Recent advances in biological separations using trapped ion mobility spectrometry – mass spectrometry

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ABSTRACT
Ion Mobility Spectrometry (IMS) is a widely used technique for the post-ionization separation and structural characterization of biomolecules. Trapped IMS (TIMS) is a relatively recent advance in the field of linear IMS that has shown advantages for the study of biological problems when in tandem with mass spectrometry (TIMS-MS). TIMS’s unique nature of holding ions using an electric field against a moving buffer gas allows for the tuning of the mobility separation by defining the scan rate as a function of the analytical challenge. TIMS can provide accurate CCS values (<0.2% RSD) and a high mobility resolving power (R up to 470). This recently commercialized technology has distinct analytical and structural biology applications. The present review focuses on advances in biological separations using TIMS-MS instrumentation for the case of isomer separations (e.g., lipids, epimers, topoisomers, positional PTMs), conformational states (e.g., peptides and proteins) and native macromolecular assemblies.

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1. Introduction

Ion Mobility Spectrometry (IMS) is extensively used in the detection of chemical warfare agents [1], explosives [2], illicit drugs [3], and has shown its potential for the structural characterization of biomolecules [4]. The separation of ions in a IMS device is based on the differences in their ion mobilities, or ion neutral collision cross sections (CCS), under the influence of an electric field (E) in the presence of a buffer gas [5]. IMS has been coupled to mass spectrometry (MS) to achieve an additional dimension of separation based on ion mobility or CCS and mass-to-charge (m/z) ratio [6]. In particular, this coupling affords several advantages over MS alone by providing measurement of ion size, separation of isomers, isobars and conformers, increased dynamic range, peak capacity and reduction of chemical noise [6]. In addition, coupling IMS to tandem mass spectrometry (IMS-MS) [7,8], such as collision induced dissociation (IMS-CID-MS), permits to fragment the precursor ions without need for preselection, since precursor ions are already separated in the IMS cell [7,8].

IMS-MS has proven to be valuable in a variety of bioanalytical applications, such as small molecules [9], lipidomics [10] and proteomics [11], thanks to high speed (millisecond timescale), higher selectivity and increased peak capacity. A common pursuit for IMS development has been focused on the increase in the mobility separation and ion transmission to solve bioanalytical challenges. These developments have led to a variety of high resolution (R > 100) IMS designs, including atmospheric pressure drift tube IMS (AP-DTIMS) [12], cyclic DTIMS [13], field asymmetric IMS (FAIMS) [14], trapped IMS (TIMS) [15], structures for lossless ion manipulations (SLIM) [16] and cyclic traveling wave IMS (TWIMS) [17].

Since the first publication of TIMS-MS in 2011 [18,19], many studies have shown the potential of TIMS-MS in a variety of bioanalytical applications, including small molecules [20–23], petrochemicals [24], lipidomics [25,26], catalysis [27], coenzymes [28,29], peptides [30–35], proteins [36–42], polymers [43,44], DNA [45–47], glycomics [48] and proteomics [49–51]. The commercialization of a high resolution TIMS-MS instrument by Bruker Daltonics Inc. in 2016 has further spurred the adoption of TIMS-MS for such applications. Different from other linear IMS variants, TIMS is based on holding the ions stationary using an electric field (E) against a moving buffer gas. The implementation of the TIMS technology yields several attractive features including (1) the ability to radially confine the ions allowing higher ion transmission and sensitivity...
In this article, we review the recent advances in biological separations and structural investigation of biomolecules using TIMS-MS. A special emphasis is placed on the capability of high resolution TIMS-MS for the separation of isomers (e.g., lipids, epimers, topoisomers and positional PTMs), conformational states resolution TIMS-MS for the separation of isomers (e.g., lipids, epimers, topoisomers and positional PTMs), and high mobility species with extremely small CCS differences permitting to separate species with extremely small CCS differences [31].

In this article, we review the recent advances in biological separations and structural investigation of biomolecules using TIMS-MS. A special emphasis is placed on the capability of high resolution TIMS-MS for the separation of isomers (e.g., lipids, epimers, topoisomers and positional PTMs), conformational states (e.g., peptides and proteins) and native macromolecular assemblies. More details on the trends in TIMS-MS instrumentation can be found elsewhere [54].

2. TIMS instrumentation

The general fundamentals of TIMS as well as the calibration procedure have been described in the literature [52,55–57]. A conceptual schematic of the TIMS analyzer is illustrated in Fig. 1a. The TIMS analyzer section is composed of a stack of segmented ring concave electrodes, where each electrode is comprised of four electrically isolated segments. A radio frequency (rf) voltage is applied to the electrodes of the TIMS analyzer to generate a radially confining pseudopotential. In addition to the radially confining rf, an axial electric field gradient (EFG) is produced across the segmented ring concave electrodes to counteract the drag force exerted by the gas flow and trap the ions in the TIMS analyzer. Ions are eluted from the TIMS analyzer region by decreasing the axial electric field (Fig. 1b). The TIMS analyzer section is also bracketed by electrodynamic ion funnels to efficiently transfer ions into and out of the analyzer region.

The commercialization of the TIMS-qTOF technology (timsTOF Pro™, Bruker Daltonics, Inc.) in 2016 includes a TRAP-TIMS analyzer in addition to the possibility to perform parallel accumulation - serial fragmentation (PASEF) (Fig. 1c) [51]. The currently commercialized TIMS-qTOF differs from the single stage TIMS design in having an additional linear trap section that allows for trapping of ions prior injection to the TIMS analyzer region, leading to near 100% duty cycle (Fig. 1c). In PASEF MS/MS scans, the selected precursor ions are synchronized with the TIMS separation (Fig. 1cE).

The advantages of a tandem TIMS analyzer (TIMS-TIMS) has been recently demonstrated for the analysis of biomolecules [41]. The TIMS-TIMS instrument consists of two TIMS analyzers connected by an interface region composed of two apertures (Fig. 1d). The two adjacent TIMS analyzers are dimensionally identical and have the same concave electrode TIMS design as presented in Fig. 1a. The specificity of this acquisition scheme is based on the capability to fragment multiple precursor ions from a single TIMS scan (Fig. 1cF).

The pursuit for the analysis of intact macromolecular assemblies lead to the development of new electrode TIMS geometries, with higher penetration radial fields while keeping the high mobility resolution. A new convex electrode TIMS geometry (Fig. 1e) was introduced in 2018, not yet commercially available, capable of trapping high molecular weight species (up to 1 MDa) in native conditions contrary to the initial concave electrode TIMS geometry utilized in the commercial trap-TIMS instrument. Although the shape of electrodes is different, ion dynamic simulation using an elastic hard sphere model from the SIMION (v 8.0) package showed similar behavior between the two geometries. The convex electrode TIMS geometry requires smaller rf amplitudes than the concave electrode geometry. This feature is particularly interesting since the rf amplitude can be a limiting factor during the TIMS operation due to potential discharges.

3. TIMS applications in lipidomics

IMS–MS was recently shown to be a useful tool for lipid class and isomer identifications. In fact, previous studies from other high
resolution IMS techniques (e.g., AP-DTIMS [12], FAIMS [58] and SLIM [59]) in shotgun lipidomics [10] have already demonstrated the capability for IMS to separate lipid isomers with a high degree of analytical selectivity. The introduction of TIMS in 2011 and recent commercialization in 2016 has enabled the community to explore lipidomic applications in a LC-TIMS-MS/MS fashion. In a recent study, the analytical power of LC-TIMS-MS was compared to LCxLC-MS for the identification and separation of common lipid classes during non-targeted analysis of human plasma [25]. The first TIMS lipid separation was achieved on a mixture of ten lipid standards including lysophosphatidylcholine (LPC), diacylglycerol (DG), phosphatidylethanolamine (PE), ceramide (CER), phosphatidylserines (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), sphingomyeline (SM), phosphatidylinositol (PI) and triacylglycerol (TG) [25]. Moreover, CCS values were calculated for each lipid and were found consistent with previous reported CCS for the same lipid species [12,60]. In addition, TIMS has been coupled to liquid chromatography (LC) for non-targeted analysis of human plasma resulting in approximately 800 molecular features with a great number of compounds with equal retention times but separated in the TIMS dimension [Fig. 2a] [25]. These findings illustrate the good orthogonality and analytical potential of combining the LC, TIMS and MS dimensions.

In another recent study, the analytical power of TIMS-MS was investigated for the separation of PC lipid isomers, comprising different double bond orientations at the same position (Fig. 2b) and different double bond locations with the same geometry (Fig. 2c) [26]. TIMS experiments at slow scan rates \(Sr = 0.012–0.016 V/\text{ms}\) delivered a nearly baseline separation for the protonated species of PC(18:1(6Z)/18:1(6Z)) and PC(18:1(9Z)/18:1(9Z)) (Fig. 2c) while the protonated species of PC(16:1(9Z)/16:1(9Z)) and PC(16:1(9E)/16:1(9E)) were well separated in their mixture (Fig. 2b). Comparison of the TIMS performance on PC(18:1(6Z)/18:1(6Z)) and PC(18:1(9Z)/18:1(9Z)) with other high resolution IMS technologies showed similar separation performance as obtained in AP-DTIMS [12] and SLIM [59], but was better than reports using high resolution FAIMS [58] for which the lipid isomers are not separated. For PC(16:1(9Z)/16:1(9Z)) and PC(16:1(9E)/16:1(9E)), the TIMS separation exhibited better performance than the separation obtained in AP-DTIMS [12] but the high resolution FAIMS [58] and SLIM [59] technologies showed better performance by resolving these two lipid species (Fig. 2b). These two examples showcase the orthogonality between FAIMS and TIMS for the case of lipids. The present data were consistent with other IMS variants, for which lipids with cis double bonds have smaller structures than those with trans double bonds and lipids with cis double bonds closer to the head group have more extended structures than those presenting cis double bonds farther to the head group. The TIMS technology appears to be sufficiently powerful when compared to the other IMS technologies and has the advantage of being commercially available for the analysis of small molecules, proteomic-like applications and small proteins (<150 kDa) in the native form.
4. TIMS applications in peptides and proteins

Since the introduction of IMS-MS, numerous studies have been focused on the capability of IMS-MS to separate biological isomeric species. However, in some cases, the IMS resolving power has been limited resulting in unresolved isomeric species in complex biological mixtures. The push for higher resolution and sensitivity has led to the developments of the TIMS technology among others, and TIMS-MS has been applied to biological isomeric separations [31,32,50]. Recently, the performance of the TIMS analyzer was demonstrated for the separation of D/L-peptide epimers in isomeric mixtures [31] (Fig. 3a). In addition, a linear quantification down to 0.25% proved the utility of high resolution TIMS-MS for real samples with large interisomeric dynamic range as compared to the traditional MS/MS approach (Fig. 3a1-3). Moreover, the advantage of using mobility separated collision induced dissociation (TIMS-CID) followed by high resolution mass spectrometry for epimer analysis has been shown to enhance their identification and further improve their detection and quantification limits [31] (Fig. 3a4).

In a recent study, the potential of native nESI-TIMS-MS for high throughput screening of peptide topoisomers was illustrated for lasso peptides with their branched-cyclic analogs [32] (Fig. 3a5). In addition, the advantages of metalation as a way to increase the analytical power of TIMS has been demonstrated for topoisomers with similar mobilities as protonated species, efficiently turning the metal ion adduction into additional separation dimensions [32]. In another recent study, the performance of the TIMS platform was demonstrated for the separation of middle down proteoforms of complete histone tails comprising methylation, acetylation and phosphorylation post-translational modifications (PTMs) [50] (Fig. 1a6). In addition, comparison of TIMS and FAIMS data exhibited a great orthogonality between these two mobility dimensions, suggesting online FAIMS/TIMS-MS as a powerful platform for proteoform analysis [50].

Previous reports have shown the advantages of TIMS-MS for the structural investigation of proteins in native and/or denaturate states [36–42]. In particular, recent studies investigated the kinetic intermediates of cytochrome c [38] (Fig. 3b1) and rice nonsymbiotic hemoglobin type 1 [39] as a function of the starting solvent organic content and pH solution conditions. In addition, candidate structures can be proposed using molecular dynamic simulations by correlating the CCS and charge state with the starting solution conditions as a way to describe a potential folding/unfolding pathways and their intermediates [36,38,39]. Moreover, the possibility of measuring CCS and HDX back-exchange rates simultaneously, permitted the identification of local fluctuations [36,38] which might be useful in the identification of structural features during protein folding/unfolding. In addition, collisional activation prior to TIMS analysis has shown advantages for the study of protein unfolding pathways [38,40,41]. In particular, previous studies assessed the structural changes induced in cytochrome c [38] (Fig. 3b2) and ubiquitin [40] using collision induced activation (CIA). In addition, the recent implementation of tandem TIMS permitted the structural denaturation of ubiquitin by mobility selecting the +7 ions in the TIMS-1 and collisionally activating the mobility selected +7 ions in the TIMS-2 [41] (Fig. 3b3).
The advantages of TIMS-MS have also been illustrated for the conformational dynamics as well as folding/unfolding pathways of DNA molecules (e.g., i-motif DNA) \[45,47\]. Recently, the conformational dynamics and DNA binding of the intrinsically disordered ATHP3 peptide has been investigated using single amino acid substitutions and TIMS-CID-MS revealing that the $\text{eRGRP}$ core is essential for stabilizing ATHP3 in complex with DNA \[46\].

Condensed-phase separations are now increasingly complemented or replaced by IMS-MS in proteomic applications due to the high speed (ms timescale) and unique selectivity. The recent commercialization of the timsTOF equipped with a TRAP-TIMS analyzer has been a great advance for proteomics due to its capability to do online parallel accumulation - serial fragmentation (PASEF) (Fig. 1e) with near 100% duty cycle. In particular, recent

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**Fig. 3.** (a) TIMS spectra showing the potential of TIMS for separation of 1–4) D/L-epimers, 5) lasso and branched-cyclic topoisomers and 6) middle down proteoforms (PTMs). (b) 1) Mass spectra and overall CCS profiles of cytochrome c as a function of the starting solvent conditions, 2) TIMS spectra of the +6 to +11 charges states of cytochrome c as a function of the organic content and the activation energy and 3) mobility selection followed by collisional activation of compact ubiquitin +7 ions in TIMS-TIMS. (c) TIMS analysis of tryptic digest of ADH, BSA, phosphorylase b and enolase using the PASEF method.
studies demonstrated the high performance of the PASEF method during complex protein [49] and human cervical cancer cell [51] digest analyses. The application of the PASEF method to complex protein digests is illustrated in Fig. 3c. Fig. 3c1 shows the complicated m/z and TIMS distribution as detected in full scan. As apparent in Fig. 3c2, sequential isolation of four mobility separated precursors have been selected after parallel accumulation. In the next step, the PASEF method has been applied by performing MS/MS on the isolated precursors leading to characteristic fragment ions for each precursor (Fig. 3c3). The fast switch time of the quadrupole enabled to target multiple precursors during the 50 ms mobility separation in the PASEF method (Fig. 3c4).

5. TIMS analysis of intact macromolecular assemblies

The advantages of coupling ion mobility spectrometry with mass spectrometry for the study of intact proteins and protein assemblies under native conditions, providing unique information about their three-dimensional structure, has been previously summarized [11,61]. In a preliminary study, the high resolution TIMS technology showed great promise for the structural characterization of intact proteins and protein complexes during native conditions, with data reported up to a ~150 kDa (Avastin, IgG) [37]. In order to increase the molecular weight and size range, new TIMS electrode geometries were studied based on the possibility to apply higher penetration radial fields, since the rf amplitude can be a limiting factor during TIMS operation, and to increase the trapping volume leading to larger mobility ranges and higher sensitivity.

A convex electrode TIMS geometry was introduced in 2018 capable of measuring intact macromolecular assemblies over a large molecular weight (MW) range approaching 1 MDa [62]. Examples of the analytical power with CCS measurements (up to 23,000 Å² with <0.2% RSD) over a large m/z range (up to m/z 19,000) were illustrated for common proteins, antibodies, protein oligomers, protein-DNA and protein-protein complexes during native analysis. The implementation of the convex electrode TIMS geometry permitted to trap species over a larger mobility range, as illustrated for tuning mix oligomers (Fig. 4a). In addition, the convex electrode TIMS design allowed for a better trapping efficiency of high m/z / low charge state species when compared to the concave electrode TIMS geometry. For example, the analysis of a monoclonal immunoglobulin G (IgG, Avastin, MW 149 kDa) antibody, the current largest trapped species using the concave electrode design (25 + to 27 +), resulted in the trapping of lower charge state (19 + to 25 +) as compared to the concave electrode design (25 + to 27 +), while maintaining the same performance (Fig. 4b).

The high performance of the convex electrode TIMS geometry was shown for very high MW non-covalent protein assemblies, including the E. coli RNA polymerase holoenzyme (479 kDa, Fig. 4c) and the bacterial chaperonin GroEL (801 kDa, Fig. 4d). In addition, the E. coli RNA polymerase holoenzyme dimer (958 kDa) was also observed and is currently the highest MW species trapped with the TIMS technology (Fig. 4e) [62]. In a recent report, the convex electrode TIMS design proved to be a powerful analytical tool for the structural investigation of protein – DNA complexes under native conditions, for which multiple IMS bands (R = 100 at Sr = 0.3 V/ms) were observed for the unbound and protein – DNA complex [63]. The ability to perform high resolution ion mobility characterization of intact macromolecular assemblies under native conditions using the convex electrode TIMS geometry opens new applications in structural and biochemical studies as well as the investigation of protein-interaction networks.

6. Conclusion

The attractive features of the TIMS technology, including high sensitivity, small size and high resolving power, has allowed substantial advances in many bioanalytical applications. TIMS instrumentation has also been subjected to significant growth since the first published work in 2011, mainly leading to the TRAP-TIMS, tandem TIMS, convex electrode TIMS and PASEF technologies. These recent advances have revolutionized lipidomics and proteomics analysis and the structural characterization of peptides, proteins and intact macromolecular assemblies. Furthermore, the
TIMS-MS platform present the advantages to perform analysis in a very short time (ms timescale) as compared to traditional LC-MS approach (min timescale) and provide information about the structure of the interest compounds through the CCS values. A rapid increase of TIMS-MS users is expected given the recent commercialization of the timsTOF equipped with a TRAP-TIMS analyzer capable of near 100% duty cycle and online parallel accumulation - serial fragmentation. Further integration of the TIMS technology with other MS analyzers and their commercialization will increase the analytical power and number of biological applications. In addition, further improvements in analytical instrumentation could lead to the integration of orthogonal technologies (e.g., online GC/LC-FAIMS-TIMS-MS) for higher specificity, higher sensitivity and increased peak capacity, further enabling the separation of complex biological mixtures. The increasing popularity combined with the flexibility of TIMS indicate that this technology will continue to evolve and come into routine use for a broad range of applications.

**Author contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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