84]. Practically, a number of studies have demonstrated that isomers exhibiting minor differences in CCS can be differentiated with excellent repeatability precision when analysed separately. Conversely, measurements of mixtures of isomers often yield only broadened IMS peaks as the differences in CCS are in the order of <2% and cannot be resolved by most IM-MS instrumentation (Figure 5) [82]. In a recent contribution, the group of McLean provided theoretical estimations for the required resolution to achieve sufficient IM separation such as small molecules and explored the necessary advances in IM resolution required in some details [85]. In particular, the

separation challenges associated with different isobaric separation problems are discussed with reference to the degree of separation required. The differences in CCS values for isomer pairs of particular classes (e.g. diastereoisomers) were estimated to vary from 0.3 up to 6.9%, and further suggest that an instrument with a resolving power of 250 should be capable of resolving approximately three quarters of the considered isomer pairs. Thus, as modelling developments and improvements in measurement accuracy established by inter-laboratory comparisons continue to inform our understanding of IMS behaviour, we expect that instrument design can mature in order to address this challenge. Nevertheless, it is clear that chemical separation (e.g. LC) remains critical in many cases for the analysis of complex sample analysis in order to correctly assign mass spectral features to individual compounds, and also to reduce ion suppression which is obviously not mitigated by ion mobility separation.

5. Formation of protomers – a potential issue in ion mobility spectrometry?

When considering a singly charged species in positive electrospray ionization mode (ESI+), the molecules have to be first protonated, and the added proton, which gives the charge, is located at the molecule site having generally the largest gas-phase basicity. However, a significant number of substances possess several protonation sites, which can lead to different isomers in the gas phase, also known as protomers. As reported in [4], the average number of protomers for more than 100,000 structures of biologically relevant compounds contained in the drug data report database was equal to 6.3 per compound, which means that the prevalence of protomers in the small molecule chemical space is important. In addition, it has been reported that the protonation sites (location of charges) can have a significant effect on the conformation of an ion [4]. In MS and HRMS, the protomers cannot be distinguished since they possess different conformation, but the m/z ratios remain identical. However, when adding an IMS dimension to the separation, the protomers can be differentiated based on their shapes, leading to several peaks observed on the mobilogram. This behaviour is well illustrated in Figure 6, showing the mobilogram of ciprofloxacin (fluoroquinolone antibiotic), which reveals the presence of two well separated gas-phase species (protomers), with their respective sites

of protonation in the positive ionization mode (on the cyclic ketone and the piperazine moieties) and their corresponding drift times [86]. In this example, these two different gas phase components, although they only differ by their site of protonation, were well separated in IMS (1.14 ms difference in drift time). The experimental IMS data of ciprofloxacin were compared with theoretical calculations using molecular modelling (density functional theory) and subsequent CCS calculations using projection approximation. Besides ciprofloxacin, there are several other examples reported in the literature.

In differential mobility spectrometry, two protomers were observed for the 4-aminobenzoic acid [87]. In this study, the authors have demonstrated that the relative abundance of each protomer depends on the nature of the solvent employed in ESI. Various proportions of acetonitrile/water and methanol/water were tested and the following conclusions were drawn: in presence of a protic solvent (MeOH), mostly O-protonation of 4-aminobenzoic acid occurs, while the presence of aprotic solvent (acetonitrile) favours the formation of N-protonated 4-aminobenzoic acid. This behaviour can be attributed to the stabilization of one of the two protonated forms in a given solvent [88]. Similar observations on the influence of ESI solvent on the relative abundance of protomers were reported for benzocaine, a local anaesthetic [89]. The authors proved that the differences in drift times observed for the two species were attributed to a different charge distribution within the molecules. They also highlighted that the permittivity of the solvent was a key point since a highly protic solvent (such as MeOH/water) facilitates the ionization of the most basic group (amide nitrogen) into the gas phase, while the carbonyl oxygen becomes energetically favourable for protonation in the presence of a less protic solvent (ACN/water), when the relative permittivity decreases.

Apart from differences observed between protomer abundance when altering the nature of the ESI solvent, different fragmentation behaviours were also reported for the protomers. This has been highlighted in [87], where some differences in the amount of residual precursor ion and in some fragment ions, as well as the presence of unique ions were observed for one of the two protomers of 4-aminobenzoic acid. These differences in MS/MS spectra of protomers were also highlighted in [90], with ciprofloxacin as a case study. The formation, separation and

characterization of the protomers of aniline and three different porphyrins in presence of different organic solvents and using polarizable CO₂ as the drift gas was shown in [91]. The observed protomers of aniline were characterized *via* structurally diagnostic collision-induced dissociation (CID), thanks to post-TWIMS CID, while the pre-TWIMS CID allows the determination of protomers ratio from equilibrium in solution or formed in the gas phase. For aniline, the two protomers, namely N-protonated and unusual ring-protonated species, exist as a ratio of 5:1 in solution equilibrium. This ratio was found to be substantially different in the gas phase at non-equilibrium conditions. The same conclusions were drawn for porphyrins, showing that pre- and post-TWIMS CID may be considered as a useful structural tool for investigating protomers in more details.

In conclusion, IMS can be considered as a valuable analytical tool for the separation and characterization of protomers. This feature could be particularly attractive for those who are interested in determining the most favourable site of protonation or deprotonation of molecules, which exhibit several acidic and basic sites. Therefore, the characterization of protomers is of great fundamental relevance, to better understand compound stability and reactivity. However, in analytical chemistry, the presence of various protomers for one single molecule is not welcome, since it will surely add complexity to the mobilogram and MS spectra, making the data interpretation difficult. Indeed, several peaks could potentially be observed for one single molecule on the mobilogram. If these different species cannot be totally resolved in IMS, the corresponding mobility peak will appear broad and distorted (shouldering) on the mobilogram. Currently, the IMS resolution is relatively low (often below 50), and therefore, the different protomers are often not separated at all and are analyzed as a single peak in IMS. However, if (as expected) the IMS resolution will be significantly enhanced with the next generation of instruments (see section 8.1.), numerous protomers will be observed for one given substance, which will create some additional issues in terms of data interpretation. Finally, the number of studies dealing with the understanding of protomers formation, separation and characterization in IMS and its prevalence is still too limited and deserves some additional work from specialists.

In addition, it is important to consider that the same effect (protomer formation) can also take place with proteins and protein complexes. This is of great interest, because of the mechanism whereby charges migrate upon gas-phase unfolding of the protein (collision induced unfolding, CIU). More details will be provided on this particular mechanism in section 7, dedicated to the characterization of large biomolecules.

6. Application of ion mobility spectrometry to the analysis of small molecules

6.1. Metabolomics and related applications

Global metabolomic studies are a challenging task that aims to analyze as much of the metabolome of interest as possible often within a single analysis [92]. For this reason, high resolution mass spectrometry in combination with chromatographic separation has become the essential tool for successful metabolite annotation and relative quantification tasks [93,94]. IM-MS in combination with LC attempts to directly address the challenge of limited metabolome coverage within a single analysis, by increasing overall method selectivity, which was outlined in recent contributions on this topic [9,95]. The high chemical diversity of small metabolites (e.g. encompassing amino acids, small organic acids, sugars, nucleosides, nucleotides, carbohydrates, sugar phosphates, vitamins, but also lipids) and their widely differing molecular properties (e.g. polarity, acid/base character) provides particular challenges for the development of IM-MS and LC-IM-MS workflows as in-source fragments, dimers and adducts can be separated by IM, which would not occur when using LC-MS alone. Thus, the correct re-grouping of these signal features and interpretation of data from such workflows remains a challenging task that has not yet been solved. The earliest literature examples using this combination for metabolomics demonstrated increased confidence in metabolite annotation via drift time or CCS alignment, but also limitations in transmission and assignment of molecular formulae to adducts [72,96,97].

Building on a landmark interlaboratory study [98], Paglia and Astarita recently published a protocol using LC-TWIMS-MS for both metabolomic and lipidomic studies using this platform, which presents details on all procedures from calibration, tuning, analysis up to data

processing including the derivation of $^{TW}CCS_{N_2}$ values [3]. Other recent works focusing on method development for global metabolomics have investigated the use of commercially available TWIMS-MS instrumentation for urine profiling [99] and DTIMS-MS for human plasma and HaCaT cells [100], whereby improvements in signal-to-noise and some confirmed examples of isobar separations have been demonstrated. Other notable recent studies have focused on non-targeted metabolomic assessment of placental samples [101], and serum profiling [102]. The potential of LC-FAIMS-MS was also explored in a very recent contribution with a broad outlook toward different “omics” approaches [103].

In addition to high resolution mass data, as commercially-available IM-MS instrumentation also includes a quadrupole and collision cell (e.g. IMS-QTOF), the potential of using both IM-MS with fragment ions associated to the precursor ion (i.e. aligned by drift time) can become part of standardized workflows in order to utilize retention time, CCS, MS and MS/MS information for metabolite annotation. However, data processing to make use of all of this information for annotating metabolites in fully non-targeted workflows is time consuming and remains a current roadblock for method development.

Aside from global metabolomic profiling, several groups have also focused on characterization of secondary metabolites including food and plant studies [104–107]. Xiao *et al.* studied the thermal degradation of beta-carotene using TWIMS-MS and putatively identified several breakdown products [107]. Causon *et al.* demonstrated preliminary developments for wine metabolomic studies using DTIMS, where excellent day-to-day repeatability precision (<0.3%) and agreement between CCS determined using standards and online measurement of real samples (<0.5%) was observed [104]. Covington *et al.* recently reviewed strategies utilized for secondary metabolism and concluded that IM-MS is likely to play a role in natural products dereplication [108]. Structural isomers and epimers of steroids, oxysterols and related hormones have also been in focus within some recent IM-MS studies demonstrating the suitability of available instrumentation for a diverse range of applications [109–111]. In all of these examples, cleaner mass spectra and use of CCS as an identification point are highlighted to have potential for increasing confidence in confirming metabolite identity. Thus,

it is of critical importance for establishment of user-friendly LC-IM-MS metabolomic workflows that the introduction of CCS as a conditional identification point for metabolite annotation involves careful establishment of consensus CCS values to populate libraries for global use. It is therefore expected that combined efforts in building reference CCS libraries for different types of IM-MS instrumentation will emerge in order to provide validated methods supporting metabolomic-related studies.

The area of drug metabolism has also been studied using IM-MS in recent contributions with particular focus on isomeric variants. Reading *et al.* studied the gas phase conformation of drug metabolites using TWIMS-MS, with a focus on correlating experimental data with computational models for pharmaceutical structural characterization (Figure 7) [112]. Lee *et al.* investigated metabolites of the anticancer drug Paclitaxel to study hydroxylation at different sites using TWIMS-MS and supported their results with theoretical calculations [113]. Beucher *et al.* studied non-steroidal selective androgen receptor modulators, which are prohibited by the World Anti-Doping Agency, but remain readily available and potentially used in livestock production for growth promotion [114]. Interestingly, the authors were able to successfully couple supercritical fluid chromatography (SFC) separation with TWIMS-MS for the analysis of bovine urine and suggested that CCS has potential within this workflow to be used as an identification parameter within a screening method.

6.2. Glycan analysis

Glycans constitute a large class of biomolecules with a broad chemical diversity. They are involved in many biological processes, such as embryonic development, cell-cell recognition, inflammation and fertilization, among others [115,116]. Glycans can be covalently bound with proteins to form glycoconjugates and hence affect protein folding mechanism and protein stabilization. An abnormal conjugation between proteins and glycans can lead to a modification of the tertiary structure having an impact on their biological response.

Structural characterization of glycans is the first step towards the understanding of their biological activity. Due to their structural heterogeneity, the elucidation of glycan structures

remains a challenging task. Liquid chromatography combined with tandem mass spectrometry has been widely used for glycan analysis [117–119]. Nevertheless, different chromatographic columns may be required to provide sufficient separation of isomers, which cannot be obtained with tandem MS, leading to a low analysis throughput. Recent advances in IM-MS have allowed the improvement of the structural characterization and separation of carbohydrates have been reported since then [5,120]. Clemmer and coworkers first published the reduced mobilities of negatively charged trisaccharides and their fragments by using a home-built drift tube instrument [121]. In 2003, Hill and coworkers separated 21 different carbohydrate standards using a non-commercial ion mobility instrument as well [122]. Overall, these results showed the capabilities and the potential of IM-MS for the analysis of carbohydrates.

The interest on IM-MS especially increased after the advent of commercial instruments. A large number of studies dealing with carbohydrate analysis in IM-MS have been reported [120] since then [5]. Resolving power is the major issue of ion mobility in the analysis of carbohydrates. Several strategies have been developed to circumvent this problem. Formation of non-covalent complexes with metal ions has been largely used to induce alternative carbohydrate conformations and thus, enhance isomer separation [123–125]. This approach has been used for comparing the effect of different cations on the CCS of several milk sugar isomers [123], to study the influence of the metal cation on carbohydrate structure. The sodium adducts presented larger CCS compared to lithium adducts, but Na^+ was not found to be a suitable cation to provide the best isomeric carbohydrate separation. Altogether, it was concluded that separation of isomers does not only depend on cation radius, but also on the stereochemistry of the compounds. Hoffman *et al.* studied a set of six trisaccharide isomers with different composition in the non-reducing-end moiety [126]. IM-MS experiments were performed in positive and negative ion modes to evaluate the influence of the ion polarity. In addition, two kinds of trisaccharide adducts were formed with Na^+ and Cl^- to improve the separation of the precursor ions. Overall, IM provided a better separation of configurational and linkage isomers in negative ion mode, compared to sodium adducts or protonated trisaccharides. Interestingly, similar ATDs were observed for $[\text{M-H}]^+$ and $[\text{M-Na}]^+$ sodium adducts, whilst an impact on the

general structure of trisaccharides was observed upon chloride adduct formation. Another additional interest about IM highlighted in this work was its ability to quantify small amounts of carbohydrate impurities from a given mixture. Product ions in a relative concentration of 0.1% could indeed be efficiently detected and quantified by analyzing the corresponding ATDs. The versatility of certain IM-MS instruments allows the fragmentation of ions before the IM cell (pre-mobility CID) [127,128] and/or after (post-mobility CID) [129,130] to provide in-depth insights about the structure of precursor carbohydrates and also to generate specific tandem mass spectra. For instance, oligosaccharide-alditols from bovine submaxillary mucin (BSM) were separated by HPLC and analyzed by IM-MS in the negative ion mode [128]. Some specific precursor ions were post-mobility fragmented to generate specific tandem mass spectra from isomeric precursor ions. CID was also performed upon IM separation giving rise to isomeric mobility peaks. The mobility resolved product ions were also fragmented in the transfer cell, generating further structural information. A more recent study from Harvey *et al.* performed a post-mobility fragmentation of hybrid and complex glycans obtained from five different glycoproteins [130].

The previous works were mainly focused on improving the separation and identification of isomeric species and the characterization of the global structure from IM measurements. However, IM can also be used as a filter, to reduce the complexity associated with the carbohydrate mass spectra [11,127,130,131]. Interactions between neutral buffer gases and ions strongly depend on the chemical nature of the analytes, leading to different mobility behaviors. May *et al.* calculated almost 600 CCS values that comprise different molecules such as lipids, peptides, carbohydrates and quaternary ammonium salts, using a drift tube coupled to a quadrupole time-of-flight mass spectrometer [11]. Above 1200 Da, these four classes exhibit different gas-phase packing trends that were clearly distinguishable by IM. In addition, multiply charged ions will exhibit higher mobilities than singly charged ones, describing specific drift time/mass-to-charge trends that can be individually extracted from the global mass spectrum. This example is clearly observed in the analysis of N-glycosides from bovine fetuin studied by Harvey *et al.* in the negative ion mode [130]. Several well-defined

regions are displayed in the driftscope, corresponding to different N-glycan charge states. It is worth pointing out that filter-role of ion mobility not only facilitates the interpretation of mass spectra but also allows the detection of minor species that could not be observed in classical MS conditions by improving the signal-to-noise ratio.

Alternative fragmentation techniques to CID are required to produce more diagnostic cross-ring fragments such as electron excitation dissociation (EED) [132], vacuum ultraviolet photodissociation (VUVPD) [133], and extreme ultraviolet light (XUV) [134] or spectroscopic techniques such as infrared multiple photon dissociation (IRMPD) [78], to produce characteristic IR spectra to elucidate the structure of the carbohydrate isomers. Further improvements in separation and identification of glycan isomers can be achieved by coupling two IMS dimensions, as recently demonstrated [135,136]. IMS-CID-IMS-MS experiments can be performed with these experimental set-ups to study the structural heterogeneity of complex glycan mixtures, where the separation of the precursor ions is not fully achieved in classical IM-MS experiments.

6.3. Lipid analysis

Lipids occupy a distinct conformational space in IM-MS analysis, due to their relatively high flexibility and low density (i.e. large CCS for their m/z). Moreover, aside from the broad class separation requirements for lipid analysis (triacylglycerides, phospholipids, free fatty acids, ceramides, sphingomyelins,...), there is great structural diversity within individual classes influencing their conformation including different alkyl chain lengths, degree of saturation, and double bond position. In this regard, IM-MS offers great potential to investigate this diversity that may not be revealed by conventional analysis. Application of IM-MS to lipidomics has been recently reviewed by Paglia et al [137,138] and the reader is referred to these two contributions for a comprehensive overview of this topic. Astarita and Paglia also recently published an advanced lipidomic protocol using TWIMS-MS in combination with liquid chromatography [3], while excellent insight into CCS calibration strategies for lipidomics using this type of instrument was recently published [139]. In a particularly novel approach, the group of Murphy

has demonstrated the use of product ion mobility for lipidomic workflows, whereby collisional activation is used to generate product ions for which a CCS value can be determined to aid with lipid identification using a TWIMS-MS platform [140,141].

Some very recent results from DTIMS studies have indicated the challenges associated with separating minor differences in CCS (e.g. double bond position isomers) due to the sub-1% differences in CCS of many isomers. Kyle *et al.* studied a wide range of lipid classes using the Agilent DTIMS instrument demonstrating the possibilities for a wide range of lipid isomer types and the separation potential offered with current resolution limitations [142]. Dried blood spots were the focus of a second study from this group involving longitudinal samples from diabetic patients also assessed by DTIMS [143]. Stable isotope-labelling was used for profiling of fatty acids using DTIMS, where the similarity of drift times was used to help associate labeled with unlabeled acids in normal and cancerous human thyroid tissue samples [144]. Impressive resolution of acyl chain- and double bond positional isomers of lipids has been demonstrated on the TOFWerk DTIMS platform [145]. A resolution of >250 was calculated for isomeric lipids measured under low-field conditions with the instrument operated with Hadamard multiplexing, which requires sophisticated signal deconvolution approaches.

As is the case for metabolomic studies, standardization of CCS values must become a key focus for building of global lipid libraries for identity confirmation and is already a targeted focus of some computational modelling studies [146]. Nevertheless, some of the subtleties of gas-phase lipid conformation have not yet been resolved between theoretical and experimental considerations and ongoing effort is needed to reconcile these aspects. The commercial availability of high-precision instrumentation utilizing different types of IMS analysers is already playing a key role in supporting such work.

FAIMS offers a complementary approach in comparison to TWIMS and DTIMS and has also been used for targeted studies of lipids by several authors for interrogating isobaric lipid species as essentially a “mobility filter” prior to MS experiments. In one such study, Steiner *et al.* used differential-mobility in combination with a state-of-the-art ozonolysis strategy to probe the structure of deoxysphingosine [147]. This type of study underlines the structural diversity

within a single lipid class that can influence biological behavior that can benefit from IM-MS as a powerful analytical approach (Figure 8). A very recent study has also demonstrated some promising results using FAIMS for resolution of a wider range of isomeric lipid classes [148]. Finally, due the complementarity of different available IM-MS analysers, the potential for revealing previously unknown biological information using IM-MS has started to emerge in recent literature and further insightful publications in this area are expected.

6.4. Environmental analysis

IMS has a long history for usage in field applications entailing rapid environmental assessments. The recent review of Marquez-Sillero *et al.* presents IMS applications focusing on environmental analysis [149]. While IMS alone has great potential for applications requiring portable analysis, IM-MS and LC-IM-MS have excellent potential to be used for lab-based screening workflows, particularly for trace analysis in complex samples. The group of Schmitz has recently demonstrated a powerful approach by combining two online separation methods (LC+LC or GC+GC) with DTIMS-MS [150,151]. Complex wastewater samples were successfully screened using an in-house CCS database with excellent repeatability of CCS and mass spectra, while their more recent study used GC+GC with atmospheric pressure chemical ionization (APCI) for the screening of various compounds, including pesticides. Further discussion of the potential of multidimensional chromatography in combination with IM-MS is presented in section 8.3. Grimm *et al.* used TWIMS-MS for the study of unknown or variable composition biological materials (UVCBs), complex reaction products, and biological materials in petroleum samples [152]. This workflow allowed mass-to-charge, drift time, and Hydrogen/Carbon ratio to be used to support compound classification and inter-samples comparisons. While such studies offer exciting results, for such approaches to become broadly accepted in standardized methods, CCS will require standardization as an identification point in order to be used alongside retention time and MS identification parameters within regulated methods.

The substantial challenge of exposomic studies, where 1000s of compounds including xenobiotics, secondary metabolites, and transformation products which remain currently unidentified, is a focus of high-throughput IM-MS studies whereby computational prediction of CCS values is suggested to be a way forward to allow rapid screening of samples [153]. Such a workflow requires substantial computational effort to predict CCS values, but some authors have presented promising results using different approaches in addressing this major challenge [154–156]. The workflow demonstrated by Zhou et al [156] was particularly in-depth as DTIMS experimental data from a total of ~400 compounds were used to generate molecular descriptors to train a machine-learning algorithm, which matched CCS values measured on their instrument with a median relative error of 3%. Alternative approaches to modelling of CCS are discussed in Section 3.3.

6.5. Clinical analysis

A number of studies have investigated the use of IMS and IM-MS for the analysis of samples of clinical origin and the potential of these technologies for such applications was recently reviewed by Chouinard *et al.* [110]. As is the case for environmental applications, standalone IMS instrumentation is considered to be attractive for clinical applications due to its portability, ease of use and low cost. Rapid sampling of exhaled breath is one example that has already explored the use of this technology since the 1980s [157]. In the case of IM-MS, some of the more promising investigations for clinical applications appearing within the last few years are aiming toward the use of IM-MS for imaging using desorption electrospray ionisation (DESI) or matrix-assisted laser desorption ionisation (MALDI) in combination with IM-MS [158–160]. Apart from these developments, many of the goals and challenges within clinical applications using IM-MS also overlap with those of the related “omic” studies, which are presented in the elsewhere relevant sections of this review.

7. Application of ion mobility spectrometry to the characterization of large biomolecules

7.1. Separation of isobaric peptides

One of the most important challenge of structural biology is the elucidation of the protein structure/function relationship. Posttranslational modifications (PTM) like phosphorylation, acetylation, or methylation can be involved in modification of the protein structure and thereby have a straight impact on their function. Identification and precise location are essential information to provide a thorough PTM characterization. For this purpose, LC has been used prior to MS/MS to separate precursor peptides, however this approach often fails to separate and identify positional variants, especially when isobaric peptides exhibit no specific reporter/diagnostic fragments [154]. A newer approach based on the implementation of IMS has been used to provide an additional separation dimension to the characterization of large biomolecules. Ruotolo *et al.* studied [154] a large set of phosphorylated and non-phosphorylated peptides using a MALDI-DT-MS mass spectrometer. The contour plots show that phosphorylated peptides exhibit lower drift times compared to non-phosphorylated peptides with the same m/z . Unfortunately, the resolving power of the technique [161–163] only allows the efficient separation of some phosphorylated variants. The separation capability of IM was further improved by using FAIMS coupled to MS [23,164]. Three different phosphorylated peptides from τ protein (VAVVRTpPPKSPSSAK, VAVVRTPPKSpPSSAK, VAVVRTPPKSPSpSAK) were efficiently separated under the influence of the asymmetric electric field and by modifying the He/N₂ ratio of the buffer gas [165]. The best separation was achieved with a mixture of gas containing 40/60 v/v of He/N₂. The identification of each phosphorylated variant was performed after ETD fragmentation. It is worth noting that ETD spectra of each individual phosphorylated peptide do not exhibit unique fragments that allow unambiguously identification of the precursor ions, thereby, an efficient separation is required prior to ETD fragmentation. More challenging PTM characterization was envisaged with the same experimental set-up, like methylated [166] and acetylated peptides [167]. Both modifications were studied on histone tails, more precisely in the 1-21 and 21-44 segments of H3 tails, and 1-25 segment of H4 respectively. Positional variants of methylated and acetylated peptides were separated using different He/N₂ or H₂/N₂ gas ratios, to improve ion separation.

Overall, very narrow peaks and a baseline separation could be achieved after addition of He to the buffer gas (from 0% to 40% v/v). It is worth mentioning that methylated or acetylated peptides are +14 and +42 Da modification, respectively over “middle down” sequences (up to 3 KDa) which means that methyl variation accounts for 0.5% of the mass, requiring high resolving power to provide an efficient separation. These results show the utility of IM-MS to improve detection of positional isomers of modified peptides and identification from a complex peptide mixture.

7.2. Adding an ion mobility dimension for improved peak capacity in high throughput large scale proteomics

Modern proteomic analysis consists of identification and simultaneous quantification of thousands of proteins present in complex protein mixtures, with many applications in molecular and cellular biology [168–170]. Up to now, most proteomic studies rely on bottom-up analysis: complex protein mixtures are digested into peptides that are further analyzed by reversed phase liquid chromatography coupled to nanoelectrospray ionization tandem mass spectrometry (nanoLC-MS/MS) platforms. Hybrid tandem mass spectrometers such as ion trap-orbitrap, quadrupole-orbitrap and quadrupole-time-of-flight (QqTOF) instruments have dominated the field of large scale high throughput proteomics. These configurations offer high data acquisition speed and high mass accuracy for peptide precursor and fragment ions. However, this classical bottom-up proteomic workflow presents a series of caveats : i) several peptides can co-elute from the chromatography column and ii) MS/MS acquisitions are mostly performed in a data-dependent (DDA) way, meaning that most intense ions are fragmented one at a time, the others being entirely discarded. Limited scan speeds and sensitivities of the mass spectrometers mostly account for this under-sampling, leading to subsequent lower sensitivities. Thus, improving the performance of new instrumentation relies on increasing the rate of MS/MS acquisition without information loss (transmission) on tandem mass spectra. Although ion trap instruments may still offer higher absolute sensitivity owing to their ability to accumulate precursor ions to a desired level prior to fragmentation (a feature that is not

available on QqTOF instruments), TOF instruments have the potential for very fast data acquisition (up to 100 Hz for QqTOFs) [171] and high in-spectral dynamic range, both of which are highly desirable for the analysis of the very complex samples [172]. IMS can then be exploited to increase speed, selectivity, and sensitivity of MS/MS experiments [2,173,174].

The integration of TWIMS into DDA acquisition schemes [14], termed high definition or HD-DDA [175] has removed the long standing limitation of low duty cycles of QqTOF instruments and leads to considerably improved sensitivity and productivity of proteomic analysis on such instruments [14]. The actual speed of HD-DDA is 30 Hz, mainly owing to the time required for on-the-fly data processing within the instrument control software. In the TWIMS configuration, charge separation capabilities of TWIMS cells are used to separate singly charged from multiply charged ions of tryptic peptides.

Data-independent acquisition (DIA) modes, in which fragment ion spectra of all detectable precursor ions within an LC-MS run regardless of any specific characteristics (intensity, charge state and so on) are generated, [176] have also been reported as alternatives to circumvent under-sampling of MS/MS experiments. MS^E is one of the first reported DIA approach and consists of two alternating MS scans recorded at low and high collision energy across the full mass range, providing precursor and product ion information, respectively [177]. The integration of IMS to DIA workflows has been referred to as high-definition MS^E (HDMS^E). IMS provides here an additional dimension of separation that increases the overall system peak capacity in LC-MS workflows [174], offering the possibility to fragment precursor ions after IMS. IMS not only allows the resolution of co-eluting isobaric species, but also enables the use of drift-time information [178] for the alignment of precursor and product ions (in addition to retention times in chromatography). This drastically increases the number of identified peptides and proteins in complex samples as compared to MS^E workflows without IMS [179]. Optimized HDMS^E approaches achieved similar proteome coverage as compared with state-of-the-art DDA methods and displayed excellent reproducibility and overlap between technical replicates [180,181]. Figure 9 presents an example of HDMS^E analysis of a 400 ng Hela digest, leading to the identification of more than 38440 peptides corresponding to 3490 unique proteins.

Thorough overview of the use of IMS-DIA-based label-free quantification and its applications in biomedical research and clinical proteomics have been reported elsewhere [182,183]. Similarly, IMS-DIA has also been reported for enhanced impurity detection in protein biotherapeutics (Host Cell Proteins, HCP), as they present a high dynamic range between the very abundant therapeutic protein and its low abundance impurities. HCPs present at a concentration as low as 1 ppm (1 ng HCP/mg mAb) were successfully identified and quantified in the reference NIST monoclonal antibody and Infliximab [13]. Recently, Haynes *et al.* described the use of a new mode of IMS-DIA acquisition: instead of performing IMS-DIA at a fixed velocity TWIMS settings, ramping the traveling-wave velocity increased drift space occupancy, amplifying resolution by 16%, peak capacity by nearly 50%, and peptide/protein identifications by 40%, opening new perspectives for variable-velocity TWIMS in proteomics analysis [184]. To summarize, IMS adds an orthogonal analytical dimension, reduces ion interference to improve reproducibility, peak capacity, and peptide identifications to rival modern hybrid quadrupole orbitrap systems.

A variant of the HDMS^E approach has been proposed using a TIMS device [185], introducing the concept of parallel accumulation–serial fragmentation (PASEF) consisting of sub-millisecond quadrupole serial selection and fragmentation of multiple precursors in single 50 ms TIMS scans [185]. PASEF enables hundreds of MS/MS events per second at full sensitivity. The PASEF workflow presents a series of advantages among which: i) no loss of sensitivity, ii) the resulting spectra are fully precursor mass resolved and iii) good compatibility with reporter ion based chemical multiplexing strategies (such as iTRAQ or TMT). A 10-fold gain should be achievable by PASEF in shotgun proteomics experiments, as a consequence of increased sequencing speed, without a decrease in sensitivity. However, modeling suggests that a combination of targeting more precursors and targeting weak precursors repeatedly will be most effective. The PASEF principle could be applied to any IM-MS configuration with the required sub-millisecond scan speed in the MS read out.

7.3. Ion mobility spectrometry for the characterization of gas phase protein structures

ESI-IM-MS has been mostly used to study the 3D architecture and conformational properties of many proteins and biomolecular complexes, through the measurement of their averaged CCS [28]. However, over years, the question “is protein conformation altered from the solution to the gas phase of the mass spectrometer?” became central to avoid misinterpretation of IM data. Several studies that compared IM measured CCS to either calculated CCS from PDB structures or in vacuum molecular dynamics (MD) generated CCSs brought partial answers to this question. One common observation is that when the charge increases, the molecules undergo Coulomb-induced unfolding to adopt extended helical structures [186,187] and ultimately string-like extended structures at very high charge states [188,189]. At low charge states, however, the proteins usually remain compact, with sizes that are expected for condensed phase native structures. Thus, depending on the type of proteins/complexes of interest, the gas phase collapse and/or unfolding of proteins is more or less pronounced. It is now generally accepted that 3D structures of globular proteins (like TRAP, TTR, SAP, integral membrane proteins) [190] survive the transfer into the gas phase without major compactions or unfolding at low energies [191], as attested by the number of success stories based on agreement between IMS-measured CCSs and calculated ones. More recently, Devine *et al.* [192] showed that non-globular proteins (like monoclonal antibodies or proteins with flexible hinge regions in between more structured domains) and RNA molecules undergo strong compaction in the gas phase (CCS values measured underestimate the physical size of the solution structure and modeled data of the biomolecule), highlighting a potential caveat in studying these specific biomolecules with this technique. However, IMS is not directly sensitive to protein secondary structure, and observing a compact structure does not guarantee that the secondary structure accurately reflects that of the native form. To go ahead with keeping secondary structures from the solution to the gas phase, optical/IR spectroscopy, a direct method that allows distinguishing helices from β -sheets, has been coupled to IMS [193]. In this configuration, IMS is used to select the global shape of proteins, followed by IR spectroscopy to probe their secondary structure. On two reference proteins, myoglobin (85% of α -helix) and β -lactoglobulin (60% β -sheets), IMS combined to IR showed that secondary along with tertiary

structures are retained in the condensed gas-phase. When IMS is combined to gas-phase infrared multiple photon dissociation (IRMPD) spectroscopy, gas-phase Coulomb-driven unzipping to a defined secondary structure is a general feature of all highly charged protein ions [194].

7.4. ion mobility spectrometry for proteins and protein-ligand complexes

7.4.1. Ion mobility spectrometry for intrinsically disordered proteins

Intrinsically disordered proteins (IDPs) have been the scope of many IM-MS studies (for review see [195,196]). Indeed IDPs, which are associated with a wide range of major disorders including cancer and amyloid-related disorders, are flexible proteins that can adopt many different conformations in order to bind to different physiological partners. This conformational change along with absolute conformation(s) characterization and detection of populations of conformations can be uniquely monitored by IM-MS. Bernstein *et al.* [197] performed IM-MS experiments on α -synuclein that indicate how the size of its conformations differed with charge state at initial solutions of neutral and acidic pH. IM-MS has also been used to study the structure of the DBD (DNA-binding domain) of the tumour-suppressor protein p53 with and without the functional zinc [198]. IM-MS showed smaller CCSs (12.3% in CCS) for the p53 without zinc than for the zinc-bound form. Other research using IM-MS to study IDPs has examined changes in conformational equilibria of the intrinsically disordered HMGA (high-mobility group A) chromatin factors [199]. IM-MS measurements of wild type and of a C-terminal truncated (shorter) HGMA2 were recorded. Despite the increase in size of WT with respect to the C-terminal truncated HGMA2, IM-MS revealed a more compact structure for the C-terminal shorter form, indicating that the presence of the C-terminal tail is here responsible for further compacting HGMA2, which could be correlated to a reduction in oncogenic activity. D'urzi *et al.* reported the use of IM-MS to characterize the complex between the intrinsically disordered N_{TAIL} domain and the phosphoprotein X domain (P_{XD}) from measles virus (MeV). IM-MS revealed distinct conformers, yielding the estimation of the solvent-accessible surface area (SASA) in solution and the average CCS in the gas phase. Computational modeling of

the complex in solution, based on experimental IM-MS constraints, were generated and provided the first attempt to modeling the entire N_{TAIL} domain bound to P_{XD} at atomic resolution. IM-MS is also well-positioned to assess IDP/ligand interactions and conformations induced upon ligand binding. Dickinson *et al.* have demonstrated the use of IM-MS to characterize the effect of two inhibitors RITA and Nutlin-3 on their respective binding partners: p53 and MDM2 [200]. While no Np53:RITA complex was observed in native MS experiments, an alteration in the conformational distributions (through variations in ^{DT}CCS_{He} values) adopted by Np53 in the gas phase was observed. This finding supports the hypothesis that RITAs mode of action proceeds via a conformational change in p53, which was further confirmed by a combination of complementary biophysical techniques. Similarly, DTIMS analysis revealed that, in the absence of Nutlin-3, every charge state of N-MDM2 shows at least two conformations. Again, upon binding to Nutlin-3, the protein undergoes a compaction event similar to that observed with RITA and Np53. This multi-technique approach highlights the inherent disorder in these systems; and in particular exemplifies the power of IM-MS as a technique to study transient interactions between small molecule inhibitors and IDPs. For instance, Knapman *et al.* reported the use of IM-MS to probe the conformational characteristics of two IDPs, apo-cytochrome *c* and apo-osteocalcin, which are both structured when bound to their respective substrates, but disordered in their unbound-states [201]. In both cases, the intrinsically unstructured apo-states exist as multiple conformations with larger cross-sectional areas than their holo-analogues, suggesting that IDPs do not preclude the formation of preferred conformations. Together, these examples illustrate the scope and utility of IM-MS for studying the characteristics and properties of IDPs, whose analysis by other techniques is limited. Another feature that can be monitored by IM-MS is the tendency of IDP to form amyloid fibrils, the protein aggregates involved in the onset of neurodegenerative diseases such as Parkinson's and Alzheimer's (for review see [202]). Due to their dynamic, polydisperse, and polymorphic nature, these oligomers are very challenging to characterize using traditional condensed-phase methods. IM-MS based approaches can be used to study amyloid formation, providing information about the assembly pathway and the size and shape of

presumably toxic oligomers. IM-MS was first used to separate, analyze and measure CCSs of individual oligomer species present in equilibrium [202]. A remarkable IM-MS study is reported by Leney *et al.* [203] and compares the oligomers formed from wild-type $\beta 2m$ (beta-2-microglobulin) with those formed from a variant of the protein (H51A), during in vitro fibril assembly. While H51A was shown to exhibit a two-fold increase in the lag-time of fibril formation, no differences were observed by IM-MS for the oligomers of the two proteins (similar CCSs). Real-time native MS was the only technique able to highlight the differences in the kinetics of the lag phase between the two protein variants. Similarly, IM-MS was used to investigate the formation of heterogeneous pre-fibrillar, oligomeric species involving human amylin (also known as islet amyloid polypeptide, hIAPP) and the amyloid-beta peptide ($A\beta$ -40), two pathogenic factors in Type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD), respectively [204]. Oligomerization of human islet amyloid polypeptide (IAPP), being considered as a pathogenic process in type II diabetes, has also been extensively investigated by IM-MS [205,206]. Time-course MS and IM-MS were reported to probe the early aggregation states of an amyloidogenic endcapeptide derived from amino acid residues 105-115 of the human plasma protein transthyretin [207]. Early $A\beta$ oligomers, which are thought to be responsible for the neurodegeneration of AD patients, have also been analyzed using IM-MS [31]. IM-MS revealed for instance that full-length $A\beta$ 42 aggregates faster and is more cytotoxic than the shorter $A\beta$ 40 variant, which could be related to a compact stable tetramer formation for the $A\beta$ 40 and a rather "open tetramer" that can evolve to an hexamer for the $A\beta$ 42. More recently, Illes-Toth *et al.* have characterized by IM-MS a specific population of α -syn oligomers, which was shown to have the ability to induce intracellular aggregation [208]. Structurally, the MS compatible oligomers populated a range of species from dimers to hexamers. Lower-order oligomers were structurally diverse and consistent with unstructured assemblies. Higher-order oligomers were shown to be compact with ring-like structures. The observation of this compact state may explain how this natively disordered protein is able to transfer pathology from cell to cell and avoid degradation by cellular proteases.

IM-MS provides valuable data on the ion's overall size and shape, but it does not yield direct information about the underlying internal structure. To obtain such data, IM-MS was recently used as a preselection tool for a further analysis using orthogonal, structure-sensitive gas-phase techniques, like action fluorescence resonance energy transfer (FRET) spectroscopy [209] or gas phase infrared spectroscopy [210]. IM-MS can also be successfully applied for high-throughput inhibitor screening. IM-MS is capable of high-throughput binding screening with a rate of up to 5000 molecules per day [211,212], providing not only conformational characteristics but also binding stoichiometries and specificities.

To summarize, IM-MS provides a useful insight into events that occur during the early stages of aggregation including delineating the structure of the monomer, identifying oligomer distributions, and revealing mechanistic details of the aggregation process.

7.4.2. Ion mobility spectrometry for protein-ligand interactions

The study of protein–ligand interactions is of particular interest for the pharmaceutical industry, especially in the context of drug development. Native MS has there also been demonstrated to be a pivotal technique providing relevant information about stoichiometry of interaction, site-specificity of ligand-binding and, in appropriate cases, affinities (through dissociation constant, K_D , measurements) between the target protein and the ligand [212,213]. As the binding of ligands often induces a conformational change of the protein, the hyphenation of IMS to native MS could bring an additional level of conformational characterization, in addition to the classical binding screening (for review see [213,214]). A first solution to track the conformational change of a protein upon ligand binding is thus to perform IM-MS and measure CCS values for the free protein and the ligand-bound form. However, this simple and basic idea is not straightforward as ligand binding not always induce major global conformational changes, but rather very localized conformational changes with exceedingly low impact on the global CCS measurements (below 5% CCS differences). In addition, the resolution of the IMS cell is here of paramount importance. As a consequence, careful optimization of the IM cell to distinguish between protein (P) and protein:ligand (P:L) are required to resolve CCS changes

lower than 2% for P:P and P:L complexes [29,68,215]. Despite these limitations, the use of IM-MS to discern conformational tightening on the c-MYC:MAX leucine zipper formation due to ligand binding has been previously reported by Harvey *et al.* [216] and along with these findings, provides an exciting prospect as a method for screening inhibitors to conformational dynamic systems. Collision induced unfolding (CIU) experiments have proven to be an interesting IM-based alternative to tackle systems involving very subtle conformational changes (<2%). CIU is an IMS-based gas phase method that aims at collisional heating of ions, typically through the application of an accelerating electric field that results in higher-energy collisions with background gas. Ions undergo unfolding and the resulting conformers are separated in the IMS cell. Two ions that share similar initial CCS values can be readily differentiated through the CCSs and collision energies associated with the unfolded protein forms populated during CIU. The comparison of CIU fingerprints (IM drift times as a function of accelerating voltages) obtained for P and different P:L systems can help to differentiate conformational populations of very close CCS (<1%, [217]). Ruotolo first showed that CIU experiments allow differentiation of the effects of ligand binding not normally observed by MS alone on wild-type (wt) and disease-associated variants of tetrameric transthyretin (TTR) [218]. CIU experiments were able to show that the natural thyroxine ligand of TTR stabilizes wt TTR but also disease-associated TTR variants. Similar experiments were reported for ligand binding on model proteins like FK-binding protein, lysozyme or myoglobin [219]. More recently, CIU experiments have been reported for the identification of kinase inhibitors that specifically bind to the inactive conformation of the protein. CIU patterns allowed distinguishing between inhibitors with similar K_D values, suggesting the use of IMS-based CIU fingerprints as a new assay capable of both rapidly detecting inhibitor binding and classifying the resultant kinase binding modes. Since then CIU experiments have been reported in advanced drug discovery workflow [220], highlighting the stabilizing role of lipids in the context of membrane protein complexes [221] or the cooperative stabilization effects upon sugar binding in the concanavalin A tetramer [222]. CIU appears as a method allowing to “resolve the unresolved through gas-phase measurements” [213].

7.4.3. Ion mobility spectrometry for membrane proteins

IM-MS is also a well-established strategy to study the global conformation of membrane proteins and the characterization of their interactions with ligand/lipids [223–225]. Membrane proteins are involved in many vital cellular processes, such as trafficking and signal transduction [226,227]. Due to the high hydrophobicity associated to their structure, membrane proteins are transferred into the mass spectrometer encapsulated within detergent micelles to preserve their native-like structure [167]. Detergent aggregates strongly suppress ionization in ESI and need to be removed to perform MS analysis. Therefore, ionization source conditions such as pressure, temperature, and acceleration voltages are critical for membrane protein analysis. The protective role of detergent aggregates was clearly showed in the case of the transmembrane complex BtuC2D2 [223]. After increasing the number of collisions to produce the release of the complex from the micelle, the intact tetramer can be observed. The same ion channel (BtuC2D2) and a transporter (KirBac3.1) were characterized by Robinson and coworkers [224] to compare the average CCS of both tetramers after release from the detergent micelles. Different activation energies were applied and the overall size of both tetramer remained constant. The same experiment was performed upon activation of their respective trimers. In this case, KirBac3.1 trimer exhibited one single distribution that remained constant through ion activation, however, a bimodal distribution was observed for the BtuC2D2 trimer when the acceleration voltage was set to 240 V being consistent with two conformer populations. Experimental CCS of BtuC2D2 and KirBac3.1 trimers were smaller compared to the theoretical ones, suggesting that trimer structures collapsed after the removal of one subunit.

CCS measurements played a critical role in the modeling strategy of full length OmpA (FL-OmpA) dimer structure [167]. Native MS data showed monomeric and dimeric populations while further IM-MS experiments revealed compact dimers and extended and compact monomers. The same experiment was carried out for the N-terminal domain (TM-OmpA), where only the monomer structure could be observed. These results combined with native MS

confirmed that dimer interface was located in the C-terminal 188-276 amino acid sequence. A low-resolution structure for the FL-OmpA dimer assembly was proposed based on IM-MS data as well as cross-linking constraints. The theoretical model of the FL-OmpA dimer is sustained by the good agreement between experimental and theoretical CCS.

Amphipatic polymers (APols) have been shown to be a suitable alternative to detergent aggregates for preserving membrane protein water solubility [228]. Watkinson *et al.* studied the influence of several APols on the structure and activity of three outer membrane proteins (Omp), namely PagP, OmpT, and tOmpA [229]. The catalytic activity of these three Omp strongly varies as a consequence of amphipols interactions, despite the fact that circular dichroism data indicate that PagP, OmpT, and tOmpA native structures is preserved in the amphipols. The structures of the released Omp were assessed by ESI-IMS-MS. The experimental CCS of the lower charge states were in good agreement with theoretical CCS from protein data bank (PDB)-coordinates indicating that APols tested in this study do not induce any significant structural changes.

Membrane proteins have shown strong interactions with lipids [167,230]. They play an essential role in membrane protein structure, folding, and activity [230,231]. Laganowsky and coworkers studied the selectivity of lipid binding to membrane proteins by using native MS and IMS. The stability of the mechanosensitive channel of large conductance (MscL), aquaporin Z (AqpZ) and the ammonia channel (AmtB) was assessed upon lipid binding by monitoring the unfolding process of the membrane proteins as a function of collision voltage (CIU experiments). CIU fingerprints revealed that upon the addition of CDL and PG, the stabilisation energy of AmtB dramatically increased. This result is based on the increase of the collision voltage required to induce unfolding process of the lipid complexes compared to the apo structures. In the case of AqpZ and MscL, the same stabilization effect was observed after the addition of the different lipids. These results emphasize the interest on CIU experiments to study the selectivity of membrane proteins toward lipids, and how this interaction can modulate their function. In this case, the trap cell was used for releasing membrane proteins from detergent micelles as well as to induce protein unfolding. To avoid the presence of detergent

aggregates in the trap cell, “in-source” fragmentation was performed by increasing the source temperature up to 120 V [225] (Figure 10), thus detergent aggregates are removed before the entrance of the ions in the trap cell. Ions are subsequently activated (CIU experiments) with or without previous ion selection (IMS-MS or MS-IMS-MS respectively) to monitor the unfolding process of membrane proteins as a function of the activation energy. Stabilization of AmtB upon lipid binding could be evidenced by comparing the transitions of the CIU profiles. The “in-source” fragmentation allowed performing MS-IMS-MS CIU profiles to evaluate the stabilization energy provided upon lipid binding.

Overall, for all these works, experimental CCS for membrane proteins were measured through the use of well-defined IM calibration protocols [232,233]. However, membrane protein ions exhibit lower mobility compared to soluble proteins with similar molecular weights [223] as a result of their low charge state. In a recent paper from Allison *et al.*, it was reported that the average mobility of membrane proteins was about 30% lower than soluble proteins used for TWIMS calibration [190]. Similar native-like membrane protein standards should be used to provide an accurate CCS measurement. Due to the lack of available membrane protein standards, they suggest to use larger calibrants for reducing the error associated with the long-range extrapolation of the calibration function.

7.5. Ion mobility spectrometry for large protein assemblies and its integration to structural biology programs

For protein analysis, IM-MS is mostly used to characterize conformational states or to monitor conformational changes induced upon any modification of the protein environment (experimental conditions, oligomerization, ligand binding, RNA/DNA binding, protein partner binding, etc. [41]). As the number of publications reporting the use of IM-MS for protein characterization has tremendously increased in the past 10 years, it is no more possible to draw an exhaustive picture of the possibilities of IM-MS for protein conformational characterization. IM-MS can be used to determine changes in mobility and thus conformation and CCS, properties such as conformational dynamics, folding and unfolding intermediates,

ligand-induced conformational changes, aggregation intermediates and quaternary structures (topology) [2,28,41]. We thus tried to focus on some relevant works performed over the past 10 years that could serve as case studies.

Native MS has been particularly useful to study the dynamics of assembly or disassembly of several macromolecular machineries, giving direct access to the stoichiometry of intermediates of assembly/disassembly through accurate mass measurement. When combined with IMS, native IM-MS enables monitoring of global conformational changes occurring during the assembly/disassembly process through accurate CCS measurements. Plots representing the CCS as a function of mass are often used to highlight assembly/disassembly events. In such representation, CCS is expected to follow a linear increase as a function of the compound mass, thus events that happen at constant CCS further suggest a major conformational change. An example of such IM-MS application is given by Van Duijn et al [234], who reported the use of native MS in combination to IMS for the study of the *E. coli* Cascade complex (405 kDa) and the *P. aeruginosa* Csy CRISP-complex (350 kDa) immune systems. They monitored Cascade complex disassembly either under gas-phase or in-solution dissociation experiments using native IM-MS. The results allowed to create subunit connectivity models of both the Cascade and Csy CRISPR immune systems. They compared experimentally measured CCS_{EXP} of Cascade sub-modules with values calculated using molecular modeling on the basis of the cryo-EM structure of Cascade. Finally, the interaction between Cascade and its target ssDNA was next monitored by IM-MS, highlighting a small but significant structural change for Cascade upon its association to ssDNA as evidenced by the measured CCS_{EXP} (3 nm² experimental compared to 9 nm² expected from calculations), which can only be explained by an altered conformation of the complex.

Several case studies based on structural information derived from IM-MS to build three-dimensional models have been reported, including the analysis of the full clamp loader complex ($\gamma\delta\delta'\psi\chi$, 254 kDa) in interaction with a single-stranded DNA (ssDNA) binding protein (SSB4) [235] (Figure 11), the analysis of the trp RNA binding attenuation protein (TRAP) [41],

the study of ribonucleoprotein particles (sno/sRNPs) [236] or to decipher the conformation and assembly of viruses with molecular weights ranging from 1.8 MDa up to 18 MDa [237–239]. Most of these studies were performed on TWIMS instruments. However, the group of Fernandez-Lima recently reported for the first time the use of TIMS instruments for accurate CCS measurements on protein complexes [240]. As CCS values alone give no detailed information on subunit structure within the complex, IM-MS analyses can be coupled to gas-phase activation techniques including collision induced dissociation (CID) and more recently surface induced dissociation (SID) to generate sub-complexes, SID being more adapted to retain native-like structures in the gas phase [241,242]. In addition, crosslinking strategies have been proven to be valuable to capture transient complex, protein complexes modified with either charged crosslinkers or tags, but not those treated with neutral crosslinkers, exhibiting enhanced stability in the gas phase relative to CIU [243].

Overall, these studies of large heteromeric complexes analyzed by IM-MS, coupled with integrative modelling, highlight the potential of such an approach to reveal structural features of previously unknown complexes of high biological importance.

IM-MS can be considered today as a complementary low-resolution technique for structural biology. Indeed, hybrid approaches in structural biology have gained considerable interest for uncovering the molecular architectures of large and transient biological systems. MS-based methods, and in particular IM-MS, and structural electron microscopy can complement conventional tools, such as X-ray crystallography and NMR spectroscopy [244,245]. Today, IM-MS is often applied to the structural elucidation of protein assemblies that have failed high-throughput crystallization or NMR spectroscopy screens. In the interpretation of the IM data for multiprotein complexes, there are two main steps to interpret IM for multiprotein complexes: the conversion of drift times into CCS values, and their comparison with model protein architectures.

Such IM-MS and MS/MS measurements are often integrated with other sources of structural information to build comprehensive models of intact multiprotein complexes that remain

refractory to more classical structural biology techniques. For instance, CCS restraints from IM-MS have been successfully combined to distance constraints from chemical crosslinking approaches to determine the best model among hundreds or thousands of computationally generated models for the study of the proteasomal base [246] or for the outer membrane protein A from *Escherichia coli* [247]. Combining IM-MS to solution hydrogen/deuterium exchange MS (HDX-MS) seems also appealing and has been reported for the characterization of flexible serpins [248] or on the Kai circadian complex [249]. For this latter system, native MS revealed that the complex formed between two of the components (KaiB and KaiC) is a dodecamer; HDX-MS provided their binding interface and finally the structural restraints derived from HDX were then combined with IM-derived CCSs and used as input for the HADDOCK web server to generate models of the KaiC6B6 complex. IM-MS is an emergent technology for structural proteomics, capable of assessing multiprotein topologies from complex mixtures using minute amounts of sample. Computational analysis is a key aspect of IM-MS data interpretation, and developments in this area are likely to be rapid over the next 5 years.

7.6. Biopharmaceuticals

MS performed under non-denaturing conditions (native MS) has emerged as a promising technique for mAb-related compound characterization [250,251]. As a logical evolution, hyphenation of IMS to native MS was also rapidly implemented for biopharmaceutical analysis. Since IMS provides a global view of conformational differences, it is a good choice for preliminary investigations of mAb conformations, where the shape and size of the molecule are of interest. IMS can either be used qualitatively mostly to address heterogeneity issues, but also in a quantitative way, to determine CCS values or to directly evaluate the relative amounts of the different species present in solution from IMS data. IMS can also serve to address more fundamental issues through collision induced unfolding (CIU) experiments, in which collisional heating is applied in the gas-phase to unfold the protein, which is subsequently separated by IM and then analyzed by MS. A typical CIU plot presents IM drift

times as a function of increasing collisional energies applied in the region in front of the IM cell [252]. CIU fingerprints represent the amount of protein unfolding as a function of collisional energy applied. Those unfolding patterns have proven to be highly sensitive to small variations in protein structure that cannot be easily resolved by IMS alone. Figure 12 summarizes the IMS-based information that can be obtained for biopharmaceutical characterization.

IMS for disulfide bridge heterogeneity - The first application of IMS for mAb structural characterization was reported in 2010 for the rapid characterization of disulfide variants in intact IgG2 mAbs [253]. IMS revealed 2 to 3 gas-phase conformer populations for IgG2s, whereas a single gas-phase conformer was revealed for either an IgG1 or a mutated IgG2, providing strong evidence that the observed IgG2 gas-phase conformers are related to disulfide bond heterogeneity. Similarly, Jones *et al.* have used IMS to rapidly provide information on the higher order structure of a series of wild type and mutant IgG2 mAbs [254].

IMS for global conformational characterization - Given that IM is fast and gives a global view of mAb-related compound conformation, several groups have addressed the global conformation of mAb, ADC or bispecific antibodies compounds using IMS, attempting to attribute CCSs to given mAb isotype conformations and even to particular conformational heterogeneities. Pritchard *et al.* used this approach to detect three monomer conformations and two dimers for a commercial rhGH-specific antibody [255]. A quantitative analysis of the ATDs revealed that ~80% of the species present in the sample was in the second monomer conformation. Pacholarz *et al.* reported structural differences between IgG1 and IgG4 based on IMS measurements [38]. Interestingly, the $^{DT}CCS_{He}$ values obtained on a linear drift tube IM instrument in helium (61.1–83.2 nm²) were in agreement with those measured on TWIMS instruments by other groups for different IgGs, which demonstrates the robustness of the CCSs calculated from properly calibrated IM-MS data. However, the experimentally IMS-estimated CCS values were shown to be significantly lower than the CCS determined either from crystal structures or from MD simulation [38,192]. This has recently been explained by the flexibility

of the hinge region which collapses in the gas phase, such that the measured CCS of the mAb is substantially less than both the predicted CCS from its crystal structure and the in vacuum MD simulation [192].

Real-time IMS to monitor dynamic processes – IMS has also been employed to monitor dynamic processes such as the IgG4 Fab-arm exchange that produces bispecific mAbs, constituted of two different half-antibody arms, each of which recognizes a distinct antigen, a particularly valuable property for therapeutic applications. In combination with time-resolved native MS, IMS was used to monitor the formation of the bispecific mAbs in real time [256], the CCSs of the bsAb (66.9 nm²) being intermediate to those of the original mAbs (65.6 and 69.6 nm²).

IMS for ADC drug conjugation heterogeneity assessment - IMS can also be of great interest for the characterization of drug conjugation heterogeneity/homogeneity for antibody-drug conjugate characterization. IMS can provide several information, among which the global conformation of all detected species including conjugated and unconjugated forms or the global conjugation profile and average drug-to-antibody-ratio (DAR) through a semi-quantitative interpretation of IMS data (integration of ATDs areas). Debaene *et al.* reported the IMS characterization of brentuximab vedotin, the reference cysteine ADC [257]. The different ^{TW}CCS of the multiple drug loads were calculated, evidencing slight conformational changes that occur upon payload binding. The average DAR determined from IMS was 3.7 ± 0.1 (vs. 4.0 expected), consistent with values obtained from other MS methods. In addition, hydrophobic interaction chromatography (HIC) fractions were collected and analyzed individually by IMS, revealing conformational homogeneity of each drug load species. Marcoux *et al.* next performed a similar study on trastuzumab emtansine (T-DM1), the reference lysine ADC already on the market [258]. The detailed structural picture provided by IM-MS was complemented by molecular modeling. As for the cysteine compound, native IMS highlights the increase in heterogeneity that occurs when trastuzumab is conjugated with DM1. The

1453 resulting drug distribution profile and average DAR (3.4 ± 0.2) are also consistent with values
1454 obtained from other MS methods (average DAR of 3.5). Recently, extensive conformational
1455 characterization by IMS of a new generation site-specific ADC enabled detection of different
1456 IM drift times for unconjugated mAb and the DAR4 site-specific ADC [259]. Again, average
1457 DAR values along with drug load distributions could be estimated. Altogether, these studies
1458 demonstrate the capabilities of native IMS to directly access site-specific ADC conformational
1459 information through the measurement of IM drift times and CCS calculations.

1460
1461 *Qualitative IMS for comparability studies* - IMS is a forefront methodology for qualitative batch
1462 to batch comparison. In case of comparability studies between originators and biosimilars
1463 (copy versions of mAb), IMS can address global conformational identity. IMS has been
1464 employed to highlight conformational heterogeneity in a biosimilar version of trastuzumab.
1465 Different IM drift times were observed for trastuzumab and its biosimilar version, which could
1466 be attributed to the presence of additional species bearing 0 or 1 glycan per Fc on the biosimilar
1467 version [260]. Similarly, a combination of MS-based approaches, including native MS and IMS
1468 were used to compare the originator compound Remicade (infliximab, Janssen) to its biosimilar
1469 product Remsima (Celltrion) [261]. Native IMS revealed similar IM drift times for mAb
1470 fragments, monomers, and dimers in both sample. Extensive comparative studies of
1471 monoclonal antibody drug products representing different IgG subclasses, manufacturers, and
1472 lots were also reported by Fergusson *et al.* [262] using a set of IMS-based metrics to assess
1473 reproducibility and robustness issues of IMS. IMS enabled detection of differences between
1474 monoclonal antibodies with the same target protein but different production techniques, as well
1475 as products with different targets. All of these studies conclude that native IMS analysis
1476 presents the advantage to require only a minimal (when manual buffer exchange is used) or
1477 no (when size exclusion is coupled to IMS using volatile salts) sample preparation, along with
1478 a global conformational characterization of the studied mAb-related compounds.

CIU experiments and variable-temperature IMS to study mAb unfolding patterns - IMS methodologies based on CIU experiments have been reported to address rapid conformational characterization of mAbs [263]. CIU experiments provide specific unfolding fingerprints that can be used to distinguish similar conformations, for instance mAb isotypes (IgG1 vs IgG2 vs IgG3 vs IgG4) with different numbers and/or patterns of disulfide bonding and levels of glycosylation. CIU experiments have also been reported for site-specific ADC characterization [259]. Even if different IM drift times were obtained for the ADC and its unconjugated mAb counterpart, they both exhibited similar intact CCS, which did not allow distinction between a mass effect or a real conformational change induced upon drug conjugation. CIU experiments were thus performed on both ADC and its unconjugated mAb version. Different CIU unfolding patterns were obtained for the unconjugated mAb compared to the site-specific DAR4 ADC, revealing increased ADC stability towards unfolding as compared with the unconjugated mAb. Here, CIU experiments allowed circumventing the lack of IM resolution through the establishment of unfolding patterns. CIU experiments were also used for comparability studies between biosimilar and originator [261]. Pisupati *et al.* compared the CIU patterns of the originator Remicade and its biosimilar version Remsina. Both compounds revealed remarkably similar patterns. While CIU patterns were globally similar (two unfolded states at nearly identical voltages for each CIU transition), a marked difference in the variance associated with the CIU fingerprint for Remsima at low collision voltages was observed. CIU experiments can thus be used for batch-to-batch comparison or more generally for comparability studies. Due to its higher reproducibility compared to traditional collision cross section IMS studies, CIU experiments remain a promising technique for advanced analytical characterization of protein therapeutics [262].

Variable Temperature IMS (VT-IM-MS) were also reported to address mAb conformation and conformational transitions induced upon IM cell temperature variations [264]. In this work, the thermal stability of three types of intact IgG molecules (with the same antigen binding specificity) has been probed by variable-temperature IM-MS. In these experiments, mass spectra and IM plots were acquired, while the temperature of the helium gas in the drift tube

was increased (from 300 to 550 K). While the native mass spectra showed no significant change as the temperature was increased (other than improved desolvation), the $^{DT}CCS_{He}$ of the IgG1 and IgG4 isotypes decreased because of compaction. The maximum collapse occurred at a higher temperature for IgG1 (400 K) than for IgG4 (360 K), in agreement with solution-phase studies in which IgG1 was found to be more thermally stable than IgG4. Differences in the extent of unfolding (an increase and widening of $^{DT}CCS_{He}$) among the IgG subclasses were also observed at high buffer gas temperatures, IgG4 unfolding at a lower temperature and to a greater extent than IgG1. VT-IM-MS provides insights into mAbs structural thermodynamics and can be considered as a promising tool for thermal-stability studies for proteins of therapeutic interest.

In conclusion, IMS is an interesting methodology for the characterization of intact antibodies and related formats (bispecific, ADCs) and may be useful to routinely fingerprint higher order structures.

Reproducibility of IMS measurements and mobilograms, as well as the suitability of IMS to differentiate between and/or characterize different monoclonal antibody drug products has been evaluated by the FDA [262], evidencing that the use of summed charge state datasets and the analysis of metrics beyond drift time should be preferred for a more comprehensive comparative study between different monoclonal antibody drug products. Also the added value of CIU experiments, that have proved to be more reproducible than classical IMS measurements (IM drift times and CCS), offers new possibilities for mAb-related compound conformational characterization. IMS measurements can finally be extended to investigate conformational changes induced upon antigen binding to individual mAb isoforms in order to gain more structural insights.

7.7. Oligonucleotides

IM-MS applied to oligonucleotides has been mainly used to gain conformational insights of such highly polymorphic macromolecule. Indeed, besides the classical and widely known

double-stranded DNA sequence (duplex), a plethora of other helical structures can be found, such as hairpin, pseudoknot, triplex, cruciform (Holliday junction) and G-quadruplex structures. Most of these DNA conformations have been studied by IM-MS and molecular dynamics, highlighting a more dynamic and complex behaviour in the gas-phase as compared to proteins [265].

One of the first research article was published by Clemmer *et al.* [266]. They reported a conformational study of an oligonucleotide comprising ten thymine nucleotides (T_{10}). The authors highlighted that the gas-phase conformations observed by IM-MS were strongly dependent of the charge states: lower charge states corresponded to globular conformers (lower CCS) and higher charge states to elongated forms (higher CCS). Moreover, by comparing the experimental CCS (Ω_{EXP}) with calculated CCS (Ω_{THE}) of conformers generated by modelling technique (by using one of the first parametrisation of EHSS model), [48] a strong dependency was found between the gas-phase conformations and the number and especially the locations of charged sites. It should be noted that charge states observed by IM-MS reflect the extension of the neutralization of the ionizable groups. Whereas in proteins, the ionizable groups are represented by the N- and C-terminal residues plus the basic and acidic side chains, in nucleic acids they are represented by the phosphodiester linkages that are all potentially and equally ionizable. In the case of T_{10} , neutralization could occur along the nine phosphate groups with a number of combination equal to $9!/n!(9-n)!$, thus several protomers could arise for each unique charge state. Authors calculated theoretical CCS (Ω_{THE}) for several different charge site assignments and they found that conformers having the same charge state (-5) showed a compact conformation ($\Omega_{\text{THE}} = 501 \pm 19 \text{ \AA}$) when having close located charges, and a most open conformation ($\Omega_{\text{THE}} = 608 \pm 21 \text{ \AA}$) when charges were more evenly distributed throughout the oligomer. Open conformation was found to match better with the experimental values ($\Omega_{\text{EXP}} = 627 \pm 6 \text{ \AA}$) and thus it was accordingly assigned [266]. Although the authors themselves questioned the absolute accuracy of the calculations, the concepts highlighted on number and locations of charged sites affecting the conformers shape were

fundamental to understand how different from solution nucleic acids might behave in the gas-phase.

Duplex stability and its behaviour in gas-phase has also been investigated by IM-MS and molecular modelling; initial contributions in this direction came from Bowers group [267–269]. First studies involving $d(CG)_n \cdot d(CG)_n$ duplexes [267] demonstrated that a globular form is the dominant conformer for up to 8-mer length oligomer, whereas for longer duplexes only the helical structure was observed, keeping quite good memory of the solution structure. Then, the effect of the oligomer sequence composition on the overall conformation of the duplex was investigated by studying a series of 14-mer duplexes containing both C·G and A·T pairs in different sequences [268,269]. It turns out that the A·T pairs were preferentially broken over C·G pairs and they appear more easily disrupted when placed at the ends of the duplex, while forming bubble if placed at the middle of the sequence. Therefore, overall results showed that duplex helicity was usually maintained in the gas-phase on the ms timescale but the structures stability was dependent of the length of the oligomer, its bases composition and its sequence order. However, as highlighted in [270], the structural comparison between the duplex native forms in solution and the conformations assumed in the gas-phase remained questionable, since the experiments were performed in non-native solution conditions. Indeed, a more recent contribution on duplex nucleic acids behaviour in the gas-phase upon native electrospray mass spectrometry came from the Orozco and Gabelica groups [270]. A compelling study on short (12-bp) and long (36-bp) DNA and RNA duplexes was performed by ESI-DTIM-MS, and a significant compaction of the duplex nucleic acids was observed for ions sprayed from native solution conditions. Ω_{THE} of the structures adopted in solution were obtained upon MD simulation carried out in water and then compared to Ω_{EXP} ($^{DT}CCS_{He}$) that were found significantly lower than the predicted values. To investigate the behaviour of the duplexes in the gas-phase, an extensive molecular modelling study was performed, including DFT and semi-empirical calculations for small helices and short duplexes plus MD simulations for long duplexes. Finally, authors were able to demonstrate that the compaction experimentally observed by ESI-IM-MS was related to the self-solvation of the phosphates groups occurring

through new phosphate-phosphate interactions that undermine the Coulomb-driven expansion of the duplexes. These metastable conformations arose in part during the desolvation process and rearranged by self-solvation while in the gas-phase, thus resulting significantly compacted compared to the starting structures [270]. Similarly, Devine *et al.* described the gas-phase compaction of two 35-mer RNAs having similar mass, but different sequences and secondary structures [192]. Reference Ω_{THE} values of the two 35-mer RNAs were obtained from an NMR and a crystal structure, respectively. Nevertheless, ESI-TWIMS-MS of the RNAs yielded to identical Ω_{EXP} ($^{\text{TW}}\text{CCS}_{\text{N}_2}$) that were significantly lower than the predicted values, hence accounting for a severe compaction of the RNAs in the gas-phase. However, beyond mentioning the collapse of the RNAs, authors did not investigate further on the RNA behaviour in the gas-phase by molecular modelling studies.

A quite different gas-phase behaviour was observed for higher-ordered oligonucleotide structures, namely triplex and G-quadruplex structures. In [271] Ω_{THE} obtained upon an extensive (90 μs) MD simulation were compared with Ω_{EXP} ($^{\text{DT}}\text{CCS}_{\text{He}}$) obtained by ESI-DTIMS experiments, to validate the theoretical data and assess the stability and the structure of a triplex in the gas-phase. A very good agreement was found between theoretical and experimentally derived CCS. A 12-mer and 18-mer triplexes were used for the study and the overall results suggested that the gas-phase structures maintained an excellent memory of the solution structures, together with a well-preserved helicity, even though a significant number of structural alterations were observed. Indeed, a slight compaction of the triplex (in respect to the solution structure) was observed for the most abundant charge states, but no triplex disruption was observed in any case.

Even a higher DNA structure stability in the gas-phase was observed for the G-quadruplex structures that were monitored by both ESI-IM-MS and molecular modelling [272–275]. G-quadruplexes are characterised by the formation of G-tetrads (four planar guanines held together by Hoogsteen hydrogen bonds) that stack on top of each other to form rigid and well organised structures, thanks also to the presence of cations placed in-between the G-tetrads that further stabilise the structure. These particular DNA conformations can be formed, starting

from G-rich oligonucleotides, they can be intra-, bi-, or tetra-molecular, and they are related to cellular ageing and transformations. Evidences of G-quadruplex structure preservation in the gas-phase were reported by several groups [276,277] and mainly related to the preservation of the inner cations in-between the G-tetrads [278]. Moreover, MD simulation (0.5 – 1 μ s) studies validated this preservation and proved the ability of the G-quadruplexes to keep an excellent memory of their solution structure [279], with a general excellent agreement between theoretical and experimentally derived CCS [39,268,272]. Then, the stability of G-quadruplexes in the gas-phase was furthermore validated after testing a series of potential ligands that were found to form stable complexes maintained also upon transfer in the gas-phase [280].

8. The future of ion mobility spectrometry

8.1. Some potential solutions to improve ion mobility resolution

One of the major constraints of IMS is the limited resolution of commercial instruments. This low separation power obviously limits the applicability of IMS, as it remains difficult to differentiate structurally related compounds (species having mobilities differences of less than 1-2% are hardly distinguishable today [215]). Besides resolving isobaric compounds, a higher resolving power could also contribute to increasing the dynamic range of the measurements [8].

To circumvent this limitation, there are many IMS prototypes that have been proposed by academic laboratories and providers of MS instrumentation, which are well described in the recent review of McLean and May [9]. However, it is important to keep in mind that achieving higher resolution comes at the expense of mobility range and/or sensitivity reduction. In this section, only the technical solutions that have the potential to be transformed into commercial products, at least from our point of view, will be described. Indeed, we have to keep in mind that the speed of translating IM-MS prototypes into commercial instruments in the last 5 years has increased dramatically.

As described in the first sections of this review, the DTIMS resolution is directly proportional to the square root of drift tube length and applied electric field. Therefore, there have been a lot of attempts to increase these parameters in DTIMS. However, when increasing drift tube length, the transmission may be drastically reduced (ion losses), or only a very narrow mobility range could be investigated, thus limiting the molecular coverage. In addition, a drift tube length of several meters, as described by Kemper [281] may be impractical for common laboratory space and requires very high voltages. To avoid the use of high voltages, the TWIMS technology was found to be more adapted in combination with long IMS cell. Recently, the group of Smith *et al.* have constructed a 13 m long serpentine TWIMS drift path, including 44 “U” turns to limit the required laboratory space [73]. In addition, the 13 m tube was included in a structure for lossless ion transmission (SLIM), to assist in ion transmission and manipulation, thus enabling a very limited loss in sensitivity. They demonstrated a baseline resolution for two isomeric sugars and also employed this new device for resolving peptides of an albumin tryptic digest. According to the authors, the resolution was increased by a factor of 9, in comparison with commercial DTIMS instrument having a tube length of 80 cm.

Another promising strategy to improve the current resolving power of IMS may be the use of a cyclic TWIMS design to limit laboratory space. Using this approach, very high resolution can be achieved after an important number of cycles (up to 12) on a circular TWIMS drift tube of around 1 meter [9]. One of the major limitations of the circular drift tube geometry may be related to the fact that the ions of high mobility of the next cycle can lap over the ions of low mobility from the previous cycle. Since the TWIMS operates in a time dispersive manner, this point is less critical, but one of the main limitations of this design remains that the measurable mobility range decreases with every cycle needed to improve the resolution.

Another strategy to improve IMS resolution is to modify the gas nature since the latter could strongly impact the separation power in FAIMS. In particular, Shvartsburg and co-workers have improved FAIMS measurements by using non-traditional buffer gases (mixtures of He/ N₂ or H₂/N₂). They showed that when helium rich-gases and elevated electric fields were used, the resolution was significantly increased, allowing the separation of previously unresolved

conformers of a proteolytic digest [282,283]. To avoid the electrical breakdown which is inevitable when a high proportion of He is used, mixtures of N₂ and up to 90% H₂ were also successfully employed [284]. Some other authors have also demonstrated that drift gas with high polarizability (carbon dioxide or argon) allow achieving better resolution and peak capacity in DTIMS and TWIMS, due to the extended interactions that take place [81,285]. As example, Eberlin *et al.* show a separation of disaccharide isomers in TWIMS with carbon dioxide, while it was not possible with nitrogen [286].

8.2. Improving orthogonality between ion mobility and mass spectrometry

Besides its limited resolving power, another important constraint of IMS is the lack of orthogonality with MS, since the shape of molecules in a gas phase (IMS separation criteria) is strongly correlated to its mass (MS separation criteria). In this sense, FAIMS offers a better orthogonality to MS, due to a different operation principle, in comparison with DTIMS, TWIMS and TIMS. Some authors have clearly demonstrated that the gas nature in IMS could also impact selectivity, in addition to the improvement of resolving power discussed in section 5.1. The most remarkable example to date has been reported by Hill *et al.* who demonstrated the separation of enantiomers in DTIMS, by doping the drift gas with a chiral selector (2-butanol) [287]. Some other research groups have also tested different drift gases (i.e. nitrogen, helium, argon, methane) in DTIMS for separating peptides from a tryptic digest, but they showed that selectivity was less affected by the gas nature, when the ions belong to the same biomolecular class [288].

In FAIMS, the selectivity could be drastically modified when adding different polar modifiers (alcohols, acetonitrile, acetone...) to the drift gas (nitrogen) [289,290]. This was well illustrated by Varesio *et al.* who considered a mixture of eight pharmaceutical compounds having all a mass of 316 Da, as a case study. As illustrated in Figure 13, with pure nitrogen as transport gas, the resolving power for the eight compounds with the same nominal mass was close to zero and the eight analytes experienced a relatively similar differential mobility in the 10 V compensation voltage window [291]. When adding only 1.5% organic modifier, the separation

was drastically improved, particularly with isopropanol. The separation enhancement was probably due to the formation of transient clusters between the analytes of interest and the polar modifier molecules. In this example, the overall IMS separation was quite good, despite the fact that the drugs possess the same masses, because their chemical properties were different as well as their interactions with the modifiers. In another study [292], 30 drugs of abuse were investigated in FAIMS using various modifiers (i.e. acetone, acetonitrile and methanol) in the drift gas. The best IMS separation was achieved with 1.5% methanol, but this example also demonstrates the limited resolving power of FAIMS (the maximum peak capacity was less than 20). Despite the poor peak capacity, some isomeric metabolites such as hydroxylated or desmethylated ones were partially resolved, and glucuronide metabolites were differentiated from the parent drug, preventing the overestimation of the latter in case of in-source fragmentation. Finally, Chen *et al.* have also used FAIMS for the detection and quantification of nine structurally related amphetamine-type stimulants in urine in presence of dopants (i.e. MeOH, propanol, butanol, hexanol, heptanol) and various transport gas (i.e. nitrogen, helium, argon, carbon dioxide) [293]. The authors concluded that the combination of He and MeOH allows achieving the best sensitivity and selectivity for the investigated mixture of compounds.

All the above mentioned studies illustrate the potential of using alternative drift gas natures or adding modifiers to the drift gas in IMS and DMS to tune selectivity. It is also clear that there are further combinations of gas and additives that could be explored to improve orthogonality between IMS and MS and some additional studies in this particular field will be helpful to make progress. However, it is also important to keep in mind that some of the current instruments on the market are not compatible with a variety of gases and modifiers and can only be used with pure nitrogen as drift gas (i.e. Waters VION, Bruker TIMS...).

8.3. Combining two-dimensional LC and ion mobility - mass spectrometry (4 dimensional analytical approach)

Because the samples to be investigated in the -omic field (i.e. lipidomics, proteomics or metabolomics), in natural products analysis or in biopharmaceuticals characterization are extremely complex, there is a need to develop analytical strategies able to separate the highest number of compounds. On the chromatographic side, comprehensive 2D-LC strategy has emerged over the last few years, as a promising (but complex) alternative to very high-resolution 1D separation [294–299]. Beyond separation techniques, IMS and HRMS become ever popular and powerful, particularly for untargeted analysis of complex samples. To simultaneously take advantage of these chromatographic and mass spectrometric advances, Stephan *et al.* [105,151] have recently combined in series 2D-LC and IMS-HRMS, enabling the characterization of the most complex samples in four dimensions. According to the authors, there are many constraints when implementing such a complex analytical setup. In particular, there is a need to improve the software for managing the LC+LC-IMS/MS setup and above all the data treatment. Nevertheless, they have successfully applied the strategy to characterize plant extract (*Ginkgo Biloba*) and achieved impressive performance, with a peak capacity of 8,700 for an analysis time of only two hours [105]. In addition, for each peak observed on the 2D-LC chromatogram, the CCS and m/z values were also available, as illustrated in Figure 14. Stephan *et al.* have also applied the same strategy for the analysis of wastewater [151]. They performed the analysis of wastewater sample in “regular” LC-IMS/MS and discovered 22 different pollutants using a database containing about 500 drug-like compounds and pesticides. When using the newly developed LC+LC-IMS/MS strategy, 53 different compounds were identified using the exact mass and CCS values in the database. In the future, it is expected that this type of approach will be investigated for the characterization of -omic samples, as the number of detected features could certainly be strongly enhanced when using these multiple separation dimensions. Another application could be the analytical characterization of biopharmaceuticals, including monoclonal antibodies, antibody-drug conjugates, and bispecific antibodies. The addition of IMS to the more commonly used 2D-LC-MS setup would be of particular interest because it could help to differentiate isobaric compounds that are not chromatographically separated [298]. Recently,

an innovative multidimensional analytical approach combining comprehensive on-line two dimensional chromatography that consists of hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC) to ion mobility and mass spectrometry (IM-MS) was applied for performing analytical characterization of ADCs under non-denaturing conditions. This promising approach allows obtaining in-depth and detailed information on ADC samples that cannot be reached from HIC-UV or non-denaturing mass spectrometry alone. The all-in-one analytical strategy described here permits a deep, straightforward and rapid characterization of complex ADC samples [300].

8.4. Improvement of data interpretation workflows and software

As underlined in the previous sections, there are currently a number of new IM-MS techniques and technologies under development with the aim of further improving the performance in terms of resolution, sensitivity and orthogonality between IMS and MS. However, one of the remaining limitations of IM-MS instrument is often represented by the software, which requires intensive development for improving speed and user-friendliness, especially when technical possibilities continue to grow.

Indeed, when performing LC-IM-MS experiments, the data are extremely complex as they are acquired on many different dimensions (i.e. retention times in LC; drift times in IMS; exact mass, MS/MS spectra and signal intensity in MS). Not all softwares are well developed for 4-dimensional (retention time, drift time, m/z , abundance) alignment of molecular features (entities) across different samples. This means that doing high-quality statistical interpretation during non-targeted (discovery) analysis can be extremely challenging or even impossible without innovation using open-source tools.

In addition, files processing of large data sets may be critical. In particular, peak picking (feature finding) can be very slow and algorithms optimization is still a topic of prime interest. In this regard, Vivo-Truyols and co-workers reported the Bayesian approach for features finding in GC-HRMS [301] or GCxGC-FID [302], that could be envisaged for application in LC-IM-MS data treatment in the future in order to improve the peak picking process throughput.

For processing MS/MS data alongside IMS information (e.g. LC-IMS-QqTOF/MS), some solutions are proposed by the IM-MS instruments providers. For example, Waters proposes the MS^E approach, which records data without discrimination or preselection, while Agilent uses an approach called "alternating frames", where two MS analyses (high energy and low energy) can be obtained within a single acquisition in order to associate MS/MS and MS spectra via drift time alignment. Such approaches can be already implemented into analytical methods, but software-based extraction of all the information together is awaiting for further developments and it will also require careful validation for non-targeted workflows. Another important area of software development regards the data deconvolution strategies, which allows the extraction of additional information from partially resolved ion mobility data, taking into account the orthogonal information obtained from ion fragmentation taking place after the ion mobility cell. This promising approach can allow the reconstruction of ion mobility spectra for individual species, especially for the challenging topic of isomer differentiation, as reported in [66].

9. Conclusion

This review paper summarizes the current status of IM-MS and main application fields where the technology has been employed to date. As discussed, the main benefits of IM-MS are the following: i) Improving the confidence in peak annotation thanks to the use of CCS values in addition to retention time, exact mass and fragment spectra, ii) Reducing the complexity of MS/MS spectra, and rapid association of fragments with their monoisotopic precursors by taking into account the mobility alignment, iii) Increasing the overall resolution of the analysis, since MS and HRMS are obviously unable to separate isobaric compounds, iv) In-depth studies of the gas phase conformation of large biomolecules such as proteins and oligonucleotides and comparison of calculated and experimental CCS values. Among the applications reported in this contribution, adding an IMS dimension looks particularly attractive in metabolomics for improving peak annotation confidence. It can also offer a higher resolving power for a few families of compounds having relatively high flexibility

and containing a substantial number of isomers. Among them, lipids and glycans are particularly well adapted to IMS and have been studied extensively during the last few years. Finally, due to the inherent complexity of protein biopharmaceuticals and oligonucleotides, there will certainly be even more interest for IMS in the coming years in order to increase the amount and quality of information that can be obtained from their analysis.

As illustrated throughout this paper, the field of IMS is very active and instrumental advances are being regularly published. In this regard, one of the remaining bottlenecks of IMS is its limited resolution. Therefore, there is a need for longer IMS drift cells, or alternative IMS designs, as an order of magnitude improvement in resolving power would clearly revolutionize the field [16]. Another important limitation of the technology is the poor orthogonality between IMS and MS. To improve this aspect, the addition of dopants (small amount of organic modifier) to the IMS drift gas appears as a promising strategy, but this solution is unfortunately not compatible with all IM-MS instruments. Finally, even though the sensitivity achieved in IM-MS has been drastically improved over the last few years, there could still be some sensitivity loss when adding an IMS dimension prior to MS. To avoid, or at least limit this issue, Smith *et al.* have recently developed some IMS structures with lossless ion manipulations (SLIM), which seems certain to be implemented in the next IMS generation [303].

The authors have declared no conflict of interest.

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11. Figure captions

Figure 1: Number of papers published since 1995 in the field of IMS, IM-MS and LC-IM-MS. In this figure, the 2 years gap, mean all papers published within 2 years. Source: Scifinder scholar 2017. Date of information gathering: June 2017.

Figure 2: Schematic representation of a three-dimensional analysis, including a liquid chromatography separation, followed by ion mobility discrimination and time of flight mass spectrometry detection. A 3D representation of the data was also included in this representation.

Figure 3: Schematic representation of the commercially available IMS technology, classified as time-dispersive methods (DTIMS, TWIMS), space-dispersive methods (FAIMS, DMA) and ion trapping methods (TIMS). This figure was adapted from [2,5,18,24], with permissions from the Publishers.

Figure 4: Schematic representation of the three atomistic models employed for estimating gas-phase conformations, allowing calculation of CCS values. A: projection approximation (PA), a non-explicit method working by projecting the “shadow” of the ion onto a plane to find the average area of all the possible orientations of the molecule under investigation. B: exact hard sphere scattering (EHSS), a hard sphere collisions based method that computes the multiple scattering events without taking into account the long-range interactions. C: trajectory method (TM), an explicit model which accounts for both long-range interactions and multiple scattering events. The arrows represents the buffer gas atom trajectories.

Figure 5: (a) Calculated absolute CCS differences required to satisfy various peak-to-peak resolution conditions based on experimental CCS data for positively-charge ions based on conditional resolution model for current Agilent DTIMS instrument. Symbol key: metabolites

(filled squares), peptides (open stars), and lipids (open circles). A peak-to-peak R_s value of 0.6 reflects conditions allowing both CCS values to be reliably determined for co-eluting isobaric species, while 1.5 represents baseline resolution. (b) Corresponding calculated percentage CCS differences for the same data. Reprinted from J. Chromatogr. A, Vol. 1416, Causon, T. J., Hann, S., Theoretical evaluation of peak capacity improvements by use of liquid chromatography combined with drift tube ion mobility-mass spectrometry., pp 47-56 (ref [82]). Copyright 2015, with permission from Elsevier.

Figure 6: Intensity vs. drift time for the protomers of ciprofloxacin, with the respective sites of protonation on the cyclic ketone and the piperazine moiety in the positive ionization mode. CCS values of the two protomers were also reported in this figure. This figure was adapted from [86], with permission from Waters corporation. However, a modification was applied by the authors of this application note on the original figure (Figure 5) found in [86], since one of the hypothesized protonation site was wrongly assigned in their original work.

Figure 7: CCSs of drug glucuronide metabolite structural isomers with different adducts and polarity. (A) Structures of drug glucuronide metabolite structural isomers. Overlays of representative arrival time distributions at 50 V drift voltage for (B) naringenin-4'-glucuronide (4') and naringenin-7-glucuronide (7), MW (Da) = 448.5 and for (C) β -3-estradiol-glucuronide (3) and β -17-estradiol-glucuronide (17), MW (Da) = 448.5. ATD full width half maxima and DTCCSHe averages are inset. Errors quoted are the standard deviations from at least two interday repeated measurements. Reproduced from Reading, E., Munoz-Muriedas, J., Roberts, A. D., Dear, G. J., Robinson, C. V., Beaumont, C., Anal. Chem. 2016, 88, 2273–2280 (ref [112]). Copyright 2016 American Chemical Society.

Figure 8: ESI(+) results from a mixture of D3-labeled hydrolyzed HEK cell lipid extracts spiked with unlabeled synthetic standards, SPH m18:1(4E)(3OH) and SPH m18:1(14E)(3OH). (A) Ionograms showing separation in voltage space of the $[M+H]^+$ ions formed from the two lipid

isomers. Each of the synthetic lipids spiked into the extract produced a peak in the black trace (m/z 284) and similar features were observed for the labeled HEK293 extract shown in the red trace (m/z 287). (B) Ozonolysis (OzID) spectra obtained for synthetic standard (black spectrum) and the D3-labeled extract (red spectrum) corresponding to the first feature in the ionogram (i.e., CV = 17 V ozone reaction time of 100 ms). (C) OzID spectra obtained for synthetic standard (black spectrum) and the D3-labeled extract (red spectrum) corresponding to the second feature in the ionogram (i.e., CV = 20 V ozone reaction time of 15 s). OzID product ions characteristic of the locations of the carbon-carbon double bonds are indicated by closed circles and enable assignment of the position of unsaturation to (Δ 14)-position (B) and (Δ 4)-position (C). Adapted from [147], with permission from the American Society for Biochemistry and Molecular Biology.

Figure 9: HDMS^E analysis of 400 ng Hela digest. IMS additional dimension provides an increasing number of identified peptides and proteins (38440 and 3490 respectively) with a FDR of 1% at a protein level using PLGS algorithm.

Figure 10: Workflow utilized for IMS-MS analysis of membrane protein-lipid interaction by Laganowsky *et al.* Proteins are released from micelles by rising the source temperature and subsequently activated in the trap cell. The in-source fragmentation allows performing of MS-IM-MS analysis on specific ions.

Figure 11: A general picture of the interaction of the full clamp loader and SSB4 before and after the addition of ssDNA. Mass spectra and the corresponding countour plots are depicted for a) γ 3 δ δ' ψ χ -SSB4 and b) γ 3 δ δ' ψ χ -SSB4-SSDNA₂. The three dimension structure proposed for c) γ 3 δ δ' ψ χ -SSB4 and e) γ 3 δ δ' ψ χ -SSB4-SSDNA₂ complexes from molecular modeling. Measured drift time (d) for the 36+ charge state of γ 3 δ δ' ψ χ -SSB4(blue) and γ 3 δ δ' ψ χ -SSB4-SSDNA₂ (red). A significant increase in measured collision cross section of the clamp loader-SS4 is observed upon DNA binding. Reprinted from J. Mol. Bio., Vol. 425, Politis, A., Park, A.

Y., Hall, Z., Ruotolo, B. T., Robinson, C. V., Integrative Modelling Coupled with Ion Mobility Mass Spectrometry Reveals Structural Features of the Clamp Loader in Complex with Single-Stranded DNA Binding Protein, pp 4790-4801 (ref [235]). Copyright 2013, with permission from Elsevier.

Figure 12: Insights about biopharmaceutical mAbs and ADC characterization based on IM-MS data. a) Analysis of global conformation differences induced by disulfide bridge heterogeneity (Reproduced from Bagal, D., Valliere-Douglass, J. F., Balland, A., Schnier, P. D., Anal. Chem. 2010, 82, 6751–6755. (ref [253]). Copyright 2010 American Chemical Society.) b) Benchmarking of different conjugation strategies by IM-MS, c) CIU fingerprint to evaluate gas-phase unfolding process, d) analysis of protein aggregation (oligomerization), e) characterization of the global conformation of gas-phase ions.

Figure 13: Separation of a mixture of 8 pharmaceutical compounds having a nominal mass of 316 by DMS coupled to a QqQLIT MS. DMS separation: (a) without polar modifier (nitrogen), (b) with 1.5% of MeOH as modifier, (c) with 1.5% of EtOH as modifier, and (d) with 1.5% of i-PrOH as modifier. With kind permission from Springer science + business media: Anal. Bioanal. Chem., Real-time 2D separation by LC × differential ion mobility hyphenated to mass spectrometry, Vol 402, 2012, 2555-2564, Varesio, E., Le Blanc, J. C. Y., Hopfgartner, G. Figure 3 (ref [291]).

Figure 14: Heat map of an LC + LC-IM-qTOF measurement of a *Ginkgo biloba* extract (a). The separation on the second dimension column of one fraction collected between 4 and 8 min (eluting from the second dimension between 8 and 12 min) from the sample is zoomed in (b). 2D plot of m/z vs. drift time extracted from 9.63 to 9.76 min (c). The zoomed in image shows the separation of two peaks with the same m/z in the IM dimension (d). With kind permission from Springer science + business media: Anal. Bioanal. Chem., A novel four-dimensional

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2878 Jakob, C., Hippler, J., Schmitz, O. J. Figure 4 (ref [105]).
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12. Short biography of authors

Dr Valentina D'Atri obtained her PhD in industrial and molecular biotechnologies at the University of Naples Federico II, Italy. She is now Postdoc at the University of Geneva, Switzerland. Her expertise includes mass spectrometry, ion mobility-mass spectrometry, molecular modelling and separation science, including HPLC, UHPLC, and HILIC. Her current research activity is in the field of biopharmaceutical proteins; in particular, her interest focuses on the analytical characterization of mAbs, ADCs, and fusion proteins by LC-MS and LC-IM-MS.

Dr Tim Causon obtained his PhD at the University of Tasmania, Australia. He is now Senior Scientist in the group of Instrumental Analytical Chemistry and Metabolomics at the Department of Chemistry of the University of Natural Resources and Life Sciences in Austria. His research interests centre on mass spectrometry, ion mobility-mass spectrometry and separation science, including analytical method development for metabolomics, as well as fundamental analytical studies.

Dr. Oscar Hernandez Alba has joined the BioOrganic Mass Spectrometry Laboratory (LSMBO) at the Hubert Curien Pluridisciplinary Institute (IPHC, University of Strasbourg, CNRS UMR7178) in Strasbourg as a postdoctoral researcher. He obtained his PhD degree at the University of Paris-Sud. His thesis was focused on developing a multimodal approach based on a single mass spectrometer, which incorporates ion mobility spectrometry and infrared spectroscopy in order to characterize the structure of mobility- and mass-selected molecular ions. He is currently working on the characterization of biopharmaceuticals and noncovalent protein/protein complex using native mass spectrometry and ion mobility spectrometry.

Aline Mutabazi graduated from the School of Pharmaceutical Sciences of the University of Geneva as a pharmacist in 2016. After a master thesis about the development and validation of a LC-MS method for the analysis of neonicotinoids in honey, she joined Jean-Luc Veuthey and Davy Guillarme's team in January 2017 to begin a Ph.D. thesis focused on ion mobility applied to the analysis of pharmaceutical compounds.

Prof. Jean-Luc Veuthey is professor at the School of Pharmaceutical Sciences, University of Geneva, Switzerland. He also acted as President of the School of Pharmaceutical Sciences, Vice-Dean of the Faculty of Sciences and finally Vice-Rector of the University of Geneva. His research domains are: development of separation techniques in pharmaceutical sciences, and more precisely: study of the impact of sample preparation procedures in the analytical process; fundamental studies in liquid and supercritical chromatography; separation techniques coupled with mass spectrometry; analysis of drugs and drugs of abuse in different matrices. He published more than 300 articles in peer-review journals.

Dr. Sarah Cianférani holds a PhD degree in analytical chemistry from the University of Strasbourg, France. She is now a CNRS research director and heads the BioOrganic Mass Spectrometry Laboratory (LSMBO) at the Hubert Curien Pluridisciplinary Institute (IPHC, University of Strasbourg, CNRS UMR7178) in Strasbourg. Her expertise focused on developments in structural mass spectrometry (native MS, ion mobility, H/D exchange and crosslinking) and applications of advanced native mass spectrometry and native ion-mobility mass spectrometry methodologies for biological noncovalent complex characterization, and especially biopharmaceuticals (mAb-related compounds). She is a co-author of more than 110 scientific papers related to mass spectrometry analysis of proteins.

Dr Davy Guillarme holds a PhD degree in analytical chemistry from the University of Lyon, France. He is now senior lecturer at the University of Geneva in Switzerland. He authored 190 journal articles related to pharmaceutical analysis. His expertise includes HPLC, UHPLC,

2935 HILIC, LC-MS, SFC, analysis of proteins and mAbs. He is an editorial advisory board member
2936 of several journals including Journal of Chromatography A, Journal of Separation Science, LC-
2937 GC North America and others...
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