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TRENDS

Ion mobility–mass spectrometry as a tool to investigate protein–ligand interactions

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Abstract Ion mobility–mass spectrometry (IM-MS) is a powerful tool for the simultaneous analysis of mass, charge, size, and shape of ionic species. It allows the characterization of even low-abundant species in complex samples and is therefore particularly suitable for the analysis of proteins and their assemblies. In the last few years even complex and intractable species have been investigated successfully with IM-MS and the number of publications in this field is steadily growing. This trend article highlights recent advances in which IM-MS was used to study protein–ligand complexes and in particular focuses on the catch and release (CaR) strategy and collision-induced unfolding (CIU).

Keywords Ion mobility–mass spectrometry · Protein–ligand complexes · Native mass spectrometry · Noncovalent complexes · Catch and release · Collision-induced unfolding

Introduction

Proteins are highly abundant biological macromolecules and the *workhorses* in living cells. The structural elucidation of proteins is essential to unravel the function and better understand the various biological processes they are involved in. Over the last two decades mass spectrometry (MS) and related techniques emerged as an additional method in the structural biology portfolio and are today used on a routine basis to study noncovalent protein–ligand and protein–protein complexes [1]. Although this *native* MS was initially perceived rather critically by the community, it has been shown by now that characteristic structural elements in proteins up to the entire native-like folded structure can be retained in the gas phase [2]. In addition, protein–ligand interactions can remain intact under certain conditions, even for complexes that are largely held together by hydrophobic interactions [3, 4]. An analysis of the mass-to-charge (*m/z*) ratio typically provides information about the stoichiometry, connectivity, and topology of protein complexes while the charge state obtained after electrospray ionization (ESI) can be used as a measure for the overall shape [5]. Protein–ligand binding affinities can also be determined by ESI-MS, which in many cases were shown to agree well with values from established solution-phase methods [6, 7].

Ion mobility–mass spectrometry (IM-MS), which combines MS with ion mobility separation (IMS), is a useful tool to obtain further information on the gas-phase structure of biomolecules and their assemblies [8]. IMS can be considered as an analogous technique to electrophoresis in solution, in which the gas-phase ions are separated according to their collision cross section (CCS) and charge. As a result, the additional dimension of size and shape, which is orthogonal to the *m/z* measured in MS, is obtained.

A typical *native* IM-MS experiment starts with the soft ionization of the protein complex via nano electrospray ionization (nESI), which results in the formation of intact complex ions with relatively few net charges [9]. The ions are then guided by a weak electric field through the ion mobility cell, which is filled with an inert buffer gas, such as helium or nitrogen (Fig. 1a). During their migration, ions with a compact shape undergo fewer collisions with the buffer gas and therefore traverse the drift cell with a higher velocity than ions with

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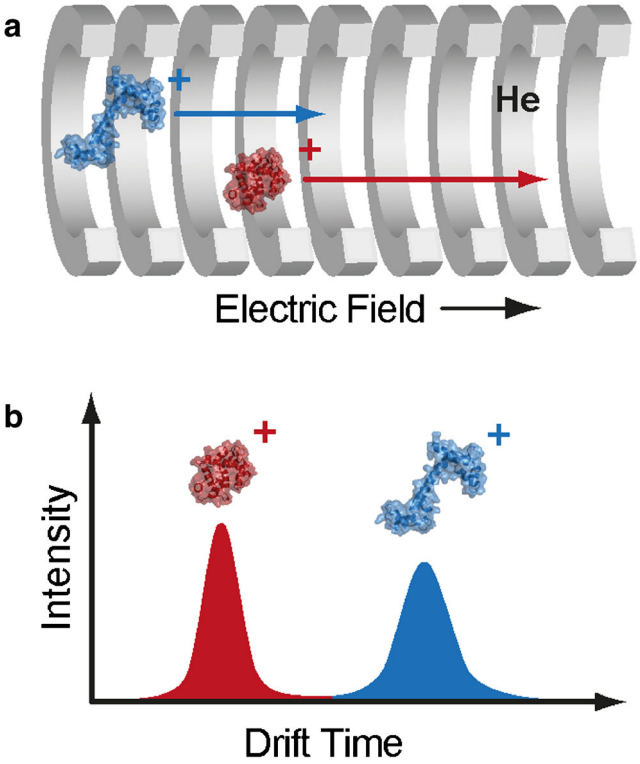

Fig. 1 Principle of an ion mobility separation (IMS). **a** Schematic representation of an ion mobility cell. The cell is filled with an inert buffer gas, such as helium, and ions are guided through the cell by a weak electric field. Compact ions (*red*) undergo fewer collisions with the drift gas than more extended ions (*blue*) and traverse the cell faster. **b** The drift time of each ion package is recorded and plotted for all analyzed species as an arrival time distribution (ATD)

a more extended structure (Fig. 1b). The last section of the experiment is (usually) a quadrupole and/or time-of-flight mass analyzer (ToF) followed by a detector. As a result, the investigated molecules are not only separated according to their *m*/*z* as in conventional MS but also according to their size and shape. When using a classical drift-tube IMS setup, the particular drift time of an ion can be further converted into a rotationally averaged collision cross section with the Mason–Schamp equation [10]. This CCS is instrument-independent and can be compared to theoretical values obtained from X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR), or model structures [11].

The two most important and most frequently used types of ion mobility spectrometry are drift tube (DT) and travelling wave (TW) IMS. In DT IMS, a homogeneous, linear electric field is used, and CCSs can be determined directly from the measured drift time and the applied experimental conditions [12]. TW IMS, on the other hand, uses traversing pulses that guide the ions through the ion mobility cell. As a result of this inhomogeneous electric field, the ions follow complex trajectories during their migration through the IMS cell. This leads to a good resolution and duty cycle, but does not allow the determination of absolute CCS values. Instead, CCS values can be estimated using species with similar physicochemical properties and known CCS as calibrants [13]. Another very recent development is trapped IMS (TIMS). Here, ions are trapped in a flowing buffer gas and subsequently eluted as ion packages, which leads to a very high IMS resolution [14]. TW and DT IM-MS instruments have been commercially available for a couple of years now, and as a result, the number of applications in the field of protein complex analysis has increased tremendously [15, 16].

In the following sections, different aspects of IM-MS as a tool to investigate noncovalent protein–ligand complexes are discussed. This includes an overview of recent efforts in this area and highlights two examples of special importance. Finally, an outlook with future prospects and possible research directions is given.

Ion mobility–mass spectrometry of protein–ligand complexes: state of the art

The study of protein–ligand interactions is of particular interest for the pharmaceutical industry, especially in the context of drug development. Millions of compounds are tested in high-throughput screening (HTS) campaigns to identify binders to target proteins. However, these large compound libraries imply time-consuming experiments and the obtained hit rates are often very low. In recent years, the screening of lower molecular weight fragments, often referred to as fragment-based drug design (FBDD), evolved as a promising alternative to HTS [17]. Owing to better chemical tractability, small fragments have the advantage of smaller libraries and generally higher hit rates [18]. As fragments bind usually with lower affinity to the target protein (micromolar to millimolar range), fragment hits are often chemically extended (*fragment growing*) or combined (*fragment linking*) to obtain a high affinity lead compound [17].

The binding of metal ions [19] or ligands [20–22] often induces a conformational change of the protein. This can be readily detected by IM-MS, which makes it an attractive tool for the investigation of protein–ligand interactions. Although IM-MS has not yet found its way into routine application in the pharma industry, it has gained considerable attention in recent years and several studies in this field demonstrated its enormous potential. Membrane proteins and their complexes with lipids have, for example, been investigated on various occasions and unique insights into the effect of lipid binding on the protein stability or the role of the detergents in structural preservation have been obtained [23, 24]. In another example, a screening scaffold with IM-MS was established to identify small molecules which inhibit the formation of amyloid fibrils, as occurs in neurodegenerative disorders such as Alzheimer's or Parkinson's [25].

A particular powerful approach to identify and quantify protein–ligand (PL) binding is *catch and release* electrospray ionization–mass spectrometry (CaR ESI-MS) [26–29]. Initially used as an MS-only experiment, Klassen and co-workers developed this approach further and used it in combination with IMS for detailed and conclusive screening studies on carbohydrate libraries [30]. The target protein is first incubated with a compound library and the formed protein–ligand complexes are subsequently analyzed using ESI-MS (Fig. 2a). The attached ligands are *caught* by the protein and can be in principle identified via the increase in molecular weight of the corresponding protein–ligand complex. In case of isomeric species or if ligands do not differ significantly in mass, however, this approach often does not provide an unambiguous result. CaR ESI-MS solves this problem by a controlled *release* of the ligands from the proteins in the mass spectrometer using collision-induced dissociation (CID). MS or IM-MS, often in combination with further fragmentation, can subsequently be used to unambiguously identify the dissociated ligands.

A recent study on the norovirus P particle demonstrated impressively how the CaR ESI-MS assay can be applied to identify new inhibitors in a fast and straightforward fashion and even provided a qualitative ranking of their binding affinities [31]. Within a 146-compound carbohydrate library, 28 binders were identified, including several milk oligosaccharides as well as bacterial oligosaccharides, which have not been known to bind to noroviruses. Isomeric ligands were successfully distinguished on the basis of their drift time (Fig. 2b, L1 and L5; L11 and L51, respectively). Remarkably, the obtained intensity of each individual species in the CID spectra was in good qualitative agreement with the binding affinities of the corresponding ligands.

Another remarkable approach in the context of PL binding is collision-induced unfolding (CIU) [32], which can be regarded as the gas-phase analogue of calorimetry experiments in solution. Instead of measuring the heat that is released upon binding of the ligand, here the heat that is required to unfold the complex by collisional activation is measured. Following ionization and transfer into the gas phase, one particular charge state of the protein–ligand complex is *m/z*-selected, e.g., using a quadrupole mass filter (Fig. 3a, b). This precursor is subsequently activated in a collision cell by

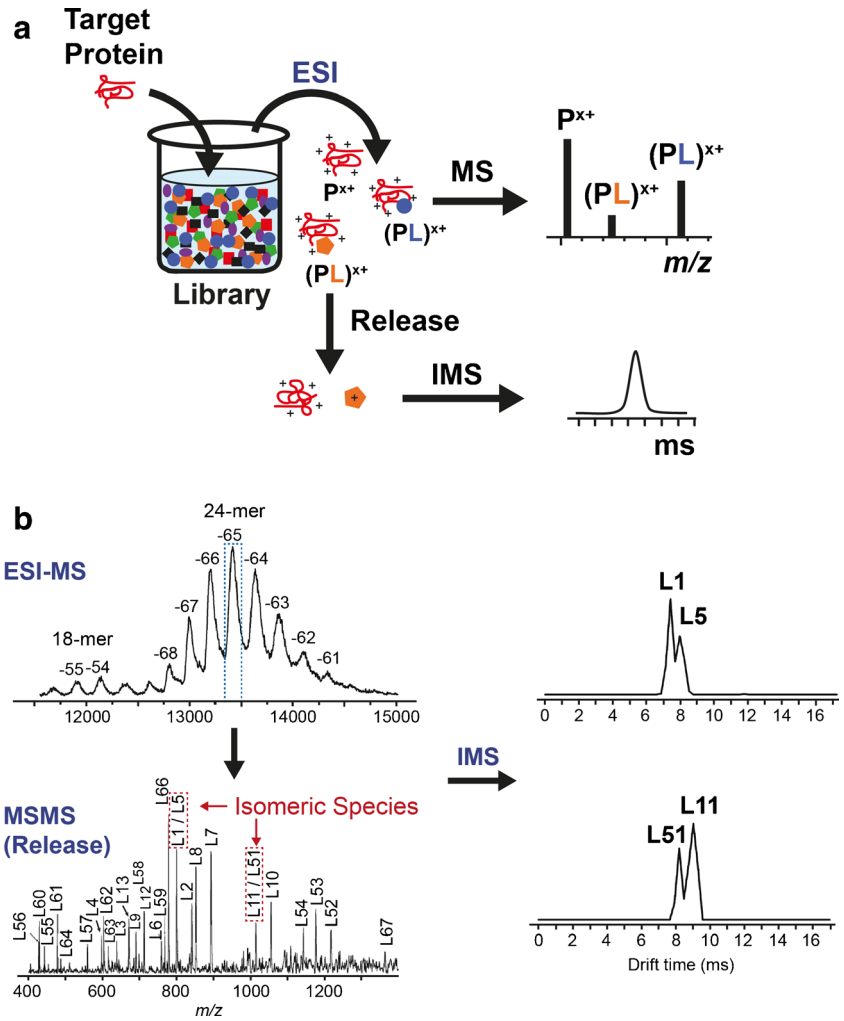

Fig. 2 **a** Scheme of the catch and release electrospray ionization–mass spectrometry assay (CaR ESI-MS). Reprinted with permission from [30]. Copyright 2012 American Chemical Society. **b** Mass spectrum of the norovirus P particle incubated with a compound library. Charge state −65 is selected and subjected to collision-induced dissociation (MSMS). Released ligands are assigned by their molecular weight. Ligands L1 and L5 as well as L11 and L51 are isomeric and can only be assigned using IMS. All four ligands bind to norovirus P particle, L1 and L11 reveal a higher affinity compared to their isomeric analogue. Adapted with permission from [31]. Copyright 2014 American Chemical Society

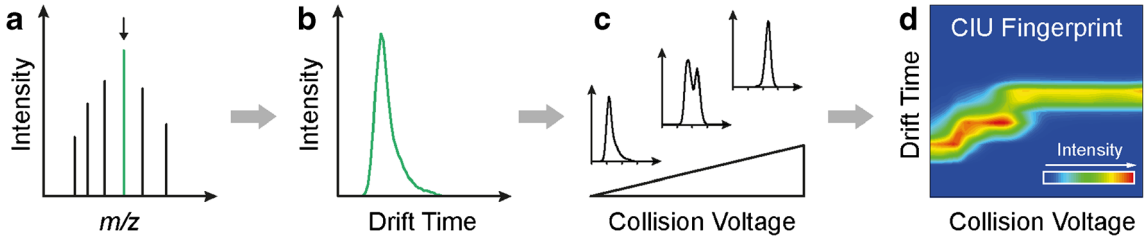

Fig. 3 Schematic description of collision-induced unfolding (CIU). **a** One charge state of a protein–ligand or protein-protein complex with its corresponding arrival time distribution (**b**) is selected as precursor from the mass spectrum. **c** The precursor species is activated by gradual increase of the collision voltage, which induces unfolding and finally dissociation of the complex. **d** Collision voltages are plotted as a function of the measured drift time resulting in a characteristic CIU fingerprint

stepwise increase of the acceleration voltage, which induces unfolding and eventually dissociation of the complex (Fig. 3c). The applied voltages are plotted against the drift time or CCS of the species of interest, which leads to a unique fingerprint for each PL complex (Fig. 3d).

By comparing the fingerprints of apo-proteins to protein–ligand complexes, different aspects can be investigated. For example, CIU showed a great potential to be used for the characterization of functional protein domains [33] and stability analysis [34–36]. Recently, CIU was used in combination with CID to establish an assay for the distinction of two Abelson kinase inhibitors [37]. While type I inhibitors bind to all kinase conformations, type IIs favorably interact with the inactive (closed) state, which makes it advantageous for disease treatment. In the resulting CIU fingerprints significant differences in specific sections were observed for both inhibitors (Fig. 4). The corresponding averaged IMS spectra and the number of conformations in these sections are unique for each of the two inhibitor types. Thus, analogous experiments with another data set of unknown inhibitor candidates resulted in a clear assignment to one of the two types of binding. In a very recent work, CIU was furthermore successfully applied to elucidate the subunit topology of human albumin. In particular, domain-specific binders and different multiprotein constructs were studied and showed for the first time that CIU can be used for the systematic analysis of the unfolding pathway of a multiprotein complex [38].

Outlook

The number of publications in which ion mobility–mass spectrometry (IM-MS) is used to investigate biomacromolecules and their noncovalent assemblies has increased vastly in recent years. Especially for large multimeric and even notoriously difficult membrane protein complexes, IM-MS was used with great success and can today be regarded as an additional tool in the structural biology portfolio [39–41]. With the currently increasing number of commercially available instruments and the accompanied steady increase in IMS resolution, this trend is likely to continue in the future.

However, IM-MS can also be applied successfully for the analysis of much smaller protein–ligand complexes. This in combination with its high-throughput capabilities makes IM-MS an exceptionally interesting technique for the pharmaceutical industry. Recent progress on the experimental side today enables the analysis of several hundreds of samples per day or the simultaneous screening of tens of structurally related

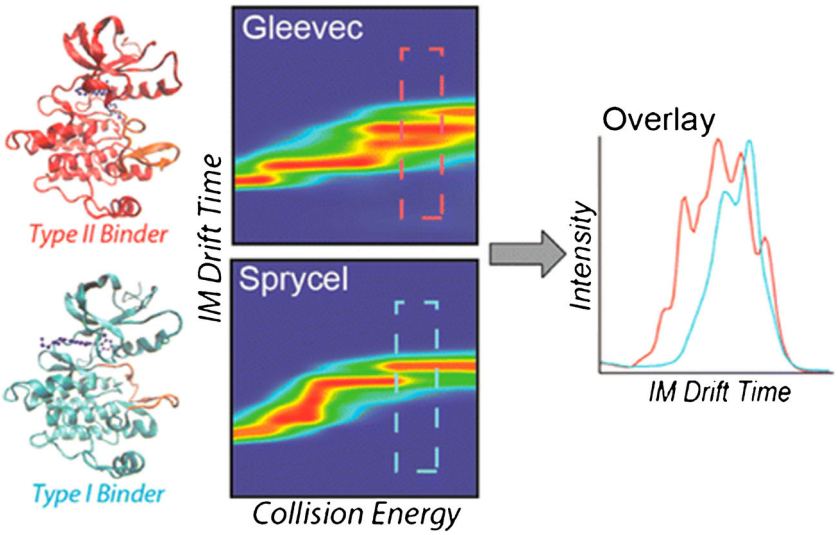

Fig. 4 Application of CIU to discriminate two inhibitors that bind in a conformation-selective fashion. Depending on the type of binding, significant differences in specific sections of the CIU fingerprints are observed. Reprinted with permission from [37]. Copyright 2013 American Chemical Society

ligands using CaR-ESI-MS. Likewise, a series of software packages, such as Amphitrite [42], UniDec [43], or CIUsuite [44], have been developed and can be readily applied for the automated analysis of large and complex datasets. However, there are still a couple of challenges arising, e.g., from non-specific ligand binding or signal suppression during the ESI process, and it can therefore be cumbersome to identify "real" binders. A few approaches are already available to identify nonspecific binding, such as the *reference method* [45] or algorithms to calculate and subtract a nonspecifically bound fraction [46]. These approaches could be incorporated in software tools for data analysis and combined with routine protocols in which standardized control samples are used. This would make the identification of nonspecific binders more straightforward and would enable the automation of IM-MS screening experiments. At the moment IM-MS is rather an orthogonal tool to confirm "hits" obtained with other methods; however, the way is paved for it to soon be fully implemented in screening routines in the pharmaceutical industry.

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Compliance with Ethical Standards

Conflicts of interest The authors declare no conflicts of interest.

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