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Advances in Ion Mobility-Mass Spectrometry Instrumentation and Techniques for Characterizing Structural Heterogeneity

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Abstract

Over the last decade, the field of ion mobility-mass spectrometry (IM-MS) has experienced dramatic growth in its application toward ion structure characterization. Enabling advances in instrumentation during this time period include improved conformation resolution and ion sensitivity. Such advances have rendered IM-MS a powerful approach for characterizing samples presenting a diverse array of ion structures. The structural heterogeneity that can be interrogated by IM-MS techniques now ranges from samples containing mixtures of small molecules exhibiting a variety of structural types to those containing very large protein complexes and subcomplexes. In addition to this diversity, IM-MS techniques have been used to probe spontaneous and induced structural transformations occurring in solution or the gas phase. To support these measurement efforts, significant advances have been made in theoretical methods aimed at translating IM-MS data into structural information. These efforts have ranged from providing more reliable trial structures for comparison to the experimental measurements to dramatically reducing the time required to calculate collision cross sections for such structures. In this short review, recent advances in developments in IM-MS instrumentation, techniques, and theory are discussed with regard to their implications for characterization of gas- and solution-phase structural heterogeneity.

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Ion Mobility (IM) Resolution and Ion Sensitivity Improvements. Biomolecular ion structure characterization by ion mobility spectrometry (IMS) originated in the mid 1990s.[1, 2] For such studies, the mobility measurement was used to determine an ion's collision cross section according to Equation 1.[3]

$$\Omega = \frac{(18\pi)^{1/2}}{16}\frac{ze}{(k_B T)^{1/2}}\left[\frac{1}{m_I}+\frac{1}{m_B}\right]^{1/2}\frac{t_D E}{L}\frac{760}{P}\frac{T}{273.2}\frac{1}{N_0} \quad 1)$$

In Equation 1, t_D is the drift time or the drift region transit time of the ion. ze, m_I, and m_B are the overall charge of the ion, the mass of the ion and the mass of the buffer gas, respectively. E and L represent the electric field in the drift region and the length of the drift region and k_B and T are Boltzmann's constant and the temperature of the buffer gas. P and N_0 are the pressure of the buffer gas and the neutral number density at STP, respectively. Ion conformation information was originally obtained by comparing collision cross sections from experiments with those calculated for theoretical three-dimensional structures. The theory behind such comparisons developed nearly in concert with the experimental measurements.[4-6]

It can be argued that improvement in comparisons between collision cross sections obtained for computer-generated trial structures and experimentally determined values do not scale directly with increased IM resolving power. For example, the reproducibility of low-resolution mobility measurements (typically ± 2%) often exceeds the variability in ion structure size observed in extended molecular dynamics simulations (MDS) for matching low-energy conformations. However, for the purpose of characterizing gas- or solution-phase conformer heterogeneity, higher resolving power in the mobility dimension significantly enhances structural studies. Such enhancements range from the determination of the degree of co-existing solution structures for biomolecular species to the determination of structural heterogeneity resulting from induced or spontaneous conformational transformations in the gas- and/or solution-phases. As an example of the utility of increased mobility resolving power for structure ensemble characterization, consider Figure 1. Multidimensional IM separations showed that

conformational types of gas-phase protein ions resolved by a single IM measurement step actually consist of many separate, unresolved conformers exhibiting unique mobilities.[7] Another example is the need to adequately resolve the structural heterogeneity associated with complex mixtures such as those encountered in 'omics investigations.[8-10] Indeed, such requirements have in part influenced IM-MS instrumentation development focused on improving the resolution of the mobility-based separation.

The resolving power (R) of a traditional IM measurement is described by Equation 2.[11]

$$\boldsymbol{R} = \left(\frac{\boldsymbol{LEze}}{\boldsymbol{16\ k_B T\ ln\ 2}}\right)^{\boldsymbol{1/2}} \quad 2)$$

Here, R represents the ratio of the ion's drift time (t_D) to the width of the peak at half-maximum height. Shortly after the application of IM-MS techniques for the characterization of biomolecular ion structure, researchers began to explore the development of instrumentation that would exploit instrumental parameter settings (Equation 2) to achieve high-resolution measurements for biological ions.[12, 13] Although, high resolution measurements were reported nearly 20 years ago, maximizing R by changing instrument geometry and operational settings (L, E, and T from Equation 2), was shown to reach a point of diminishing returns resulting from unmanageable operational conditions and spatial requirements. These challenges provided the impetus for developing different strategies for achieving high resolution IM separation capabilities. Notably, Equations 1 and 2 are applicable to measurements performed on constant-field drift tubes. However, because collision cross sections can be obtained from other mobility measurements, the discussion below is not limited to instrumentation employing traditional drift tube geometries. Although much has been accomplished in the area of resolution improvement for the technique of field-assymetric ion mobility spectrometry (FAIMS) [or differential mobility spectrometry (DMS)],[14] this technique is not discussed here as the purpose of this review is to describe techniques for which collision cross sections can be determined to provide a description of structural heterogeneity.

Recently, established and new mobility-based measurements were shown to significantly extend the achievable resolving power of mobility measurements in IM-MS instrumentation. Although traveling wave IM (TWIM) measurements were demonstrated more than a decade ago,[15] recent improvements in electric field application and pressure settings yielded a four-fold improvement in mobility resolving power.[16] Again, with regard to established technologies, de la Mora recently showed the utility of high-resolution differential mobility analysis in distinguishing conformations of charge-reduced protein and protein complex ions.[17] Within the last few years, new high-resolution IM techniques were demonstrated with MS analyses. One example was Trapped Ion Mobility Spectrometry (TIMS) which utilized a flow of gas applied along the ion separation axis, RF confining fields, and a variable DC gradient.[18] By scanning the DC gradient, ions of different mobilities could be sequentially removed from the ion trap and detected. Recently Park and coworkers demonstrated high resolution measurements on a TIMS device.[19] Resolving power as high as ~200 were obtained for the analysis of peptide ions. Overtone Mobility Spectrometry (OMS) was also presented as an alternative mobility-based separation approach.[20] As described OMS utilized periodic ion gates equally spaced along the mobility separation axis which served to allow only the transmission of ions of select mobilities (i.e., ions that traversed intervening regions of a segmented drift tube in the gate time period). Recently, Clemmer and coworkers utilized an OMS-type separation with a circular drift tube to achieve resolving power values in excess of 1000.[21] These represent the highest resolving power measurements to date for mobility-based separations. Together, these advances in resolving power provide a means for fuller characterization of the structural diversity within a sample.

With improvements in resolving power, came attendant problems of ion signal strength. For example, OMS resolving power was shown to scale with the number of ion gates each of which produced ion losses.[20] Because of the low duty cycle associated with traditional, pulsed IM measurements, efforts for improving ion sensitivity began in earnest nearly 3 decades ago.

Hill and coworkers described the first Fourier Transform IM measurements in 1985 and suggested the rapid generation of mobility distributions with sufficient signal-to-noise (S/N) levels would provide enhanced detection capabilities for GC separations.[22] Later, for low-pressure IM measurements, ion storage and confinement methods were introduced. These included the use of 3D trap,[23] linear ion trap,[9] and ion funnel[24] devices that were coupled to the drift tube. Additionally field-focusing drift tube designs were explored.[25] Currently the search for improved ion utilization in IM-MS instrumentation continues apace. Recently Russel and coworkers described a method for obtaining accurate collision cross sections from a periodic focusing IM instrument.[26] The researchers demonstrated that with the inclusion of a single dampening factor in the collision cross section calculation, accurate cross sections could be obtained. In other recent experiments, Payne and coworkers reported improved data processing techniques for removing artifacts in Hadamard Transform IM-MS measurements.[27] Application of the algorithm was found to significantly improve the sensitivity of the measurement. Smith and coworkers proposed the concept of an ultimate ion utilization device using structures for lossless ion manipulation (SLIM) such as that shown in Figure 2.[28] One hundred percent ion transmission through linear and bent SLIM configurations was demonstrated. With such minimal ion loss, one can consider the possibility of ultra-high resolution IM separations. Clemmer and coworkers also utilized RF ion confinement and improved drift tube design to allow the performance of OMS measurements without the many gridded lenses employed in previous drift tube configurations.[29] Finally, Fernandez and coworkers reported a novel approach that could significantly enable IM-MS techniques coupled to atmospheric pressure ion sources such as direct analysis in real time (DART).[30] In the new design a repeller point electrode was directed toward the entrance of a resistive glass drift tube to generate ion focusing fields for effectively guiding the ions into the mobility measurement device.

Studies of small molecule structural heterogeneity. From a relatively early time point, IM-MS was demonstrated as a means for separating ions within complex mixtures.[31, 32] Here,

the mobility measurement was shown to distinguish isobaric ions of similar type based on differences in collision cross section as well as overall ion charge. It was recognized that, for peptides, factors such as intramolecular interactions, side-chain packing, and side-chain length could affect the overall ion collision cross section leading to efforts aimed at predicting peptide ion cross sections based on a knowledge of primary sequence.[33] McLean and coworkers demonstrated that many classes of small molecules could be distinguished using IM-MS techniques.[34] These early efforts laid the groundwork for recent developments in small-molecule ion structure characterization/utilization using IM-MS techniques.

Recent experiments conducted on an IM-MS instrument that utilizes a linear, high-resolution drift tube and a time-of-flight (TOF) mass analyzer, yielded the most extensive database to date of collision cross sections for small biomolecules comprising molecular species such as quaternary ammonium salts, lipids, peptides and carbohydrates.[35] The study demonstrated the potential for observing low-abundance species in the presence of higher-abundance isobaric ions including isomers using IM-MS analysis. Paglia and coworkers demonstrated the utility of incorporating such mobility information into comparative metabolomics workflows and described searchable collision cross section databases to aid ion identification.[36] Hill and coworkers recently utilized high-resolution mobility separations to identify a new dopamine isomer obtained from striatal metabolomic extracts from genetically modified rats.[37] Similarly, experiments recently showed that mobility measurements could be used with other analytical information (LC retention time and precursor and fragment ion masses) to distinguish isomeric species in complex mixtures obtained from natural products [38] as well as to identify potential biomarkers in comparative metabolomics analyses[39]. In other experiments, de Pauw and coworkers used IM-MS techniques to distinguish isomers by employing a host-guest system for selectively shifting the t_D of one species.[40] One recent study used the structural heterogeneity of phosphorylated peptide ions doped into tryptic digests to link precursor ions with those formed upon neutral loss of H_3PO_4 resulting from collisional

activation at the back of the drift tube.[41] The revealed phosphorylated species were then subjected to ETD in a linear ion trap to obtain primary structure information.

Another active research area is the characterization of structural heterogeneity of nucleotides and oligonucleotides by IM-MS techniques. One intriguing study conducted by Kappes and coworkers coupled IM-MS measurements with photoelectron spectroscopy to characterize isomer-resolved oligonucleotides.[42] The approach enabled the determination of the origin of two isomeric classes as illustrated in Figure 3. Additionally the work implicated a sequence dependence in the formation of one of the isomer classes. A similar study showed that IM-MS techniques could differentiate isobaric oligonucleotides based on differences in ion mobilities; the added advantage of parallel dissociation[43] was also demonstrated in the identification of the isobaric nucleotides.[44] In other experiments Orozco and coworkers combined IM-MS measurements with molecular dynamics techniques to study the structures of triplex DNA; remarkably the triplex DNA ions were considered to resemble solution structures to a significant degree.[45] More recently studies using the same combination of analytical techniques characterized an oligonucleotide forming a loop-duplex structure in solution to reveal rapid conformational changes occurring upon ion desolvation.[46]

Another field impacted by structural heterogeneity characterization by IM-MS techniques is glycomics. Due to the high occurrence of the post-translational modification of protein glycosylation in living organisms,[47] glycomics analysis has great potential for biomarker discovery studies such as those in which aberrant glycosylation is believed to result from the disease process. Because glycans can exist in a variety of isomeric forms, such molecules present a challenge to biomarker discovery efforts making it difficult to disentangle isomer composition even with multistage tandem mass spectrometry (MS^n).[48] Early studies revealed that mass spectrometric glycan profiles alone could be used to distinguish control and cancer clinical samples.[49] Shortly later IM-MS measurements provided added capabilities as clinical samples could be distinguished based on the mobility profiles of observed glycan ions.[50]

Recently, a number of studies presented IM-MS techniques as a means for characterization of glycans with regard to elucidating comprising isomers. In common to the methods was the use of ion fragmentation in concert with IM-MS measurement to assess the diversity of glycan isomers present in samples. Flitsch and Eyers and their coworkers used collision-induced dissociation (CID) prior to mobility separation of monosaccharide product ions to reveal the nature of epimeric glycans attached to glycopeptides.[51] In other experiments, IM-MS measurements were used in conjunction with parallel dissociation methods to reveal the isomeric heterogeneity of glycan samples using a traveling-wave ion guide instrument[52] and a hybrid instrument coupling a drift tube with a linear ion trap mass spectrometer[53]. More recently, Hill and coworkers combined LC with IM-MS measurements of anions to characterize the complement of oligosaccharide aldiotol isomers from bovine submaxillary mucin.[54]

The application of IM-MS analysis for the characterization of heteroatom compounds in petroleum samples was first performed in 2009.[10] Because of the increased peak capacity afforded by IM-MS analysis, techniques continue to be developed for the characterization of various species in petroleum (and related) samples. Recent advances in petroleomics technique development and application range from the usage of new ionization sources to the demonstration of rapid comparisons of two-dimensional IM-MS datasets. Afonso and coworkers coupled ionization by an atmospheric solid analysis probe (ASAP)[55] with IM-MS measurements for the rapid comparison of unprocessed and processed (hydrodesulfurization) diesel fuel samples.[56] The work showed that IM-MS analysis allowed the distinguishing and tracking of two compound classes within the two sample types. In separate studies, Fasciotti and Eberlin and their coworkers used CO_2 as a buffer gas for separation of heteroatom species in crude oil and fuel samples.[57] Improvements in the separation of NO, O_2, and N class compounds were reported. Choi and Kim and their coworkers used IM-MS measurements in conjunction with high resolution MS experiments and theoretical collision cross section determinations to characterize short-chain alkyl aromatic compounds in crude oil samples.[58] In these experiments,

molecular formulas from the high mass accuracy spectra were used to propose compound structures for which collision cross sections could be computed and compared to those obtained by IM-MS measurements. The advantages of IM-MS analysis were also demonstrated for the characterization of formulated lubricants (base oils and additives) in which ASAP was employed to present a method that required no sample preparation.[59] Finally, one recent study by Chambliss and coworkers showed the application of IM-MS techniques including parallel dissociation to characterize bio oil samples;[60] such experiments represent technological inroads for IM-MS methods as demonstrated by their relatively early adoption in this emerging research area.

Although the combination of IM-MS measurements with infrared (IR) multiphoton dissociation spectroscopy was demonstrated more than 5 years ago,[61, 62] research continues in the application of this methodology. Recently Turecek and coworkers employed IM-MS measurements with IR action spectroscopy and electronic structure calculations to study the structures of peptide sequence isomers.[63] The authors demonstrated that several challenges remain with regard to the use of such techniques and noted that structural elucidation must be approached cautiously. Von Helden and coworkers combined IM-MS and IR vibrational spectroscopy measurements in a single instrumental setup.[64] This allowed for the recording of IR spectra for *m*/*z*- and mobility-selected ions of protonated benzocaine. From the IR spectra the authors were able to assign the higher-mobility dataset feature to the O-protonated ion form and the lower-m1obility feature was assigned to the N-protonated form. Similarly, Rizzo and Clemmer and their coworkers combined on-line IM-MS with IR spectroscopy to study the conformations of singly-protonated 4-residue peptide ions.[65]

Synthetic polymer characterization by IM-MS. Assessing the structural variability associated with polymer samples with IM-MS measurements commenced more than 15 years ago.[66, 67] An early seminal study used high-resolution IM separations to reduce the chemical noise associated with complex polymer samples.[68] The improved resolution allowed the

determination of the degree of structural change occurring for low-signal ion families in which, unlike peptide ions in the same *m*/*z* range, increased charge resulted in decreased ion mobilities. Early studies such as these laid the foundation for the extensive use of IM-MS techniques today to study polymer samples. One recent area enabling IM-MS characterization of polymers is the coupling of new ionization techniques with IM-MS measurements. Trimpin and coworkers recently showed the utility of the combination of matrix-assisted ionization vacuum (MAIV) with IM-MS measurements. The combination of the efficient ionization technique with the noise reduction of the gas-phase separation technique allowed for the detection of low-abundance additives.[69] Indeed ion signal levels were sufficiently large to allow for parallel dissociation of low-abundance additives within a mixture as shown in Figure 4. In separate studies, Afonso and coworkers demonstrated that ASAP ionization could be used with IM-MS measurements to characterize low molecular weight poly (ether ether ketones) (PEEK) species; the observed ions did not correspond to pyrolysis products or ion fragments as verified by comparison with MALD-TOF experiments.[70] Hercules and Geis used IM-MS measurements to help confirm mechanisms for polyurethane fragmentation reactions.[71] Charles and Pricl and their coworkers demonstrated the conformational diversity for conjugated dendrimer-linear polymer systems.[72] IM-MS afforded the recording of structural changes associated with metal ion adduct type and the number of charges. Chang and coworkers employed MDS in conjunction with IM-MS measurements to study the conformations of cyclic and linear polylactide ions associated with different stereoregularity.[73] Interestingly, a polymer length dependence for both linear and cyclic species was observed for conformational changes distinguished by stereoregularity. MDS provided information about intramolecular interactions and charge solvation that could be associated with the observed structural changes.

Structural heterogeneity revealed by native IM-MS. Nearly 20 years ago, the concept of native MS was presented as a means of characterization of protein quaternary structure by MS techniques.[74, 75] Nearly a decade after the first studies of protein complexes by MS, seminal

experiments by Robinson and coworkers revealed the capabilities of IM-MS techniques for elucidating unique structures for large ions resulting from solution protein complexes.[76] With improvements in instrumentation and techniques, a number of recent studies have presented IM-MS as a powerful means for detailing the structural heterogeneity associated with large protein complexes. A standout study performed by Heck and coworkers utilized IM-MS measurements to characterize viral capsid assembly.[77] Here, experimental and theoretical collision cross sections of small oligomers demonstrated that more diffuse structures were involved in the assembly process. In separate experiments, IM-MS measurements were performed for similar complexes and subcomplexes from two different microorganisms.[78] The approach allowed the determination of analogous subunit structures despite the disparate primary sequence of constituent proteins. Bush and coworkers used IM-MS techniques to monitor the structural heterogeneity of large anions and cations and found that both have similar structures; however, the former ion type exhibited lower charge state distributions suggesting that charge carrier emission processes play a crucial role in determining the overall charge state of the ion.[79] Grandori and coworkers utilized IM-MS techniques with circular dichroism measurements to track the assembly process of a protein complex involved in the transport of lipopolysaccharide in the periplasmic region of Gram-negative bacteria.[80] The work showed that oligomeric species form rod-like structures and protein regions transition from disordered to ordered states. In addition to the work described above, other studies utilized IM-MS techniques to monitor the effects of supercharging reagents[81], characterize intrinsically disordered protein complexes,[82] and detail the effects of stabilizing anions and cations on protein complex structure.[83]

One powerful method for studying the structures of protein complexes is to induce structural heterogeneity via collisional activation of different ion conformations.[84] In this approach, the mobilities of precursor ions are measured and subsequently these ions are activated to induce conformational changes and/or ion dissociation. Here we discuss ion

dissociation while below conformational changes resulting from collisional activation are discussed. In 2010, Ashcroft and coworkers demonstrated that IM-MS measurements could be combined with collision-induced dissociation (CID) in order to study the intermediate structures associate with viral assembly.[85] In separate studies, Robinson and coworkers demonstrated that CID of protein complex ions could be used to help refine structural information obtained from IM-MS experiments by revealing peripheral protein subunits.[86] One problem encountered in the dissociation of protein complex ions is the assymetric charge distribution that is observed in product ions where the remaining larger complex ions lose charge relative to smaller monomeric species; presumably this occurs because of the unfolding of the smaller ions to accommodate the greater charge which relieves Coulomb repulsion on the complex.[87, 88] Wysocki and coworkers demonstrated that the assymetric charge distribution problem could be avoided by dissociating protein ion complexes by surface-induced dissociation (SID).[89] Subsequently, the utility of IM-MS measurements and SID was revealed in the determination of the structural types of monomer fragment ions.[90, 91] Such experiments made it possible to compare the relative sizes of monomeric ions formed by CID or SID as shown in Figure 5. SID was observed to provide a number of ions that more closely resembled monomer solution conformations in size compared with those produced by CID. Later Wysocki and coworkers showed the value of SID performed with IM-MS measurements by revealing the stoichiometry of ribonucleoprotein (RNP) complex that assembled in solution.[92] One recent development was reported by Russell and coworkers in which cryogenic IM-MS could be used to obtain the mobilities of hydrated ions.[93] Allowing for collisional activation to control the types of hydrated ions observed, the approach was recently applied to assign a kinetically-trapped conformation type for Substance P ions[94] and has been combined with site-specific amino acid substitutions to elucidate intramolecular interactions involved in stabilizing $[M+3H]^{3+}$ peptide ions[95].

Over the last several years, the study of protein aggregation has benefitted tremendously from IM-MS studies. Early work applying IM-MS techniques for the

characterization of protein aggregation associated with disease state commenced more than a decade ago.[96] A seminal study carried out by Bowers and coworkers showed the ability of IM-MS to elucidate the structures of early multimeric species and delineate those critical for fibrilization pathways.[97] Although early studies primarily focused on multimeric species associated with amyloid beta peptide, the use of IM-MS analyses were extended to a variety of protein aggregation systems. Other studies monitored monomeric and oligomeric species associated with alpha synuclein,[98-100] transferrin,[101] amylin,[102] and tau protein[103, 104] as pertaining to fibrilization processes. Several different techniques can be highlighted from such studies. In one study, IM-MS was used to verify that autoproteolytic fragments of alpha synuclein play a key role in protein aggregation.[98] In separate studies, Ashcroft and Radford and their coworkers used CID with IM-MS measurements to compare the stabilities of amyloid beta multimer ions obtained from human and rat sources.[102] These experiments also used point mutations to determine critical intramolecular interactions and amino acid residues in the formation of compact and elongated conformational types of multimeric ions. Other experiments conducted by Bowers and coworkers monitored the effects of intermolecular interactions involving amyloid beta and tau fragments on the aggregation process.[103] Finally, experiments that utilized IM-MS for monitoring the effect of potential inhibitors to the aggregation process were also demonstrated.[105, 106]

Solution conformational changes revealed by IM-MS techniques. Much of the work described to this point highlights research in which significant efforts were undertaken to ensure that solution-like structures were sampled. The idea that mass spectrometry could probe conformational changes resulting from different solution conditions originated shortly after the development of ESI.[107] One area that showed significant amenability to IM-MS techniques is the determination of protein structural changes in solution. Morgner and Robinson and their coworkers reported recent studies showing that IM-MS could detect the interconversion of solution states of a large protein complex upon ligand binding.[108] Similar studies revealed

conformational changes in protein/DNA complexes upon ligand binding.[109] Zinzalla and Barran and their coworkers monitored the leucine zipper interaction between two proteins using synthetic peptides.[110] IM-MS measurements of the synthetic peptides alone revealed the presence of two major conformer types; however, upon incubation with an inhibitor ligand, the conformation corresponding to the leucine zipper was not observed as shown in Figure 6. One area of recent study was the effect of post-translational modification on protein structure using IM-MS techniques. Robinson and coworkers used IM-MS measurements to probe the structures of gas-phase ubiquitin ions upon protein oxidation.[111] Remarkable evidence was presented demonstrating the destabilization of the protein native state upon incorporation of a single oxygen atom. More recently, Sobott and coworkers used IM-MS measurements to track structural changes of an ion channel during its gating demonstrating the utility of the technique to monitor structural changes of membrane proteins.[112]

The examples provided above describe the monitoring of conformational changes occurring under solution conditions in which interacting or reactive species are incubated with the proteins of interest. Recently an exciting area of research was initiated in which the structures of various ions were monitored for a large number of different solution conditions.[113] In one foundational experimental embodiment, binary solution systems were utilized in which one solvent was added incrementally to the second solvent to induce and record structural heterogeneity. Clemmer and coworkers recently demonstrated the power of the technique by elucidating new solution conformations of Ubiquitin[114]. Related studies utilized a single change in solution composition as a conformational monitoring start point; here, cis-to-trans conversions of individual proline residues in polyproline were monitored in time by IM-MS techniques and the energetics associated with some conversions were outlined.[115] Relatedly, the new technique of TIMS was used to measure kinetics associated with isomerization of solution structures of AT Hook Decapeptide.[116] Williams and coworkers showed that supercharging protein complexes from aqueous solutions resulted in structural transformations

detected by IM-MS measurements.[81] Ruotolo and coworkers recently reported a novel approach illustrated in Figure 7. The method combined automated titration of sample solvent with IM-MS analysis to study the distortion of protein complexes.[117] It was reported that the approach could be used to quantify the various intermolecular interactions for unknown species. Finally, a unique study by Russell and coworkers revealed that IM-MS techniques could be used to probe the structures of proteins as they exist within lipid membranes.[118]

Gas-Phase conformational changes. More than 10 years ago, IM-MS studies showed that certain protein ion conformations underwent spontaneous structural transformations at significant timescales (ms) after their generation by ESI.[119] Such studies further demonstrated the need to better understand the process of gas-phase ion structure establishment. In 2010, the use of the relatively new OMS technique was described in the study of spontaneous structural transformations of ubiquitin ions.[120] These experiments described how OMS distributions could be used with ion trajectory simulations to estimate rate constants associated with assumed transitions. Wyttenbach and Bowers showed that IM-MS techniques could be used to simultaneously observe conformational persistence as well as transformation for different protein ions.[121] To accomplish this, the drift field was varied by nearly 5 fold and the resulting mobility distributions were recorded and compared.

In the mid 2000s, multidimensional IMS was introduced in which induced structural transformations via collisional activation were utilized as a means to study the structures of gas-phase protein ions.[122] Here we discuss recent developments in the application of IM-MS techniques for the study of structural heterogeneity arising from such induced transformations. Oldham and coworkers used IM-MS with collisional activation to study the structures of protein-ligand complexes for the wild-type protein sequence as well as several sequences with single amino acid polymorphisms.[123] Removal of a single basic residue was shown to produce significant weakening of the ion complex structure. Song and Liu and their coworkers also used IM-MS techniques with collisional activation to detail structural transformations associated with

dissociation pathways of superoxide dismutase dimer ions.[124] Foundational studies recently performed by Ruotolo and coworkers showed that protein ion unfolding events effected by collisional activation are correlated to the number of domains in the solution structures.[125] The results were compared to those obtained for ion unfolding due to Coulomb repulsion enhanced under different ESI solution conditions. Relatedly, de Pauw and coworkers used IM-MS measurements with CID to monitor conformational changes leading to dissociation of duplex DNA ions.[126] Dissociation pathways associated with different nucleotide sequences were then proposed. Experiments by Barran and coworkers showed that protein ions could be subjected to conformational changes by varying the temperature of the buffer gas in a drift cell.[127] These studies monitored collision cross sections of protein ions as a function of temperature to obtain insights into the unfolding pathways of structured and unstructured (in solution) proteins.

Nearly six years ago, the first use of IM-MS techniques to monitor protein ion structural changes associated with ion-ion reactions was reported by Badman and coworkers.[128] Since that time, other research groups began to explore the utility of this combination of analytical techniques. One interesting study described by Turecek and coworkers presented the use of IM-MS with ETD to monitor the structures of charge-reduced peptides as well as c- and z-ions produced by ion dissociation.[129] Extensive electronic structure calculations were used to complement these studies and intriguingly it was determined that these product ions were observed to preserve intramolecular interactions associated with the precursor ions. de Pauw and coworkers combined IM-MS measurements with ETD to determine the fate of intramolecular disulfide bonded peptides.[130] The mobility separation was shown to distinguish charge-reduced species into high-mobility ions resulting from proton transfer reactions and low-mobility ions resulting from cleavage of the disulfide bonds. Sobott and coworkers recently showed that conformational changes could be monitored for charge-reduced ions of large protein complexes using IM-MS combined with ETD.[131]

IM-MS measurements combined with hydrogen-deuterium exchange (HDX) techniques.

Nearly 20 years ago, the first experiments combining mobility measurements with gas-phase hydrogen-deuterium exchange were conducted.[132, 133] Although models were developed to describe the observed exchange levels,[133, 134] refined structural information from the combined IM-MS and HDX approach awaited the development of non-ergodic ion fragmentation techniques such as electron capture dissociation (ECD)[135] and electron transfer dissociation (ETD)[136]. Jorgensen and coworkers showed that ion fragmentation of deuterium labeled peptides via such techniques proceeded without significant scrambling of the label allowing the localization of the deuterium at the individual amino acid residue level.[137] These developments significantly improved the quality of the data that could be obtained from HDX experiments combined with IM-MS analysis. Here it is noted that although solution HDX combined with MS analysis is a significant and expanding field,[138, 139] the following discussion primarily describes recent gas-phase HDX measurements as they have been demonstrated to be very amenable to IM-MS measurements.

In 2009, Engen and coworkers showed that mobility measurements could be combined with gas-phase HDX using ND_3 as a deuterating reagent.[140] Different conformer types of ubiquitin ions could be distinguished by their unique mobilities and HDX levels. Rand and coworkers later showed that HDX could be accomplished for protein ions in a TWIM instrument with site-specific determination of the incorporated label.[141] Later the research group demonstrated different instrument operational modes for performing gas-phase HDX measurements with IM-MS techniques.[142] Ashcroft and coworkers monitored changes in protein ion structure resulting from solution perturbations using gas-phase HDX-MS techniques.[143] The resulting data was shown to correlate to mobility information obtained from IM-MS measurements. Valentine and coworkers demonstrated the first determination of site-specific deuterium incorporation for mobility-selected biomolecular ion conformations using a drift tube coupled to a linear ion trap outfitted with ETD capabilities.[144] The researchers then showed that

the contributions by individual amino acid residues to conformer type exchange rate could be determined.[145] Using the experimental results and a kinetics model, the authors were able to show that multiple ion conformers are likely to comprise many mobility selections as shown in Figure 8.

Theoretical developments in IM-MS techniques. The foundation for theoretical work seeking to improve structural details obtained from IM-MS measurements by comparisons to trial structures was laid nearly 20 years ago by seminal research in the Bowers and Jarrold research groups.[4-6] Since that time, a number of recent developments in theory have significantly aided or enhanced the structural information afforded by IM-MS measurements. Such developments are observed for a diverse array of research efforts ranging from models for obtaining accurate trial structures for large protein complexes to the unraveling of physical properties affecting different ion mobility approaches. Here we highlight a number of these studies. Notably, for purposes of brevity, an exhaustive discussion of improvements to molecular modeling techniques is not presented here.

Over the last several years, a number of studies revealed the theoretical underpinnings of traditional and new mobility-based separation techniques. One of the first theoretical treatments of new mobility separation strategies was performed by Shvartsburg and Smith who studied parameters affecting mobility separations in a TWIM device.[146] A consequence of the research was the determination of distinguishing characteristics of TWIM resolving power compared to that of traditional IM separations. In other experiments, data from high-resolution OMS measurements combined with results from ion trajectory simulations were used to obtain an analytical expression detailing OMS distributions.[147] It is now possible to predict the OMS distribution (peak frequency, peak intensity, and peak width) for any compound having a known mobility. In 2010, Kwasnik and Fernandez reported results for theoretical investigations into the achievable resolving power of resistive-glass atmospheric pressure IM separations.[148] More recently a model was presented describing parameters (buffer gas velocity and electric field

gradient) affecting the resolving power of TIMS measurements.[19] Interestingly, the resolving power was suggested to be related to an ion's mobility (K) and is therefore, to some degree, analyte dependent. In addition to work describing instrument resolving power, other theoretical efforts focused on determinations of ion heating in specific instrumental configurations[149, 150] as well as improvements in theoretical treatments that better account for ion-neutral collisions allowing for the generalization of such treatments to include electric fields of arbitrary strength[151].

The second theoretical area demonstrating significant growth over the last few years is associated with improving structural inferences using IM-MS data for a variety of ions. Several studies reported improvements in molecular dynamics simulations techniques to provide structures of greater relevance for comparison to IM-MS results. Bowers and coworkers used replica exchange molecular dynamics (REMD) to mimic transfer of a solvent structure into the gas-phase.[152] The solution structure was obtained using an implicit solvent model that was shown to predict reasonable structures for specific protein folds. Shortly later, a similar approach was used to obtain candidate structures for $[M+3H]^{3+}$ bradykinin ions.[153] One advantage of this approach was that a partial solution structure obtained from NMR experiments could be used to help ensure reasonable starting structures.[154] Chirot and coworkers demonstrated the utility of adaptively biased molecular dynamics (ABMD).[155] The new approach allowed for sampling of a broader range of collision cross sections. Although, unlike REMD, ABMD does not produce the lowest energy structures, the authors showed that the ion collision cross sections could be correlated to geometrical properties and it was suggested that such an approach could provide reliable structures without significant computational requirements.

For large, multi-subunit protein complexes, early pioneering work was conducted by Robinson and coworkers where protein subunits were modeled as spheres having diameters equal to that determined experimentally for ubiquitin (i.e., nearly the size of an individual protein

subunit).[76] Molecular modeling was then conducted in which the subunit spheres were translated step-wise along a polar coordinate vector associated with initial and final subunit positions while allowing for a degree of random subunit arrangement sampling. This coarse-graining strategy allowed for the determination of several protein complex subunit arrangements that agreed well with those determined from IM-MS measurements.[156] More recently, this approach was extended by utilizing IM-MS data for complexes and sub-complexes in conjunction with high-resolution structural data from the literature to model protein complex architecture.[157] Another recent report described an integrative approach that uses native MS, bottom-up proteomics (LC-MS/MS), protein chemical crosslinking and MS, and IM-MS to provide information about protein complex stoichiometry, composition and abundance of subunits, interface regions of protein subunits, and protein complex and sub-complex shape.[158] This information was demonstrated to improve the elucidation of large protein complex structures. In addition to efforts to provide better comparison structures for large macromolecular complexes, work has proceeded apace with respect to molecules of smaller size. Niñonuevo and Leary demonstrated the use of rapid protein threading predictor (RAPTOR) combined with IM-MS techniques to obtain plausible structures for homologous protein species.[159] For small molecules, McLean and coworkers demonstrated the utility of distance geometry calculations in rapidly sampling conformational space.[160] The approach was presented as a powerful means for characterizing natural products.

One of the challenges with determining reliable trial structures for proteins and protein complexes is the time required to accurately calculate collision cross sections. This challenge has spurred recent developments in computational tools for calculating collision cross sections for such three-dimensional structures. In 2011 Bowers and coworkers introduced a new method for calculating collision cross section termed the projected superposition approximation (PSA) approach.[161] The computation was performed using the projection approximation approach framework while taking into account shape effects such as pores, cavities, concavity, etc. The

PSA technique was shown to provide accurate cross section determinations while requiring significantly less computational power. Shortly later the PSA approach was demonstrated for its capability to calculate accurate collision cross sections for supramolecular assemblies exhibiting various complex shapes.[162] Other research groups have also focused on computational developments. Larriba and Hogan developed a computational approach that can account for non-specular scattering and the ion-induced dipole interaction.[163] The method was presented as a means for determining accurate collision cross sections for models in which a non-monoatomic buffer gas is used. Shvartsburg and coworkers developed a computational scheme that utilizes the scattering on electron density isosurfaces (SEDI) concept.[164] The researchers were able to significantly enhance the speed (~500 fold) of the calculation of a protein collision cross section without sacrificing accuracy. A groundbreaking study by Benesch and Baldwin and their coworkers was recently published introducing the powerful algorithm Ion Mobility Projection Approximation Calculation Tool (IMPACT).[165] This approach was demonstrated to provide accurate collision cross sections while requiring a fraction of the time that previous methods required. IMPACT is unique in that it is highly suited for "hybrid" structural investigation methods as it is able to incorporate structural information obtained from a variety of analytical techniques as well as perform the cross section calculation on such a short timescale. A demonstration of the calculation proficiency of the approach is shown in Figure 9 where collision cross sections for an entire structural database were computed in a few hours using a single processor.

Future directions for sample heterogeneity characterization by IM-MS. This review has focused on recent developments in IM-MS instrumentation and techniques that have been demonstrated for the characterization of the diverse molecular structures encountered in different samples. It is not hard to imagine that progress will continue in the categorized areas discussed above. For example, the continued search for improved IM resolution will allow the determination of an increased number of co-existing ion structures. Although improvements in

each of the areas listed above are worthy pursuits, perhaps the most significant work lies in the integration of the various approaches to achieve unprecedented structural characterization. As an example consider a combination that includes high resolution OMS or TIMS with gas-phase HDX and ion activation as well as MS/MS characterization. In this scenario, the mobility separation could be used to distinguish different solution structures of proteins and protein complexes. The use of gas-phase HDX could be used to determine the relative accessibility to exchange sites for the various conformers and this accessibility could be compared to that observed upon induced structural transitions (gas-phase or solution phase). Tandem mass spectrometry performed by non-ergodic dissociation techniques could, to some degree, reveal the location of deuterium uptake. Such data could serve to provide improved information to guide new molecular modeling techniques in order to gain a better understanding of ion structure. Finally, cross section calculations for the increased numbers of ion conformers arising from enhanced MDS techniques can be obtained using algorithms providing significantly higher computational throughput. In summary, the new developments provide a significant repertoire of technology which researchers may assemble methods in a variety of ways to accomplish unrivaled characterization of the ensemble of structures arising from specific samples.

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Figure Captions

Figure 1. The drift time distribution for $[M+7H]^{7+}$ ubiquitin ions separated by a single IM step is shown in panel a. Panel b shows the collision cross section distribution obtained upon mobility selection of a small portion of ions partially comprising the original distribution (panel a). The expanded region shows the mobility selected distribution with the distribution representing transport of a single ion conformation. Panel c shows the distributions obtained from multiple mobility selections and subsequent drift time measurement. Reprinted with permission from S. L. Koeniger, S. I. Merenbloom and D. E. Clemmer, *Journal of Physical Chemistry B*, **2006**, *110*, 7017-7021. Copyright 2006 American Chemical Society.

Figure 2. Schematic representation of a component of a linear SLIM device. Panel A shows the spacing between RF electrodes and their relative position to the DC guard electrodes. The latter electrodes prevent ion loss as they exhibit a repelling bias for ions compared with the neighboring RF electrodes. Panel B shows the parallel assembly of the lens architecture of the SLIM device. Reprinted with permission from I. K. Webb, S. V. B. Garimella, A. V. Tolmachev, T.-C. Chen, X. Zhang, R. V. Norheim, S. A. Prost, B. LaMarche, G. A. Anderson, Y. M. Ibrahim and R. D. Smith, *Anal. Chem.*, **2014**, *86*, 9169-9176. Copyright 2014 American Chemical Society.

Figure 3. Theoretical structures for dA_5^{4-} ions obtained for comparison to IM-MS experiments. The large and small circles show the positions of the Adenine nucleobase and the phoshodiester linkages, respectively. The difference in the deprotonated A5 base and protonated P4 linkage is shown on the right. For the structure on the left, all phosphodiester bonds are deprotontated. Adapted with permission from M. Vonderach, O. T. Ehrler, K. Matheis, P. Weis and M. M. Kappes, *Journal of the American Chemical Society*, **2012**, *134*, 7830-7841. Copyright 2012 American Chemical Society.

Figure 4. IM-MS distributions for a number of additives within a mixture. Ions were ionized by the matrix assisted ionization vacuum (MAIV) technique. The top, two-dimensional (2D) distribution shows the precursor ions. The mass spectrum on the left represents that obtained in the absence of the IM separation. The middle 2D distribution shows results upon ion activation of all precursor ions. Again, the mass spectrum on the left shows the integrated spectrum. The bottom 2D distribution reveals fragments for one of the precursor ions as illustrated in the inset. The mass spectra on the left shows the improved S/N obtained for an extracted spectrum using a narrow t_D range. Reprinted with permission from T. J. El-Baba, C. A. Lutomski, B. X. Wang and S. Trimpin, *Rapid Communications in Mass Spectrometry*, **2014**, *28*, 1175-1184. Copyright 2015 John Wiley & Sons, Ltd.

Figure 5. Monomer cross sections as a function of charge state produced by CID (blue symbols) and SID (red symbols) of multimeric ions. The data shown in the top and bottom panels were generated for the transthyretin tetramer and serum amyloid protein pentamer, respectively. Also shown (green line) is the collision cross section determined for the monomer native state. Reprinted with permission from M. Zhou, S. Dagan and V. H. Wysocki, *Angewandte Chemie (International ed. in English),* **2012**, *51*, 4336-4339. Copyright 2012 WILEY-VCH Verlag GmbH & Co.

Figure 6. Collision cross section distributions for dimer peptide ions formed between peptides from proteins forming a leucine zipper structure. Panel A and Panel B show results for dimers formed by peptides containing and not containing, respectively, a small molecule binding region. Top and bottom distributions in both panels correspond to conditions in which the ligand is absent and present, respectively, in solution. Candidate structures from MDS are shown. Reprinted with permission from S. R. Harvey, M. Porrini, C. Stachl, D. MacMillan, G. Zinzalla

and P. E. Barran, *J Am Chem Soc*, **2012**, *134*, 19384-19392. Copyright 2012 American Chemical Society.

Figure 7. Collision cross sections as function of collision voltage for a number of different proteins are shown as contour plots. In these plots different conformer types are defined by Roman numerals. The charge states of the activated ions are also shown. The dot/line plots show unfolding intermediates (labeled A-D) resulting from Coulombic unfolding of the same proteins. Reprinted with permission from Y. Y. Zhong, L. J. Han and B. T. Ruotolo, *Angewandte Chemie-International Edition*, **2014**, *53*, 9209-9212. Copyright 2014 WILEY-VCH Verlag GmbH & Co.

Figure 8. Panel A shows the results from a HDX kinetics simulation for $[M+3H]^{3+}$ ions formed by electrospraying the model peptide KKDDDDDIIKIIK. The deuterium uptake at different partial pressures of D_2O is in good agreement with the experimental results indicating that the simulation is accurate. A low-energy structure obtained from simulated annealing studies is also shown. The structure not only provides a match to the experimentally determined collision cross section but also to the HDX uptake rate by individual amino acid residues. Panel B shows the isotopic distribution (black trace) for the c_{12} ions after the mobility-selected precursor ions have been subjected to HDX in the drift tube and ETD in the linear ion trap mass spectrometer. The theoretical isotopic distribution (red trace) obtained from the kinetics simulations is also shown. The broader experimental distribution can be explained by the presence of multiple conformations within the mobility selection. Adapted with permission from M. Khakinejad, S. G. Kondalaji, A. Tafreshian and S. J. Valentine, *J Am Soc Mass Spectrom*, **2015**, DOI: 10.1007/s13361-015-1127-9. Copyright 2015 American Society for Mass Spectrometry.

Figure 9. Panel A shows the calculated collision cross sections as a function of mass for structures in the Protein Databank in Europe (PDBe). Panel B shows a histogram representation of an expanded region of the dataset. For a given mass, protein cross sections extend over a significant range. Panel C shows the results for the same database represented on a shape factor scale (upper trace) and a standard deviation of the shape factor (lower trace). The shape factor reveals the similarity of a structure to those at the same mass. Reprinted with permission from Erik G. Marklund, Matteo T. Degiacomi, Carol V. Robinson, Andrew J. Baldwin and Justin L. P. Benesch, *Structure* (London, England : 1993), **2015**, 23, 791-799.. Copyright 2015 Elsevier Ltd.

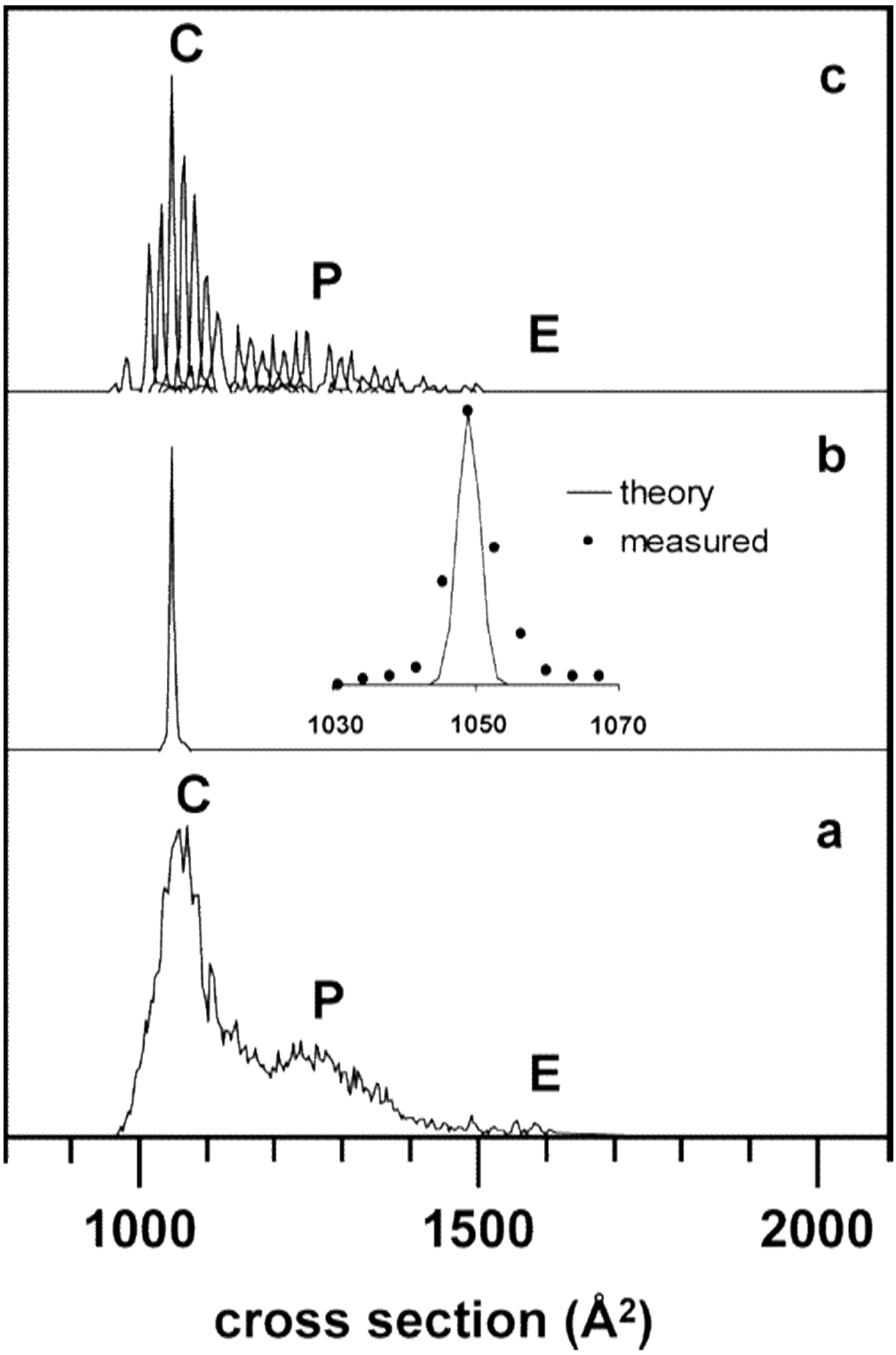


Figure 1

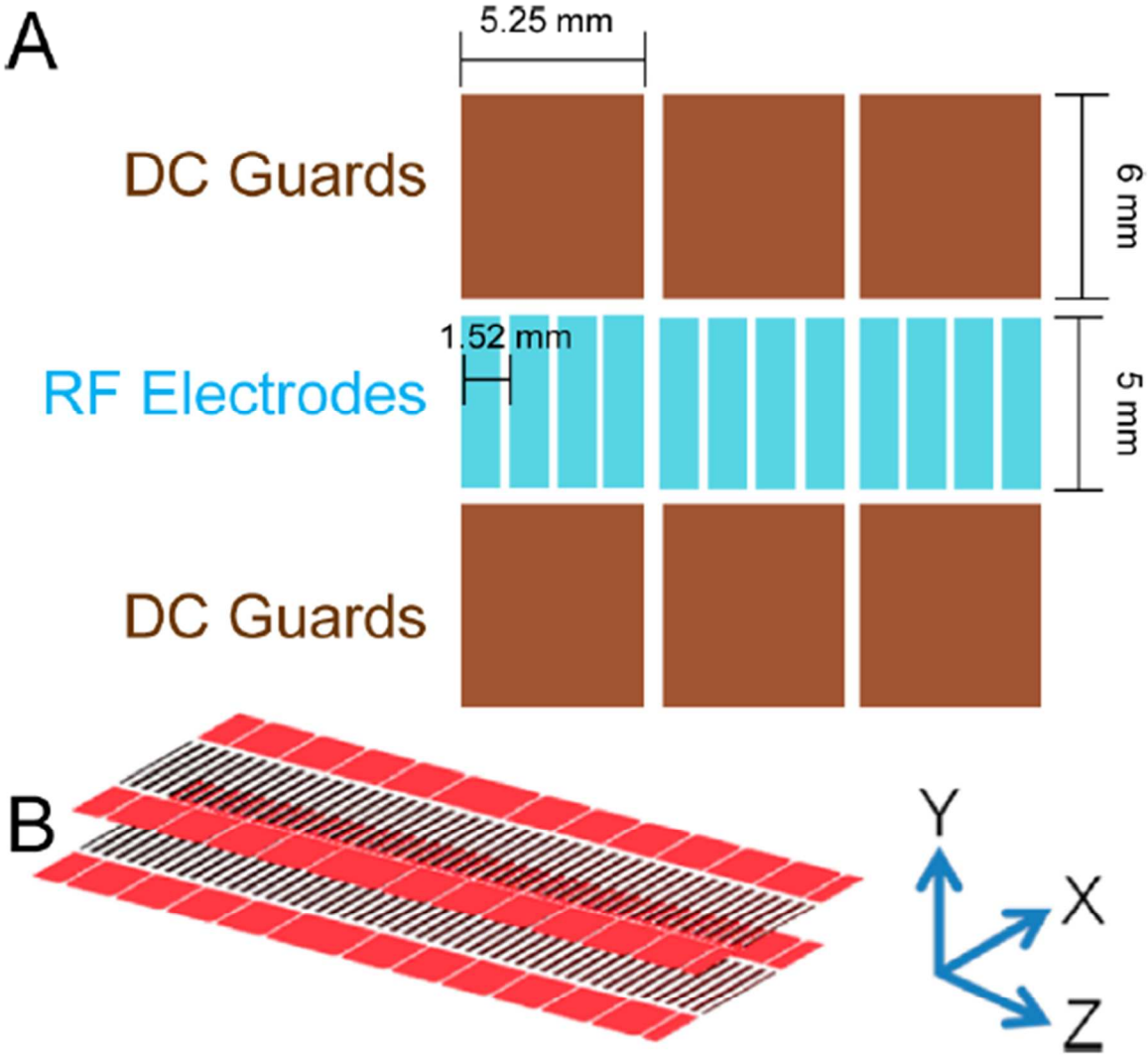


Figure 2

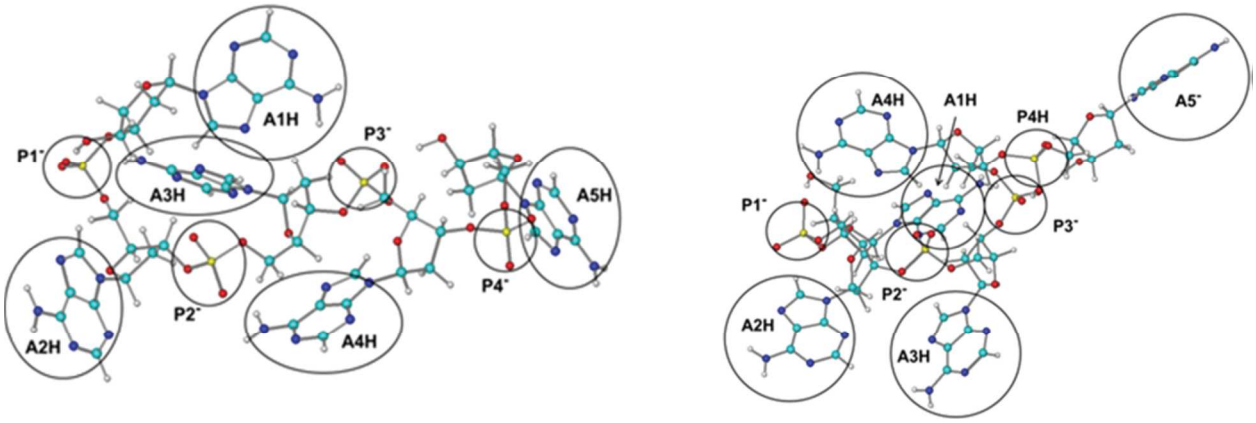


Figure 3

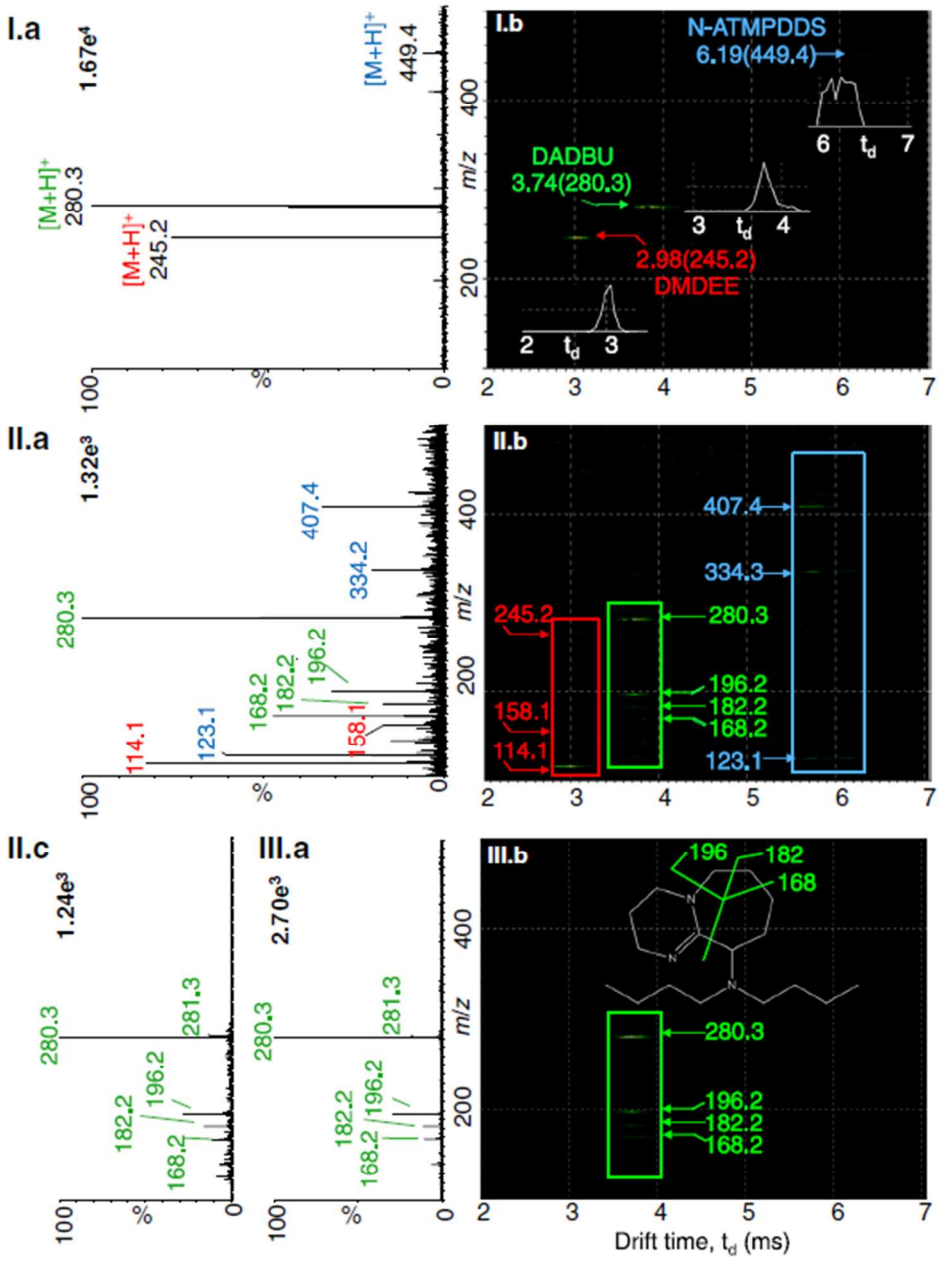


Figure 4

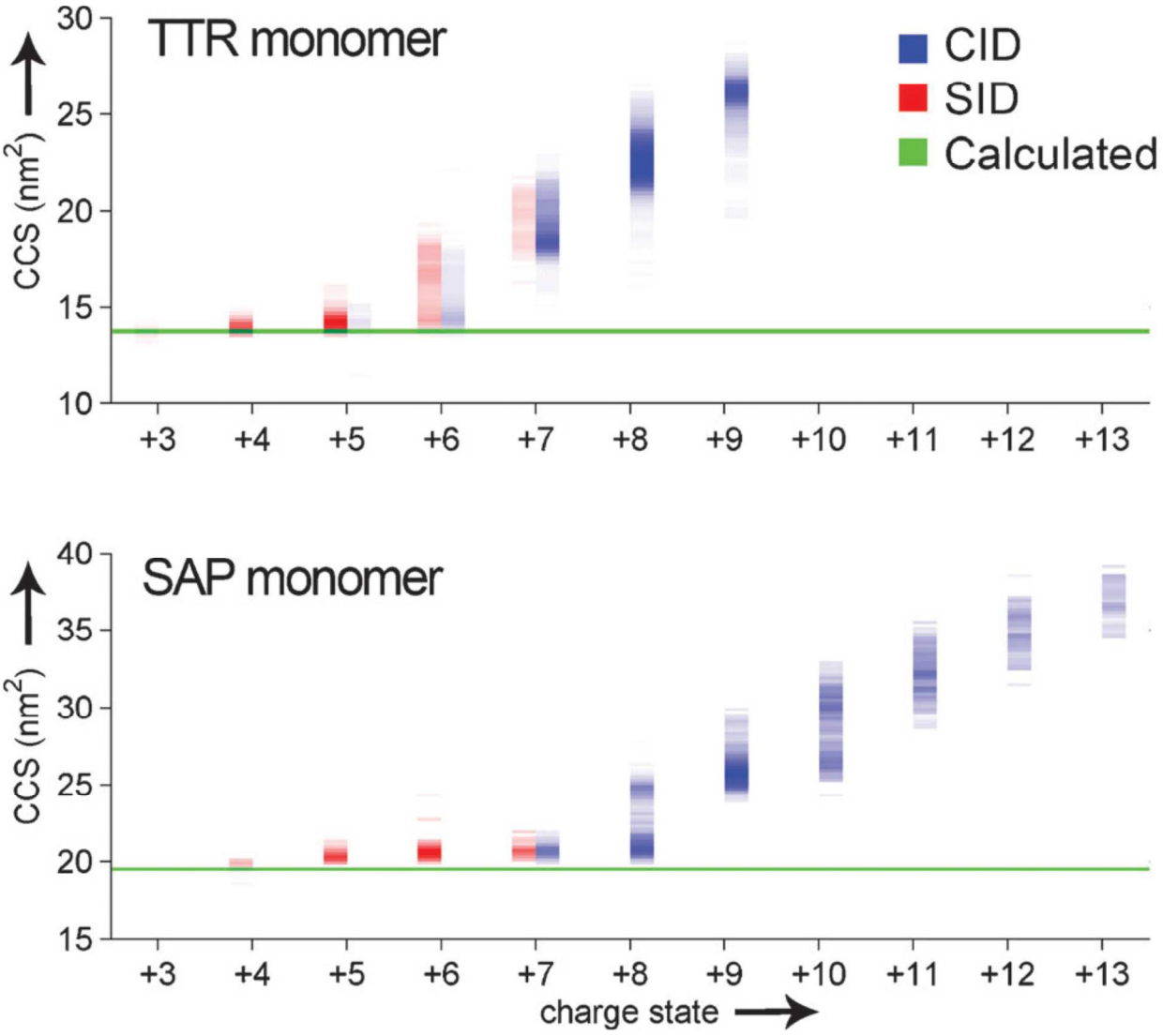


Figure 5

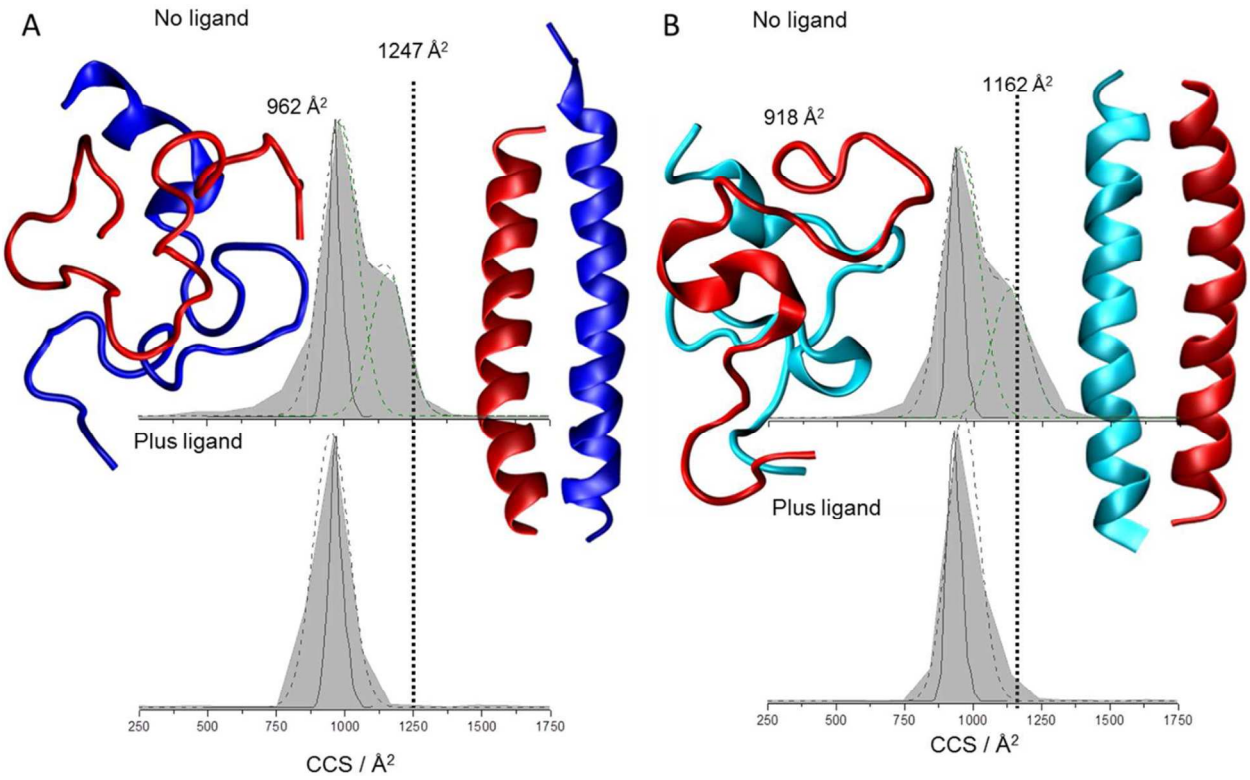
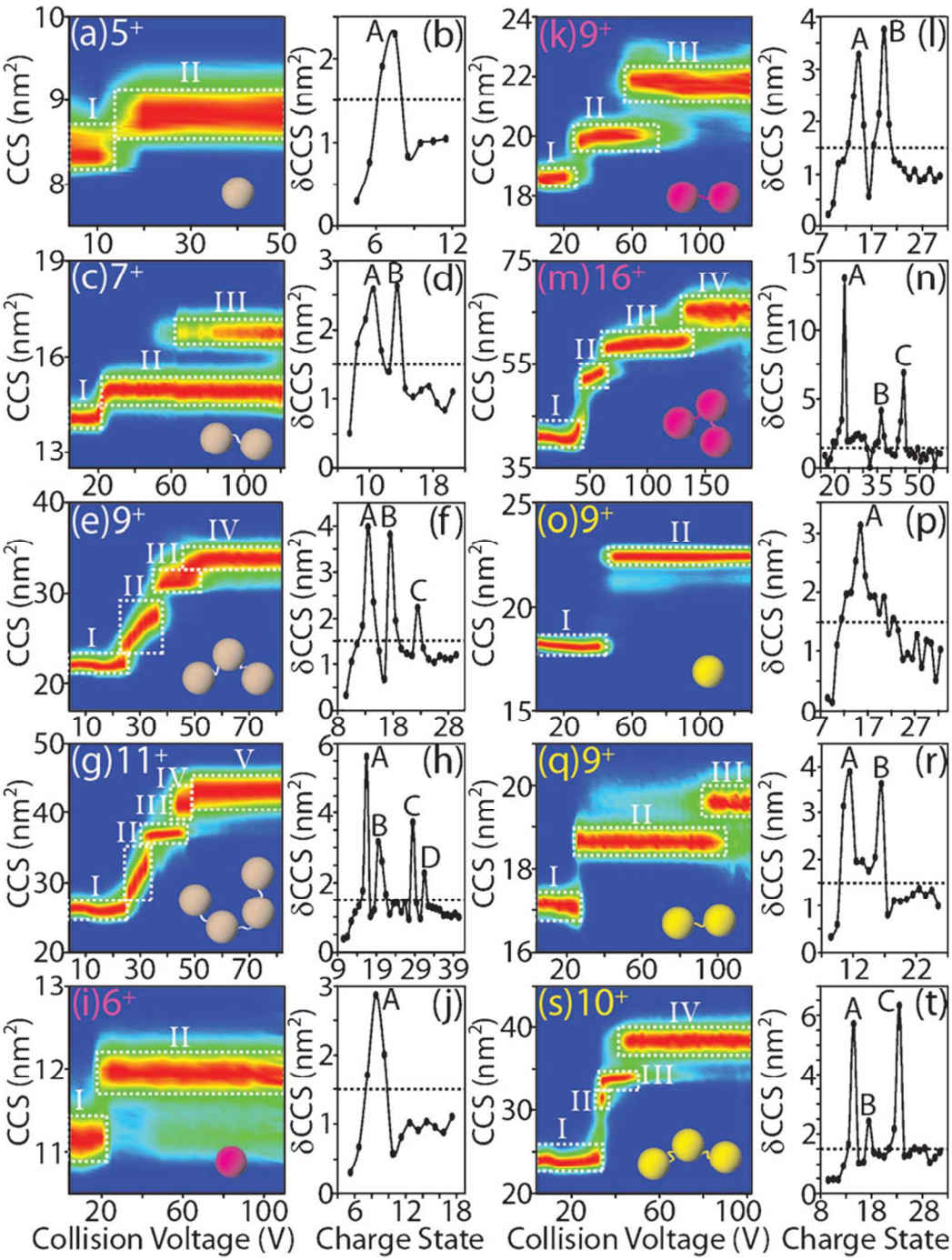


Figure 6



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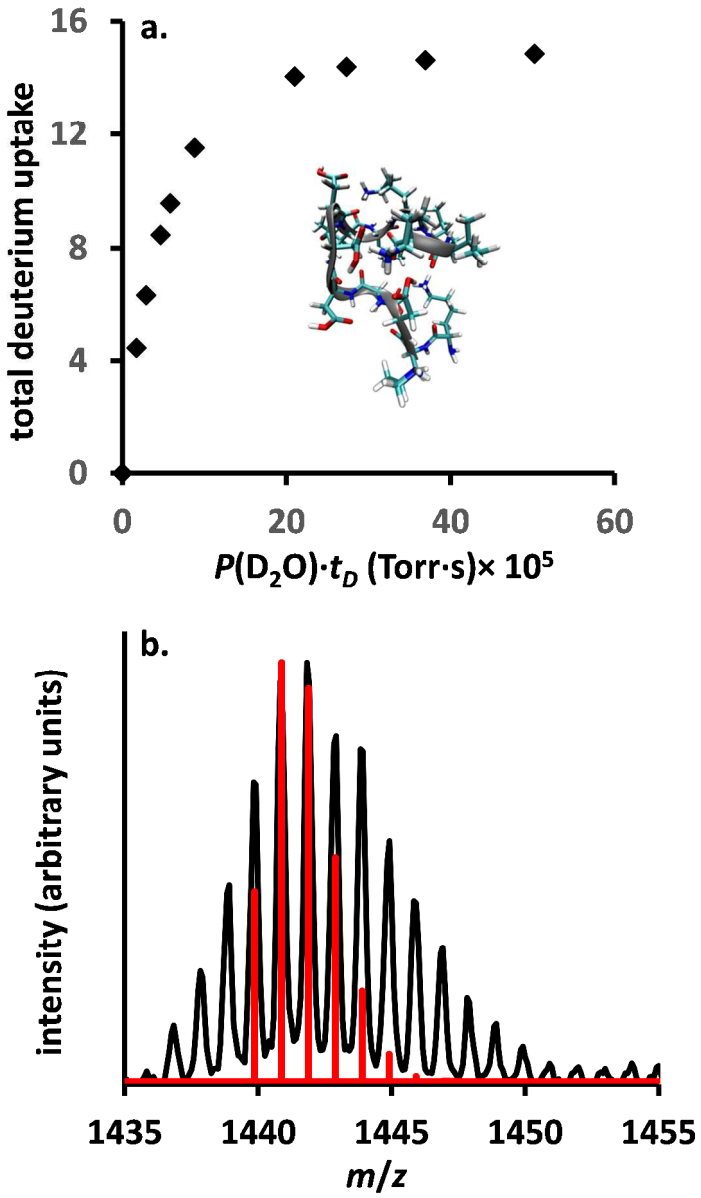

Figure 8

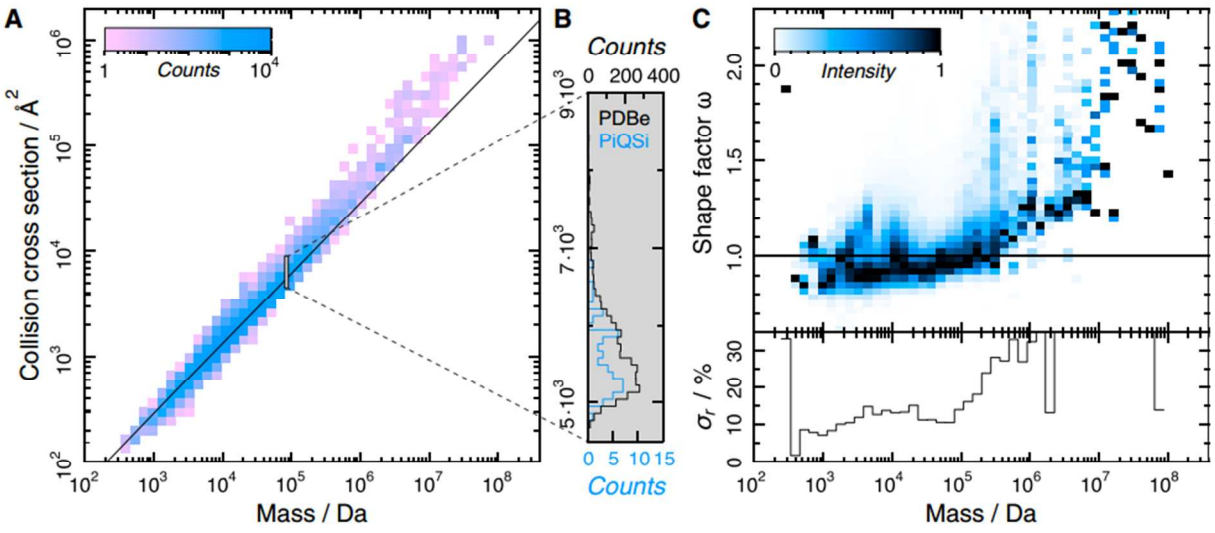

Figure 9

Table of Contents Entry

Enabling IM-MS instrumentation and techniques for characterizing sample structural heterogeneity have developed rapidly over the last five years.